The osteogenic activity of strontium loaded titania nanotube arrays on titanium substrates

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1. Introduction

Mesenchymal stem cells (MSCs) play a crucial role in bone regeneration and bony fixation of implanted biomaterials. Most of the osteoblastic cells that colonize the implant surface to induce bone growth originate from MSCs [1], and hence, in order to accomplish good osseointegration, it is critical to induce the differentiation of MSCs preferentially toward osteogenitor cells and then into osteoblasts in lieu of other cell lineages. Titanium (Ti) implants are commonly used in orthopedics and dentistry, but the metallic Ti surface fails to preferentially induce MSC commitment toward osteoblasts compared to other cell lineages to generate better osseointegration, and improvement is thus needed to meet the demand for better clinical performance. Surface nano-topography such as TiO2 nanotube (NT) coatings have been shown to alter cell behaviors such as adhesion, orientation, cell differentiation, and migration significantly [2–4] due to their similar dimensional scale with bone collagen fibrils and elasticity.
resembling that of bones [5,6]. In addition, the NT also constitutes an excellent drug delivery platform, particularly for inorganic bioactive elements such as silver and trace elements. They are stable and functional at low doses and can potentially generating long-lasting delivery effects [7–9].

Strontium (Sr) has aroused tremendous clinical interests especially after the development of the anti-osteoporosis drug strontium ranelate (SR) which exhibits pronounced effects to decrease the bone fracture risk in osteoporotic patients [10,11]. The favorable effects which have been observed from osteoporotic and normal animals [12,13] are closely related to the modulation of bone turnover toward osteogenesis [14–16]. Sr increases osteoblast replication, differentiation, and bone matrix mineralization probably via a calcium sensing receptor (CaR) dependent mechanism [14,16–18]. Sr has also been recently reported to direct the MSC commitment to the bone lineage via the Wnt/β-catenin and MAPK pathways as well as induction of cyclooxygenase (COX)-2 and prostaglandin E2 expressions while simultaneously repressing their commitment to other lineages such as adipocytes [19–23]. On the other hand, Sr can inhibit bone resorption by reducing osteoclast differentiation and resorbing activity, and induce osteoclast apoptosis through a CaR dependent mechanism [14,16,24]. Oral administration of Sr has been reported to effectively enhance bone implant fixation in osteoporotic and intact rats [25,26], but constant in situ Sr release of a proper dose directly to implant—tissue interface gives rise to better results while avoiding the potential deleterious effects associated with oral administration at high concentrations [8]. Sr has been experimentally incorporated into biomaterials such as calcium phosphates and bioactive glasses to enhance the bone formation [18,27–30]. Nonetheless, because the solubility of calcium phosphates and bioglasses changes appreciably with the amounts of Sr [31], it is difficult to achieve long-lasting Sr release at a reasonably constant rate. In this respect, a more efficient delivery platform with controlled Sr release is of clinical interest.

We have fabricated a Sr loaded nanotubular structure (NT-Sr) on Ti implants by transforming the NT into SrTiO3 while retaining the nanotubular structure [8]. This Sr releasing NT-Sr coating is expected to expedite osteointegration especially in osteoporotic patients. Although a low dose of Sr benefits bone formation, an excessively high dose may induce toxicity thereby posing deleterious effects on bone mineralization [16,31,32]. Hence, an NT-Sr coating with the optimal dose and proper Sr release is required for osteointegration. Recent investigations suggest that the NT diameter could affect the activity of bone cells and an optimal diameter is thus required for cell functionality. In this work, a series of NT and NT-Sr samples with large and small tube sizes as well as high and low Sr release amounts are prepared and the influence of the NT diameter and Sr contents on rat MSC functionalities in the absence of exogenous osteogenic factors (OS) are investigated systematically.

2. Materials and methods

2.1. Sample synthesis and characterization

Ti foils (99.7% pure, Aldrich) with the size of 10 × 10 × 1 mm3 were first polished by SiC sandpaper and then ultrasonically cleaned with acetone, ethanol, and deionized water sequentially. NT0 and NT40 were fabricated by electrochemical anodization in an ethylene glycol solution with 0.5 wt% NH4F, 5 vol% CH3OH, and 5 vol% H2O at 10 V for 1 h and 40 V for 40 min, respectively. Afterward, NT10 and NT40 were placed in 40 ml of 0.02 M Sr(OH)2 solution in a 60 ml Teflon-lined autoