

Article

Enhancing the Antibacterial and Biointegrative Properties of Microporous Titanium Surfaces Using Various Metal Coatings: A Comparative Study

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Academic Editor: Marco Ciccio

Received: 10 August 2025

Revised: 12 October 2025

Accepted: 20 October 2025

Published: 26 October 2025

Citation: Shevtsov, M.; Bozhokina, E.; Yudintceva, N.; Bobkov, D.; Lukacheva, A.; Nazarov, D.; Voronkina, I.; Smagina, L.; Pitkin, E.; Oganessian, E.; et al. Enhancing the Antibacterial and Biointegrative Properties of Microporous Titanium Surfaces Using Various Metal Coatings: A Comparative Study. *Prosthesis* **2025**, *7*, 133. <https://doi.org/10.3390/prosthesis7060133>

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Abstract

Background/Objectives: A comparative study of silver (Ag), titanium nitride (TiN), zirconium nitride (ZrN), and copper (Cu) coatings on titanium (Ti) disks, considering the specifications of a microporous skin- and bone-integrated titanium pylon (SBIP), was performed to assess their biocompatibility, osseointegration, and mechanical properties. **Methods:** To assess cytotoxicity and biocompatibility, Ti disks with various metal coatings were co-cultured with FetMSCs and MG-63 cells for 1, 3, 7, and 14 days and subsequently evaluated using a cell viability assay, as supported by SEM and confocal microscopy studies. The antimicrobial activity of the selected four materials coating the implants was tested against *S. aureus* by mounting Ti disks onto the surface of LB agar dishes spread with a bacterial suspension and measuring the diameter of the growth inhibition zones. Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) analysis of the relative gene expression of biomarkers that are associated with extracellular matrix components (fibronectin, vitronectin, type I collagen) and cell adhesion ($\alpha 2$, $\alpha 5$, αV integrins), as well as of osteogenic markers (osteopontin, osteonectin, TGF- $\beta 1$, SMAD), was performed during the 14-day follow-up period. Additionally, the activity of matrix metalloproteinases (MMP-1, -2, -8, -9) was assessed. **Results:** All samples with metal coatings, except the copper coating, demonstrated a good cytotoxicity profile, as evidenced by the presence of a cellular monolayer on the sample surface on the 14th day of the follow-up period (as shown by SEM

and inverted confocal microscopy). All metal coatings enhanced MMP activity, as well as cellular adhesion and osteogenic marker expression; however, TiN showed the highest values of these parameters. Significant inhibition of bacterial growth was observed only in the Ag-coated Ti disks, and it persisted for over 35 days. **Conclusions:** The silver-based coating, due to its high antibacterial activity, low cytotoxicity, and biointegrative capacity, can be recommended as the coating of choice for microporous titanium implants for further preclinical studies.

Keywords: direct skeletal attachment; skin- and bone-integrated pylon; SBIP; metal coatings; silver (Ag) coating; titanium nitride (TiN) coating; zirconium nitride (ZrN) coating; copper (Cu) coating; titanium implants

1. Introduction

The technology of direct skeletal attachment (DSA) for exoprosthetics of limbs has already entered the practice of traumatological and orthopedic clinics, having proven itself as a reliable approach for the rehabilitation of patients with a high level of preservation of limb biomechanics (as compared with conventional socket prostheses) and, consequently, improved functional integration and quality of life [1]. A pylon is implanted into the bone's intramedullary canal and exits through the skin, to which the exoprosthetic structure is then attached. Reliable bone fixation and biointegration of the intraosseous pylon can be achieved, as shown by numerous preclinical and clinical studies [2,3]. At the same time, the presence of a percutaneous section of the implant passing through the soft tissues of the residuum can be considered as an entry point for infection, resulting in different postoperative complications [4,5]. One approach to mitigating infectious complications is the development of an implant that allows cells to grow into its walls. This approach has been intensively investigated by the authors' team and implemented in the design of the skin- and bone-integrated pylon (SBIP) with micropores, allowing bone tissue and skin cells to effectively migrate into the pores of the implant, forming a vascularized strong tissue barrier against infection [6]. Thus, in our recent study, it was demonstrated that micropore sizes ranging from 200 to 500 μm in titanium 3D-printed implants were favorable for dermal fibroblast adhesion, while pore sizes ranging from 400 to 800 μm demonstrated favorable results (in terms of biomarker expression related to osteogenic differentiation, including osteonectin, osteocalcin, osteopontin, SMAD4, and TGF- β 1) in osteoblast cells [7]. These parameters create favorable conditions for reliable mechanical fixation of the implant and help close entry sites for infection.

Another approach for mitigating infection complications is the creation of an optional antibacterial coating that prevents the formation of a biofilm and colonization of the implant surface by bacteria [8,9].

It is this approach to which the current study is devoted. We conducted a comparative analysis of the use of antibacterial coatings on implants based on various metals (silver (Ag), titanium nitride (TiN), zirconium nitride (ZrN), and copper (Cu), which have proven to be cost-effective and reliable in traumatology and orthopedics. Materials other than silver that are potentially useful for accelerated cell growth and have been tested in various studies [10,11] include titanium nitride (TiN) and zirconium nitride (ZrN) [12–14], as well as copper (Cu) [15].

Considering that the use of metal-based coatings can significantly affect the biointegrative properties of porous titanium materials, namely, the ingrowth of bone and skin tissue cells into the pores of the implant, we investigated in vitro the effect of these coatings on

both the viability and functional properties of model cells—FetMSCs and MG-63 cells. To study the properties of the cells, we selected cell adhesion markers ($\alpha 2$ integrin (collagen-specific), $\alpha 5$ integrin (fibronectin-specific), αV integrin (vitronectin-specific), type I collagen, fibronectin, vitronectin, FAK, vinculin, paxillin), osteogenic markers (osteonectin, osteopontin, TGF- $\beta 1$, SMAD), and the activity of matrix metalloproteinases (i.e., activity of gelatinases (MMP-2, MMP-9) and collagenases (MMP-1, MMP-8)).

According to the results of the study, it was found that coatings based on copper and silver have the best antibacterial properties; however, due to the cytotoxic activity of copper and the effect on the functional properties of cells, the silver coating is favorable.

2. Materials and Methods

2.1. Synthesis, Metal Coating, and Characterization of the Ti Disks

2.1.1. Porous Titanium Samples for the Current Study

Porous titanium tablet samples (Figure 1) were fabricated with sintering technology (ADMA Products Group, Hudson, OH, USA) from surgical implant-grade titanium (ASTM F67) with pore sizes between 20 μm and 350 μm [16], in line with Poly-Orth International Standard Operating Procedure MPPS-103. Sintering was conducted using molds made of boron nitride block with cylindrical cavities with a diameter of 8.5 mm and a depth of 3 mm. The cavities were filled with high-purity titanium hydride powder that was pre-sieved to $-50/+80$ mesh (ASTM E11).

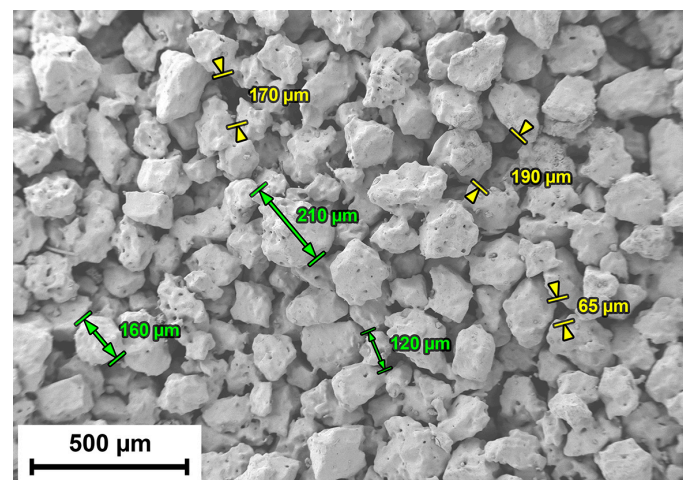


Figure 1. Structure of the sintered (control) samples.

The sintering process consisted of two stages as follows:

1. Once the 10^{-5} torr vacuum was achieved, the chamber was back-filled with high-purity argon to partial pressure between 11 psi and 12 psi. The sintering (that involved decomposition of titanium hydride to pure titanium) was carried out at 1190 $^{\circ}\text{C}$ (2174 $^{\circ}\text{F}$) for 2 h.
2. For the second stage of sintering, the vacuum pressure was reduced to 10^{-5} torr (no argon), and the temperature was raised to 1300 $^{\circ}\text{C}$ (2372 $^{\circ}\text{F}$). The sintering time was 4 h.

The tablets were removed from the cavities and then subjected to hot isostatic pressing with argon pressure of 15,000 psi for 2 h at a temperature of 954 $^{\circ}\text{C}$ (1749 $^{\circ}\text{F}$).

We used a patented combination of four key technological characteristics: porosity, pore size, porosity volume fraction, and particle size [16]. The parameter that is most distinct from other implants' systems is the porosity volume fraction (VF), which is the fraction of void space relative to the total bulk volume of the sample. In our samples, $\text{VF} = 78.2\%$. This value is associated with implants with deep porosity ($\text{VF} > 50\%$), as defined in [6].

2.1.2. Coating of the Samples (Figure 2)

The properties of the materials that are potentially useful for the accelerated cell growth are as follows: (a) natural antibacterial properties, (b) biocompatibility, and (c) if prepared properly either by composition or multilayered composites, the antibacterial components shedding quickly to prevent infection before cell growth initiates. In addition to silver, TiN, ZrN, and Cu have been trialed in various studies [13,17–20]. Each of these materials has its potential benefits and drawbacks.

Copper composites require a limited amount of copper, as copper ions can be antibacterial, but where too much copper metal is exposed to the body, it becomes an irritant causing infection [21]. For Cu, the composite aspect of the application is critical, but the technology required is in the laboratory phase, at best. While TiN and ZrN are excellent materials, they do not possess the full antibacterial properties of silver [12–14].

The technology used for coating was physical vapor deposition (PVD) [22], and the equipment used was a magnetron sputtering multi-target machine, Flexicoat series (IHI Hauzer Techno Coating B.V., Venlo, The Netherlands).

For all metals used for coating, the tablets were thoroughly cleaned with aqueous-base soap and ultrasonic equipment. Once the ultrasonic process was completed, the soap film on the tablets was removed and the tablets neutralized using a mild sulfuric acid, followed by rinsing in DI water. The tablets were then dried at 300 F for about 60 min to ensure all the water vapor was completely removed from the tablet. This method ensured two critical elements in the physical vapor deposition (PVD) process: (1) there were no organics on the tablet to cause poor adhesion of the material being sputtered to the titanium substrate, and (2) no water vapor was released during the argon pure plasma vacuum environment that could cause impure elements in the deposition of the material. Once the tablets were dry and fixed for the vacuum coating process, they were placed in a PVD imbalanced magnetron.

The silver (Ag) content of the test items was 1.00 ± 0.2 mg/cm², and the total silver content per test item was 5.0 ± 0.1 mg. Since the implantation of pylons in DSA patients is permanent, this coating specification was selected to combine the well-established bactericidal properties of silver [23] with a relatively fast dissolution of the silver layer, in order to avoid the toxic consequences of long-term exposure to silver [21]. Positive verification of this specification, patented in [16], was reported in our animal study [6], where the skin- and bone-integrated pylons (SBIPs) had a silver layer thin enough to dissolve within about 4–6 weeks after implantation. That period was sufficient for the skin to regenerate into the porous cladding of the SBIP and establish a sustainable natural barrier against infection.

The process of coating with TiN or ZrN requires similar parameters and gas combinations to coating with Ag. The power used to create a film was in the 5 kW range for a period of 1 h to build 1 micron of coating. Argon gas (non-reactive) formed the main plasma needed to create the energy required to atomize the target material. Nitrogen gas is the reactive gas that is the necessary catalyst to transform titanium or zirconium into a nitride film or coating.

For titanium nitride, we used a VT-3000 cathodic arc system with a deposit arc current set at 500 amps, argon gas at 600 sccm, and nitrogen at 600 sccm for 10 min, because a cathodic arc builds quicker than magnetron sputtering. Negative bias was set at 100 volts.

For zirconium nitride, we used a VT-3000 cathodic arc system with a deposit arc current set at 500 amps and argon gas at 600 sccm for 10 min, because a cathodic arc builds quicker than magnetron sputtering. Negative bias was set at 100 volts.

For pure copper (Cu) coating, lower power was required to create a 1-micron film, due to copper's propensity to self-sputter via the energy of the plasma transfer itself, and only

argon (Ar) gas plasma was used for the deposition. Copper coating was targeted at location #5 in the Hayzer system via magnetron at 5 kW. We used only argon gas at 500 sccm for 15 min, and bias was set at 100 volts. The power used to create the film was in the 5 kW range for a period of 1 h to build 1 micron of coating. Argon gas (non-reactive) formed the main plasma required to create the energy needed to atomize the target material. Nitrogen gas is the reactive gas providing the necessary catalyst to transform titanium or zirconium into a nitride film or coating.

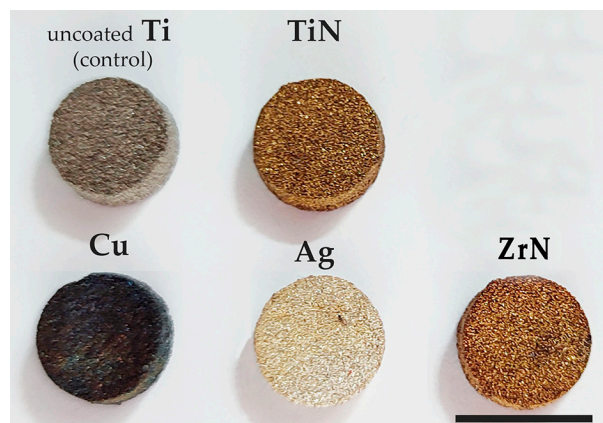


Figure 2. Coated samples: uncoated titanium medical grade; coating with titanium nitride (TiN); coating with silver (Ag); coating with zirconium nitride (ZrN); coating with copper. Scale bar, 1 cm.

2.2. Cells

Fetal mesenchymal stem cells (FetMSCs) were obtained from a shared research facility's "Vertebrate Cell Culture Collection" (supported by the Ministry of Science and Higher Education of the Russian Federation at the Institute of Cytology of the Russian Academy of Sciences (St. Petersburg, Russia)). FetMSCs were cultivated in DMEM supplemented with 10% fetal bovine serum (FBS), 1 mM Sodium Pyruvate, 6 mM L-glutamine, 4.5 g/L Glucose, 0.1 mM MEM Non-Essential Amino Acids, and antibiotics 1% penicillin-streptomycin (Gibco, Waltham, MA, USA). Human MG-63 cells were cultured in α -DMEM medium (Gibco, Waltham, MA, USA) supplemented with 10% FBS (Gibco, Waltham, MA, USA) and antibiotics 1% penicillin-streptomycin (Gibco, Waltham, MA, USA) at 37 °C and 5% CO₂. For cell dissociation, 0.25% trypsin-EDTA (Gibco, Waltham, MA, USA) solution was employed at high cell confluence ($\geq 90\%$). After 2–3 passages for increasing the number of cells, the cells were used for experimental procedures.

2.3. Scanning Electron Microscopy

To evaluate the formation of a cell monolayer on the surface of microporous titanium with different metal coatings using scanning electron microscopy (SEM), the samples were preliminarily autoclaved. Then, a cell suspension of either FetMSCs or MG-63 (5×10^6 /mL) was applied to the surface of the samples for 3 days in a CO₂ incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C. After that, a nutrient medium was added to each Petri dish, ensuring complete coverage of the implant surface. Upon completion of co-cultivation, the samples were washed with Dulbecco's phosphate buffer (PBS) (Sigma-Aldrich, St. Louis, MO, USA) and fixed in a 2.5% glutaraldehyde solution in phosphate buffer (pH = 7.0, Sigma-Aldrich, St. Louis, MO, USA). Then, after washing, the samples were successively dehydrated for 30 min in 30, 50, 70, 90, and 96% absolute ethanol. Next, conductive silver coatings of approximately 10 nm thickness were applied using a Leica EM SCD500 microscope (Leica Microsystems, Wetzlar, Germany). The morphology of the

cell monolayer was assessed using a Zeiss Auriga scanning electron microscope (Carl Zeiss, Oberkochen, Germany) in SE (secondary electron) modes.

2.4. Confocal Microscopy

The formation of the FetMSCs and MG-63 cell monolayer on the surface of microporous Ti disks was visualized employing the Olympus FV3000 confocal system (Olympus, Tokyo, Japan). Cells (0.1×10^6 /mL cells) were placed in Matrigel (0.2 mg/mL, Corning, New York, NY, USA) on the surface of Ti disks and kept overnight. After incubation, the cells were washed with PBS, stained with TMRM and LysoTracker, fixed in a 10% formalin solution (Sigma-Aldrich, St. Louis, MO, USA), and mounted using a mounting medium containing DAPI (Ibidi, Graefelfing, Germany). Unstained cells were used as controls.

2.5. Testing of Antibacterial Activities

The antimicrobial activity of the various materials for implants was tested on *Staphylococcus aureus* ATCC 29213. The disks were mounted onto the surface of LB-agar dishes and gently pressed with sterile tweezers to ensure uniform contact of the disk with the agar surface. Dishes were incubated at 37 °C for several days as indicated to allow diffusion of ions into the agar. Next, 1 mL of bacterial suspension with a density of 1.5×10^8 CFU/mL was loaded onto the surface of the Petri dish with a LB-agar and evenly spread over the surface. Then, the remaining liquid was removed and dishes were dried. The Petri dishes were incubated at 37 °C for 24 h, and the diameter of the growth inhibition zones was measured.

2.6. Analysis of the Matrix Metalloproteinase Production

The activity of matrix metalloproteinases (MMP-1, MMP-2, and MMP-9) in the culture medium on day 1 following co-incubation with uncoated and differently coated titanium samples was determined using the zymography method described elsewhere [24]. Gelatin and casein were used as substrates to evaluate the activity of gelatinases (MMP-2, MMP-9) and collagenases (MMP-1, MMP-8), respectively. A gel (10% acrylamide) contained 1.0 mg/mL gelatin or 0.5 mg/mL casein. Two micrograms of protein per lane were loaded into the gel. The protein content in the probes was measured using a Bradford protein assay. After electrophoresis, the gel was washed twice with 2.5% Triton X-100 for 30 min and then incubated in a buffer solution (50 mM Tris-HCl pH 7.6, 0.15 M NaCl, 10 mM CaCl_2 , 0.05% Brij 35) for 12 h. Then, the gel was stained with Coomassie Blue R-250 (0.25% Coomassie brilliant blue R-250 in 40% isopropanol for 2 h) and, after destaining (with 7% acetic acid for 1 h), the bands containing MMPs were developed as non-stained bands. A medium conditioned by HT-1080 fibroblasts obtained from the Culture Collections of Institute of Cytology of the Russian Academy of Sciences [RAS], St Petersburg, Russia, was used as a marker to determine MMP zones. It contains both MMP-2 and MMP-9 [25,26]. Bands of MMP-1 and MMP-8 activity were verified by molecular weight markers and in preliminary experiments by antibodies. For the quantitative assay, gels were scanned, and images were processed with QuantiScan 3 software. MMP activity was normalized to protein concentration. MMP activity, presented in arbitrary units, was analyzed with the program QuantiScan which calculated the product of the number of colored pixels of the MMP band by the color intensity. The results of densitometry were presented in the form of histograms as mean values $\pm$ SEM.

2.7. Quantitative Real-Time PCR

Total RNA from human FetMSCs and MG-63 cells lines on each material (Ag, TiN, ZrN, Cu, and non-coated Ti) was extracted with the RUpplus RNA isolation kit (Biolabmix, LLC, Moscow, Russia) according to the manufactures' protocol. The isolated total RNA was quantified using a nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA).

cDNA synthesis was performed using the M-MuLV–RH First Strand cDNA Synthesis Kit (Biolabmix, LLC, Russia). The mixture of 3 µg RNA, 50 µM oligodT primer, and DEPC-treated water was carefully vortexed, and droplets were collected by centrifuging; then it was heated at 70 °C for 2–3 min in order to melt secondary structures and the tube was placed on ice. After this, the mixture was added to the reaction mix (5× RT buffer mix, 0.1 M DTT, 10 mM dNTPs mix, M-MuLV–RH revertase (100 u/µL)), and for first strand synthesis, incubated at 42 °C for 1 h with subsequent cooling on ice for 2–3 min. The reaction was stopped by heating the reaction solution at 70 °C for 10 min. The product of reverse transcription reaction was used directly for PCR amplification or stored at −70 °C. cDNA for GAPDH and actin was used as a control for calculating fold differences in RNA levels of FetMSCs and MG-63 cells cultivated on Ti and material covered Ti disks. mRNA relative quantities were obtained using the $2^{-\Delta\Delta C_t}$ method, and forward and reverse primers specific for tested genes were designed with Pubmed nucleotide design (Primer-BLAST) software version 1.0.1 for all tested genes (Table 1). The samples were evaluated in the Bio-Rad CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s protocol. Cycling conditions were as follows: 95 °C for −5 min, followed by 45 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final cycle of 72 °C for 2 min. A melt curve was generated by heating from 65 °C to 95 °C at ramps of 0.5 °C/s.

Table 1. Forward and reverse primers specific for analyzed genes used for RT-PCR studies.

Gene	Primers (5′–3′)
Fibronectin	F: CAGCCTCTGGTTCAGACTGC R: TCTTGTCTACATTCGGCGG
Vitronectin	F: TACCCCAAGCTCATCCGAGA R: ACTGTAGCTATGGGCAGGGA
Type I collagen	F: GGTGTAAGCHCTGGTGGTTA R: CCAGTTCTTGGCTGGGATGT
α2 integrin	F: GGCTGGCCCAGAGTTTACAT R: ATCGAAAAATCTCCTAACTT
α5 integrin	F: TTCAACTTAGACGCGGAGGC R: ATCGCCCCCTCTCCTAACTT
αV integrin	F: CCTAGGCACCCTCCTTCTGA R: TCACATTGAGGACCTGCCC
FAK	F: GTCGTCTGCCTTCGCTTCA R: AGCAGGCCACATGCTTTACT
Paxillin	F: AAAGTTGCGGGGCATAGACG R: CAAGAACACAGGCCGTTTGG
Vinculin	F: GAGCAAAACCATCTCCCCGA R: CTGCCTCAGCTACAACACCT
Osteopontin	F: CAGCAGCAGCAGGAGGAG R: ACGGCTGTCCCAATCAGAAG
Osteonectin	F: TCGGCATCAAGCAGAGGAAT R: GTCCCTAGAGCCCCTGAGAA
TGF-β1	F: TGTCCAGGCAAGAAATGGCA R: AGGAACCGCAGCACTCATAC
SMAD4	F: ATGCTCAGTGGCTTCTCGAC R: CCTAGGGGAGAGCAGGAAGG

2.8. Statistics

For each of the metal coating conditions, including the control, and for both kinds of co-cultured cells (MG-63 and FetMSCs), 3 independent disks were studied, giving a total of

42 disks across 14 conditions. Means and standard deviations of [gene expression/MMPs] were computed for each condition, across each of the 4 studied time points (1, 3, 7, and 14 days after culturing). For the MG-63 and FetMSC cell groups, respectively, a one-way ANOVA tested the equality of means on the 14th day at the 0.05 level of significance. Post hoc Tukey–Cramer mean comparisons were used to identify the specific coating whose [gene expression/MMPs] were significantly different from the rest. For both cell cultures, the Cu coating's [gene expression/MMPs] were significantly lower than those of all others ($p < 0.01$). One-way analysis with the Kruskal–Wallis test was used to analyze the differences. The software program used for the statistical analysis was Statistica Version 9.2. For all experiments, distinctions were regarded as statistically reliable at $p < 0.05$.

3. Results

3.1. Cytotoxicity Profile of the Titanium Metal Coatings

To evaluate the cytotoxic properties of various metallic coatings of microporous titanium, FetMSCs and MG-63 cells were co-incubated with titanium disks for 1, 3, 7, and 14 days. Thus, when conducting light microscopy, after 24 h of co-cultivation, both cell cultures formed a monolayer directly on the surface of the implants and on the surface of the culture flask adjacent directly to the uncoated microporous titanium disk (Figure 3).

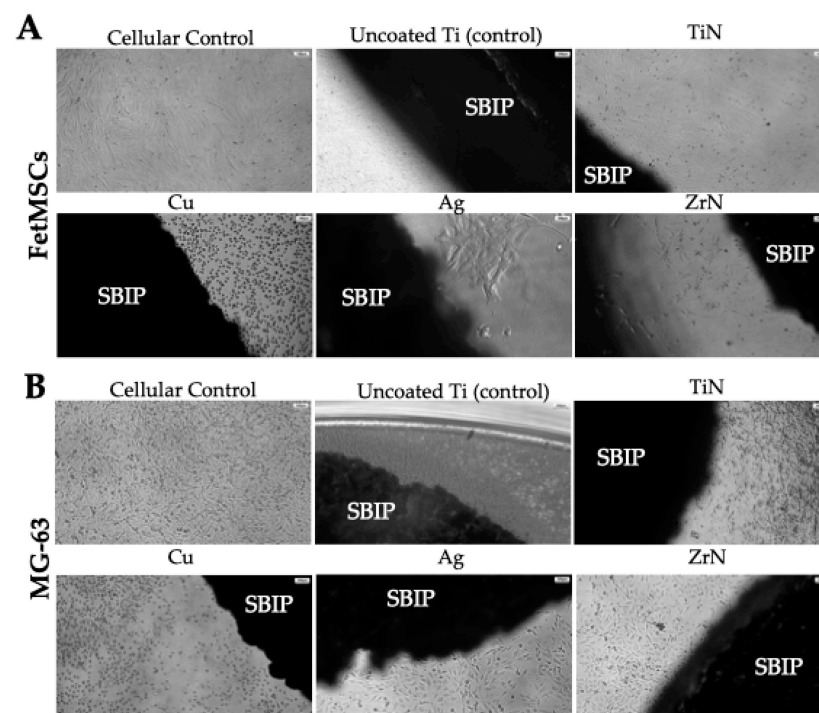


Figure 3. Light microscopy images of cells cultivated on the surface of the titan samples with various metal coatings. Cells were visualized using the inverted microscope Nikon Eclipse TS100 (Nikon, Tokyo, Japan). (A) Morphology of FetMSCs; (B) morphology of MG-63 cells. Note: Morphology of cells on the surface of a Petri dish was used as a control. SBIP—skin- and bone-integrated pylon composed of microporous titanium alloy. Scale bars, 100 μ m.

The use of silver (Ag), titanium nitride (TiN), and zirconium nitride (ZrN) did not lead to a change in cell morphology, whereas when using a copper (Cu) coating, a significant change in cell morphology was observed, which became rounded with a violation of the formation of a cellular monolayer. The obtained data from light microscopy was further supported by the results of SEM studies, which also demonstrated the cytotoxicity of copper coatings towards FetMSCs and MG-63 cells (Figure 4).

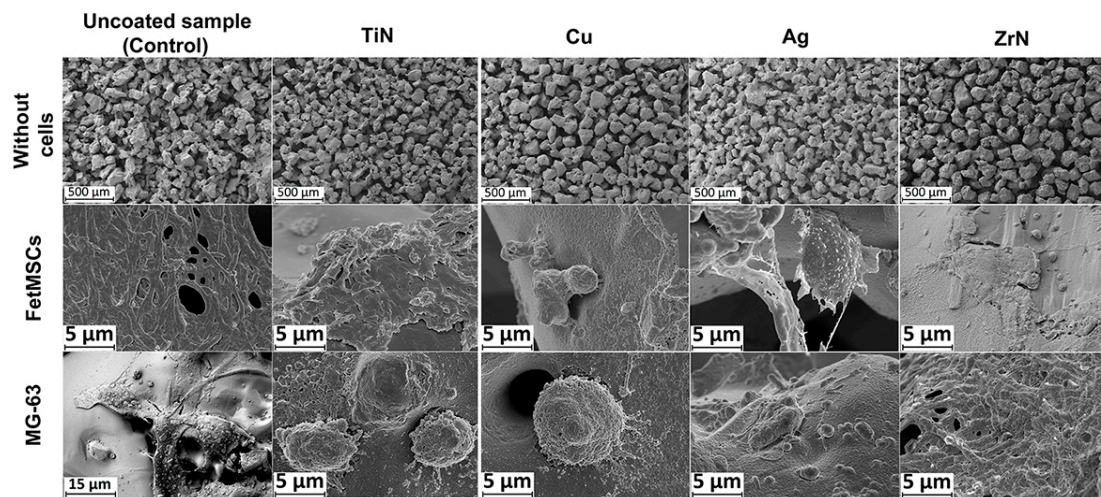


Figure 4. Scanning electron microscopy (SEM) studies of the microporous Ti disks with various metal coatings co-incubated with FetMSCs and MG-63 cells for 14 days.

Furthermore, we assessed the FetMSC cell viability on Ti disks and also detected a cytotoxic influence of Cu coatings on the cells (Figure 5). To further identify viable cells, the latter were additionally stained with TMRM (*Tetramethylrhodamin-methylester*) dye, which identifies mitochondria in viable cells.

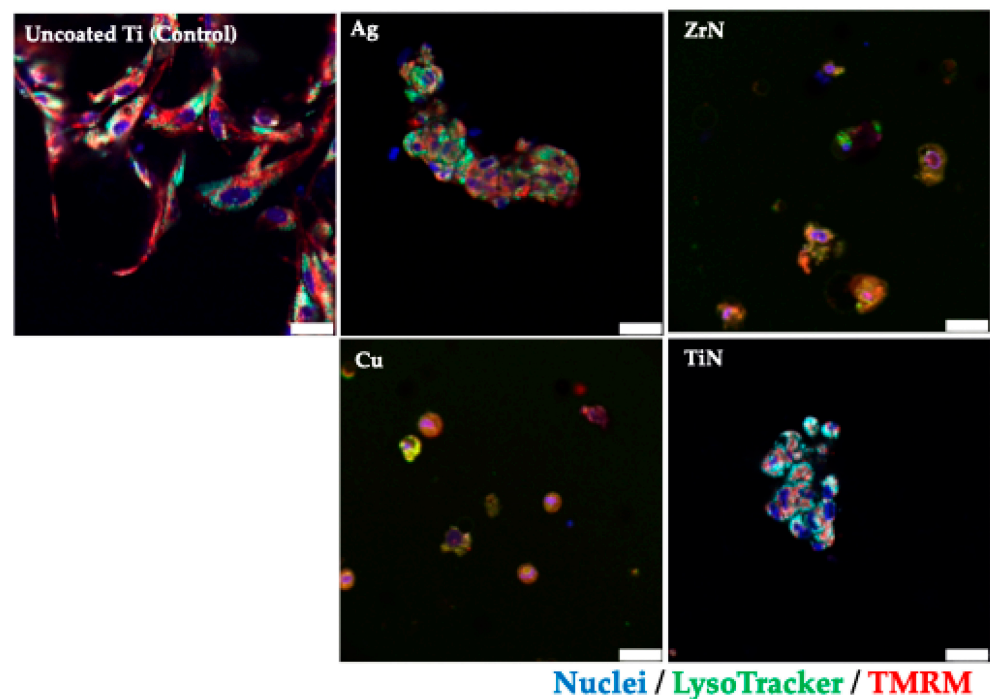


Figure 5. Confocal microscopy images of the FetMSCs co-cultured on the microporous Ti disks with various metal coatings for 14 days. Nuclei were stained with DAPI (blue), and mitochondria were stained with TMRM dye (red); lysosomes were detected with LysoTracker (green). Scale bars, 25 μ m.

When conducting the MTT test, it was found that application of the uncoated Ti disks led to a slight decrease in cell viability over the 14 days of co-incubation, which constituted 88.50 ± 0.72 % (FetMSCs) and 85.20 ± 2.25 % (MG-63) as compared to control cells cultivated on the surface of culture flasks— 91.20 ± 1.08 % (FetMSCs) and 91.13 ± 1.06 % (MG-63) (Tables 2 and 3; Figures 6 and 7). When silver (Ag), titanium nitride (TiN), and zirconium nitride (ZrN) coatings were employed, we also

did not detect significant toxicities during the follow-up period of 14 days, constituting 82.30 ± 1.78 %, 86.77 ± 1.96 %, 90.93 ± 1.27 %, and 88.00 ± 1.93 %, respectively, for FetMSCs and 82.00 ± 3.75 %, 86.33 ± 2.76 %, 86.43 ± 3.19 %, and 83.17 ± 5.28 %, respectively, for MG-63 cells (Tables 2 and 3; Figures 6 and 7). However, when copper coatings were employed, we detected significant cytotoxicity towards FetMSCs and MG-63 cells, which on day 14 constituted 47.80 ± 2.50 % and 48.77 ± 2.14 %, respectively ($p < 0.001$).

Table 2. Means and (standard deviations) of FetMSCs across different materials at different time points.

	Cellular Control	Uncoated Ti (Control)	Ag	TiN	ZrN	Cu
1d	98.93 (0.47)	96.93 (1.27)	94.87 (2.90)	96.53 (1.42)	95.67 (4.56)	90.73 (1.43)
3d	97.83 (1.40)	93.03 (1.46)	92.20 (1.73)	90.03 (0.81)	95.20 (2.23)	84.40 (4.63)
7d	95.17 (0.45)	90.90 (2.02)	87.50 (1.06)	90.30 (0.90)	91.40 (1.90)	75.97 (4.94)
14d	91.20 (1.08)	88.50 (0.72)	82.30 (1.78)	86.77 (1.96)	90.93 (1.27)	47.80 (2.50)

Table 3. Means and (standard deviations) of MG-63 across different materials at different time points.

	Cellular Control	Uncoated Ti (Control)	Ag	TiN	ZrN	Cu
1d	98.93 (0.55)	95.20 (1.06)	92.60 (2.17)	95.33 (4.31)	92.03 (2.92)	89.73 (0.45)
3d	97.67 (2.10)	94.17 (0.91)	90.80 (0.78)	93.97 (1.53)	88.70 (1.37)	84.47 (2.71)
7d	94.93 (1.05)	90.53 (1.53)	85.37 (1.68)	89.53 (1.27)	88.10 (1.06)	71.03 (2.10)
14d	91.13 (1.06)	85.20 (2.25)	82.00 (3.75)	86.33 (2.76)	86.43 (3.19)	48.77 (2.14)

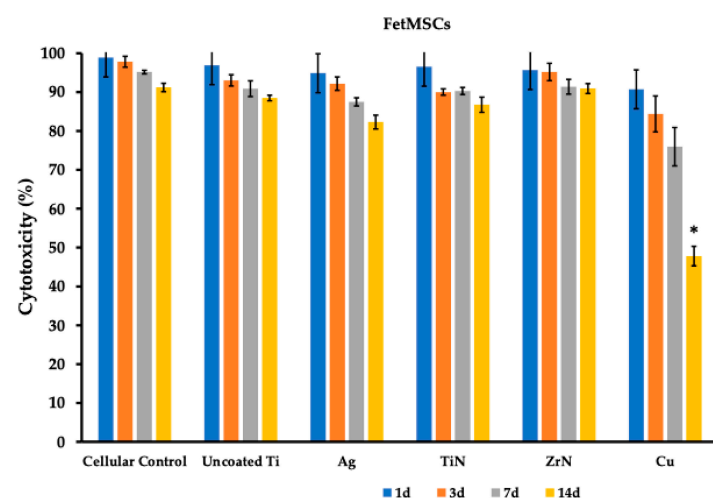


Figure 6. MTT assay of FetMSCs on microporous titanium disks with various metal coatings. Cell viability (%) was evaluated on the 1st, 3rd, 7th, and 14th day after co-incubation. Data is presented as mean (M) $\pm$ standard deviation (SD) from three independent experiments. *—statistically significant different ($p < 0.01$).

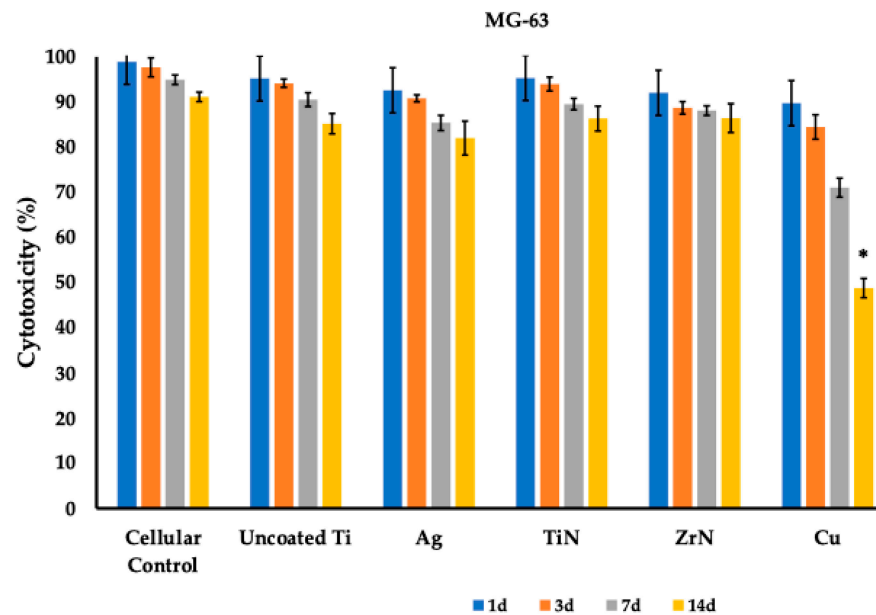


Figure 7. MTT assay of MG-63 cells on microporous titanium disks with various metal coatings. Cell viability (%) was evaluated on the 1st, 3rd, 7th, and 14th day after co-incubation. Data is presented as Mean (M) $\pm$ standard deviation (SD) from three independent experiments. *—statistically significant difference ($p < 0.01$).

3.2. Antibacterial Properties of the Microporous Titanium Metal Coatings

The antimicrobial activity of the microporous titanium disks with various metal coatings was evaluated on *S. aureus* ATCC 29213. Among all the Ti disks tested, a clear growth repression zone could be observed only for disks with silver (Ag) and copper (Cu). To evaluate whether the bacteriostatic effect will remain over a long period, the disks were mounted onto the surface of the agar in consequent series over 3–4 days. Although the antibacterial effect of Cu coatings was detected in the initial days of observation during the longer follow-up period, we did not observe any significant effect. The effect was stable and visible only for Ag coatings. Thus, as can be seen from Figure 8, the growth repression zone around the disk did not reduce over 35 days, suggesting a stable antimicrobial effect.

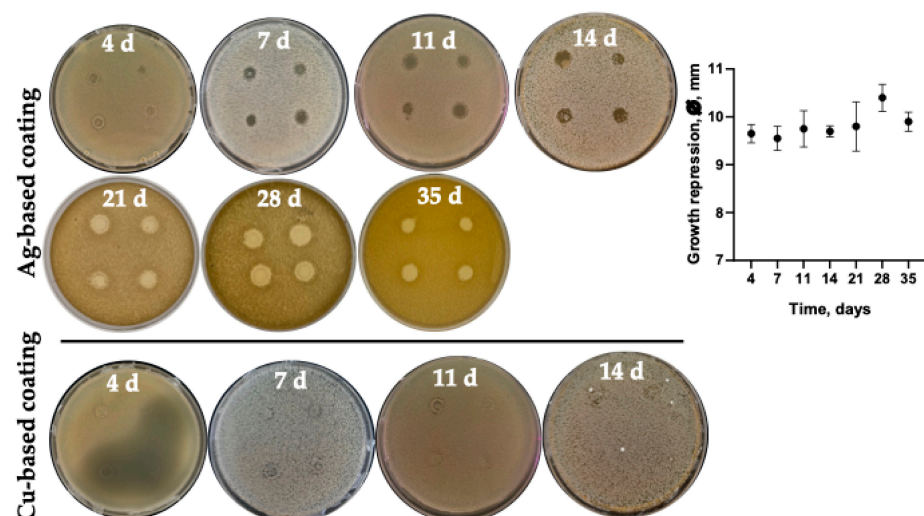


Figure 8. The growth repression zones of *S. aureus* on LB-agar after 3–4 days incubation with a mounted disk.

3.3. Induction of the Matrix Metalloproteinase Activity by Metal Coatings

Considering the important role that matrix metalloproteinases play in bone tissue remodeling and the integration of titanium implants, we assessed enzyme activity during co-cultivation of cells with microporous titanium disks with various metal coatings (Figure 9; Tables 4 and 5). For FetMSCs, we detected a manifold increase in the levels of MMP-2, MMP-9, MMP-1, and MMP-8 on TiN coating relative to the uncoated control Ti sample, constituting 4742 ± 711 (MMP-2), 1616 ± 155 (MMP-9), 7280 ± 1092 (MMP-2), 3376 ± 324 (MMP-9), 6764 ± 812 (MMP-1), and 1287 ± 206 (MMP-8) for TiN-coated samples, while in the uncoated Ti sample (control), MMP levels were as follows: 4103 ± 615 (MMP-2), 1165 ± 112 (MMP-9), 3832 ± 460 (MMP-1), and 681 ± 109 (MMP-8) ($p < 0.001$). The levels of the same MMPs on copper (Cu) and silver (Ag) coatings were similar, and on the zirconium nitride coating, they were even lower as compared to the uncoated Ti disks. As a control (base), the measurement results for MMPs in cultivation media were shown. For MG-63 cells, we also observed the increase in the levels of all studied MMPs on TiN coatings as compared to the uncoated Ti sample— 6419 ± 706 (MMP-2), 2560 ± 253 (MMP-9), 4575 ± 640 (MMP-1); 5860 ± 645 (MMP-2), 2846 ± 282 (MMP-9), 4899 ± 686 (MMP-1), and 384 ± 61 (MMP-8) ($p < 0.001$) for TiN coatings ($p < 0.001$). The levels of the same MMPs on copper, silver, and ZrN coatings were even lower as compared to the uncoated Ti control sample. As a control (base), the measurement results for MMPs in cultivation media were also shown.

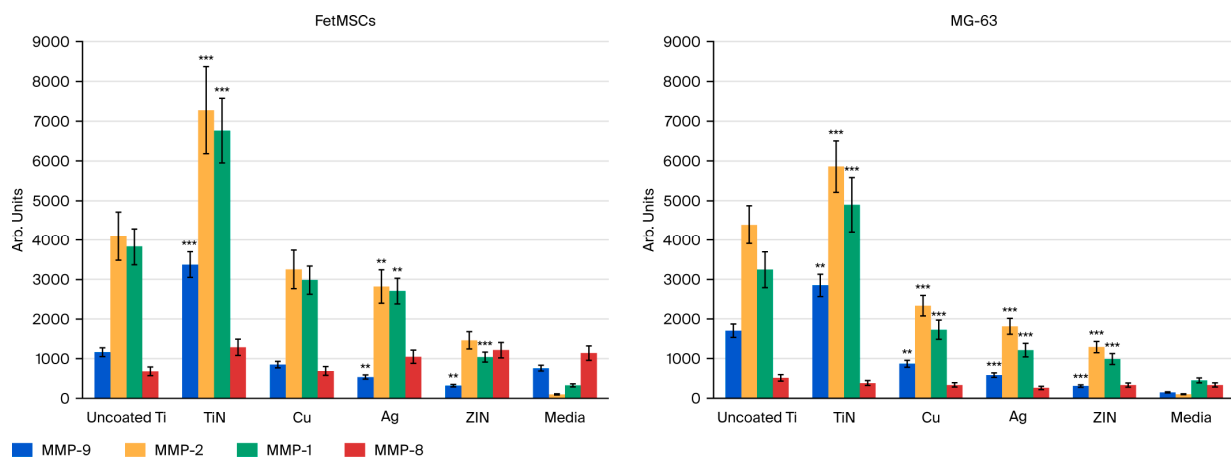


Figure 9. Analysis of matrix metalloproteinase activity (collagenases—MMP-1 and MMP-8; gelatinases—MMP-2 and MMP-9) in FetMSCs and MG-63 cells. **—statistically significant difference $p < 0.01$, ***— $p < 0.001$.

Table 4. Analysis of matrix metalloproteinase activity (collagenases—MMP-1 and MMP-8; gelatinases—MMP-2 and MMP-9) in FetMSCs. Data is presented as mean (M) $\pm$ standard error of the mean (SEM) from three independent experiments.

Samples	MMP-9		MMP-2		MMP-1		MMP-1	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Media	762	73	100	15	328	39	1140	182
Uncoated Ti (Control)	1165	112	4103	615	3832	460	681	109
TiN	3376	324	7280	1092	6764	812	1287	206
Cu	852	82	3253	488	2981	358	693	111
Ag	539	52	2821	423	2704	325	1050	168
ZrN	321	31	1464	220	1040	125	1215	194

Table 5. Analysis of matrix metalloproteinase activity (collagenases—MMP-1 and MMP-8; gelatinases—MMP-2 and MMP-9) in MG-63 cells. Data is presented as mean (M) $\pm$ standard error of the mean (SEM) from three independent experiments.

Samples	MMP-9		MMP-2		MMP-1		MMP-1	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Media	150	15	100	11	451	63	336	54
Uncoated Ti (Control)	1705	169	4393	483	3244	454	513	82
TiN	2846	282	5860	645	4899	686	384	61
Cu	869	86	2335	257	1729	242	337	54
Ag	583	58	1815	200	1215	170	261	42
ZrN	307	30	1292	142	987	138	331	53

3.4. Analysis of Focal Adhesion Markers of FetMSCs Cultured on Ti Disks with Various Metal Coatings

Gene expression of $\alpha 2$ integrin (collagen-specific), $\alpha 5$ integrin (fibronectin-specific), αV integrin (vitronectin-specific), type I collagen, fibronectin, and vitronectin genes were assessed following 72 h of co-incubation on microporous Ti disks with various metal coatings (Figure 10). Thus, after 72 h of co-incubation for uncoated Ti samples, the values of the genes constituted 0.866 ± 0.23 ($\alpha 2$), 1.033 ± 0.058 ($\alpha 5$), 0.933 ± 0.115 (αV), 1.05 ± 0.087 (fibronectin), 0.8667 ± 0.115 (vitronectin), and 0.933 ± 0.1 (type I collagen). Intriguingly, when silver (Ag) and titanium nitride (TiN) coatings were employed, we detected a significant increase in the expression of the studied genes (Figure 10).

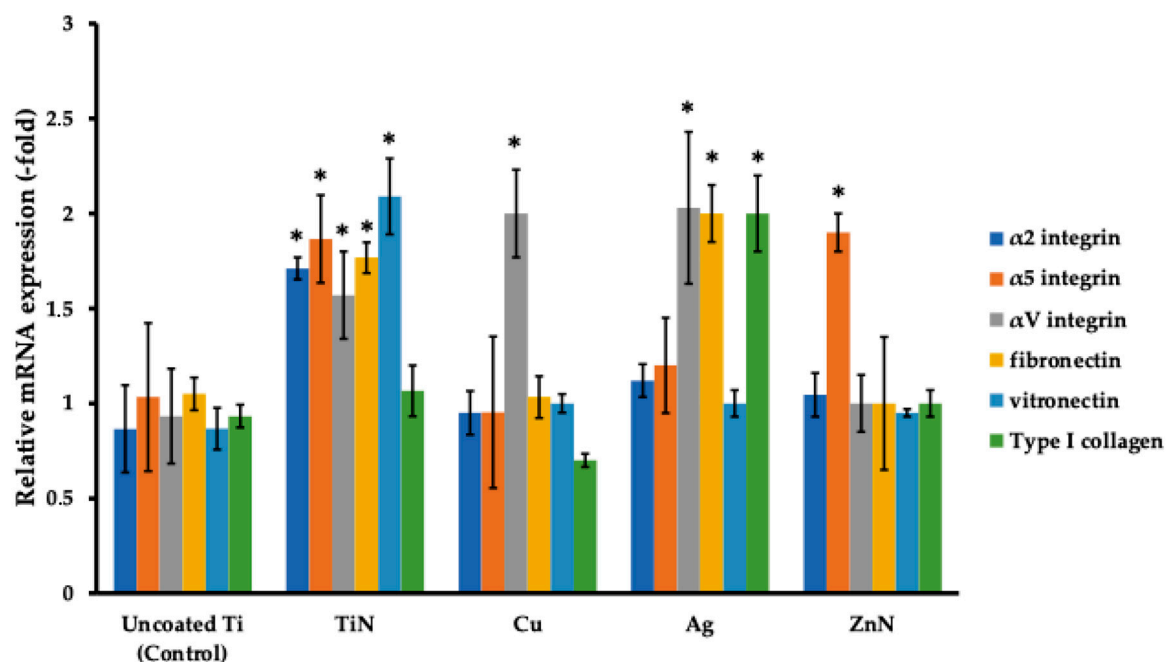


Figure 10. Comparison of expression of genes related for focal adhesion ($\alpha 2$ integrin, $\alpha 5$ integrin, αV integrin, type I collagen, fibronectin, and vitronectin) for FetMSCs co-incubated with microporous Ti disks with various metal coatings for 14 days. * $p < 0.05$ for testing mean expression levels.

3.5. Analysis of Osteogenic Markers of MG-63 Cells Cultured on Ti Disks with Various Metal Coatings

At the second stage, we evaluated the genes related to focal adhesion (FAK, vinculin, paxillin) and those related to osteogenic markers (osteopontin, osteonectin, TGF- $\beta 1$, SMAD)

for MG-63 cells after 14 days of cell co-incubation on Ti disks (Figure 11). Thus, for uncoated microporous Ti disks, the levels of expression of the studied genes constituted -1.14 ± 0.24 (FAK), 0.98 ± 0.1 (vinculin), 0.85 ± 0.21 (paxillin), 1.25 ± 0.35 (osteopontin), 1.15 ± 0.2 (osteonectin), 0.86 ± 0.196 (TGF- β 1), and 1.18 ± 0.25 (SMAD). When silver (Ag), titanium nitride (TiN), and zirconium nitride (ZrN) coatings were employed, we detected an increase in the studied genes' expressions, with the highest values for the silver coating— 3.65 ± 0.07 (FAK), 0.9 ± 0.1 (vinculin), 1.8 ± 0.14 (paxillin), 3.85 ± 0.21 (osteopontin), 2.04 ± 0.06 (osteonectin), 2.0 ± 0.283 (TGF- β 1), and 0 ± 0.21 (SMAD).

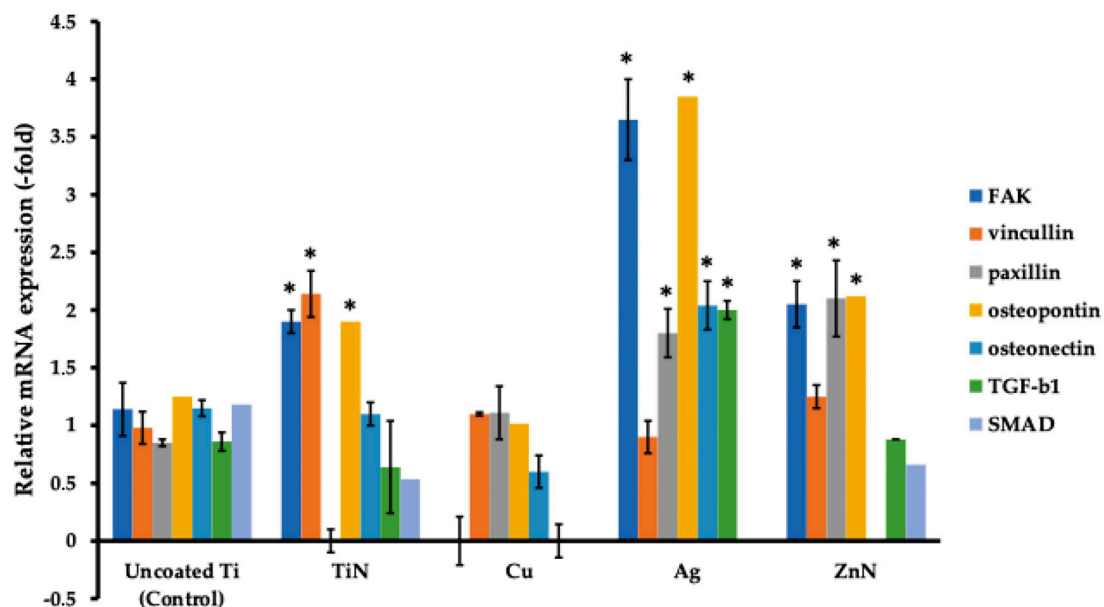


Figure 11. Comparison of expression of genes related to osteogenic markers (osteopontin, osteonectin, TGF- β 1, SMAD) and genes related to focal adhesion (FAK, vinculin, paxillin) for MG-63 cells co-incubated with microporous Ti disks with various metal coatings for 14 days. * $p < 0.05$ for testing mean expression levels.

4. Discussion

The use of metal coatings on dental and orthopedic titanium implants is an important area in modern translational research, since these coatings may exert antibacterial properties, promote osseointegration, which is important for the implant's mechanical stability, and facilitate soft tissue attachment [27–29].

The obtained data on the antibacterial activity of metal coatings of titanium disks (Figure 8), which showed the best activity for copper and silver, correspond to data presented in [23,30–32]. Although the silver coating reduced cell viability as compared to non-treated microporous titanium disks, it did not impair the cell adhesion and differentiation [33]. At the same time, use of the copper coating led to a significant increase in the cytotoxic effect of copper ions on both bone tissue cells and FetMSCs, leading to a noticeable change in cellular morphology and cell migration into the pores of the implant (Figures 6 and 7; Tables 2 and 3). Indeed, copper has been shown to have pronounced toxic activity against normal cells of the body, which significantly reduces the possibility of its clinical use. Presumably, the use of nanoformulation of copper composites will reduce the toxic effect [34,35].

To assess the processes of integration with soft and bone tissues, we assessed the expression of matrix metalloproteinase enzymes in vitro. Titanium implants do induce the expression of MMPs during the process of osseointegration [36–38]. Intriguingly, when compared to various metal coatings, the titanium nitride (TiN) coating induced

the highest expression of MMPs in FetMSCs and MG-63 cells (Figure 9). Indeed, as shown by Oliva et al. [39], TiN-coated titanium may modulate inflammation through the inhibition of the TLR4/MyD88/NF- κ B p65/NLRP3 pathway and induce extracellular matrix apposition. In parallel to the enhanced activity of MMPs, we also observed and increased mRNA production of genes related to focal cell adhesion (including FAK, vinculin, α 2, α 5, α V integrins, type I collagen, fibronectin, and vitronectin) in FetMSCs and MG-63 cells (Figure 9). The obtained data is in line with published results showing that TiN coatings also induced in vitro cellular responses with high cell adhesion molecule expression [40,41]. As was shown by Zreiqat et al., interaction between osteoblastic cells and biomaterials results in enhanced activation of Shc and the RAS/RAF/MAPK signaling pathway, as well as upregulation of c-fos signaling pathway [42].

Herein, we performed a comparative study of the most commonly used metal coatings in regard to their bioactive properties. To further increase their biointegrative properties, these coatings can be applied in combination with other bioactive molecules (including growth factors, peptides, etc.) [43–48]. Thus, in the recent study by Jiang et al., it was shown that incorporation of chitosan microspheres loaded with Bone Morphogenetic Protein-2 (BMP-2) and Platelet-Derived Growth Factor-BB (PDGF-BB) into the implant significantly enhanced osteogenic differentiation [49]. Another direction for possible research may be based on the site-selective metal coatings on the surface of the implant with various coatings on the bone-contacting surface and the skin-penetrating part of the DSA implant. Indeed, this approach showed efficacy for orthopedic titanium implants in preclinical studies [27].

5. Conclusions

When choosing the optimal microporous titanium coating for orthopedics, parameters such as antibacterial properties, cytotoxicity, and the induction of biointegration processes with soft and bone tissues should be considered. According to the results of this study, which assessed the mRNA expression of genes associated with the processes of osteogenesis and cell adhesion and studied the activity of matrix metalloproteinases, a silver coating is recommended. If the risk of infection is minimal and does not pose a threat to biointegration with surrounding tissues, the titanium nitride (TiN) coating can be recommended. At the same time, it is worth noting that a comparative analysis of titanium metal coatings was carried out only in vitro, which indicates the need for further preclinical assessments in vivo.

Author Contributions: Conceptualization, M.S. and M.P.; methodology, M.S., D.B., N.Y., A.L., E.B., D.N., I.V., L.S., E.P., E.O., A.K., G.R., M.M., V.M., M.A., S.E.C. and M.P.; software, M.S., D.B., N.Y., A.L., E.B., D.N., I.V., L.S., E.P., E.O., A.K., G.R., M.M., V.M., M.A., S.E.C. and M.P.; validation, M.S., D.B., N.Y., A.L., E.B., D.N., I.V., L.S., E.P., E.O., A.K., G.R., M.M., V.M., M.A., S.E.C. and M.P.; formal analysis, M.S., D.B., N.Y., A.L., E.B., D.N., I.V., L.S., E.P., E.O., A.K., G.R., M.M., V.M., M.A., S.E.C. and M.P.; investigation, M.S., D.B., N.Y., A.L., E.B., D.N., I.V., L.S., E.P., E.O., A.K., G.R., M.M., V.M., M.A., S.E.C. and M.P.; resources, M.S. and M.P.; data curation, M.S., S.E.C., M.A. and M.P.; writing—original draft preparation, M.S., D.B., N.Y., A.L., E.B., D.N., I.V., L.S., E.P., E.O., A.K., G.R., M.M., V.M., M.A., S.E.C. and M.P.; writing—review and editing, M.S., D.B., N.Y., A.L., E.B., D.N., I.V., L.S., E.P., E.O., A.K., G.R., M.M., V.M., M.A., S.E.C. and M.P.; visualization, M.S., D.B., N.Y., A.L., E.B., D.N., I.V., L.S., E.P., E.O., A.K., G.R., M.M., V.M., M.A., S.E.C. and M.P.; supervision, M.S. and M.P.; project administration, M.S. and M.P.; funding acquisition, M.P. All authors have read and agreed to the published version of the manuscript.

Funding: The study is financially supported by Grant R44AR079960 and 1SB1AAR086711 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH, and the Technische Universität München (TUM) within the DFG funding program Open Access Publishing.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding authors, Maxim Shevtsov and Mark Pitkin, on reasonable request.

Conflicts of Interest: Maxim Shevtsov, Danila Bobkov, Natalia Yudintceva, Anastasiya Lukacheva, Ekaterina Bozhokina, Denis Nazarov, Irina Voronkina, Larisa Smagina, Emil Pitkin, Elena Oganessian, and Airat Kayumov declare no conflicts of interest. Mykhailo Matviychuk and Vladimir Moxson report financial support from ADMA Products Inc.; Grigory Raykhtsaum and Mark Pitkin report financial support from Poly-Orth International; Michael Akkaoui reports financial support from Tanury Industries; Stephanie E. Combs reports personal fees and nonfinancial support from Roche Pharmaceuticals, AstraZeneca, Medac, Sennewald Medizintechnik, Elekta, Accuray, Bristol Myers Squibb, Brainlab, Daiichi Sankyo, Icotec AG, Carl Zeiss Meditec, HMG Systems Engineering, Janssen Cilag, and CureVac outside the submitted work. Moreover, the funders had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

BMP	bone mineral density
CaP	calcium phosphate
DSA	direct skeletal attachment
FAK	focal adhesion kinase
FetMSCs	fetal mesenchymal stem cells
HA	hydroxyapatite
ML-ALD	molecular layering of atomic layer deposition
MMPs	Matrix Metalloproteinases
MTT	3-(4,5-di methyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide
PDGF-BB	Platelet-Derived Growth Factor-BB
RT-PCR	Real-Time Polymerase Chain Reaction
RTQ	removal torque tests
SBIP	skin- and bone-integrated pylon
SEM	scanning electron microscopy
SLM	selective laser melting
SMAD4	SMAD family member 4, Mothers against decapentaplegic homolog 4
TGF- β 1	Transforming growth factor beta
TiN	Titanium Nitride
TMRM	Tetramethylrhodamine-methylester
ZrN	Zirconium Nitride

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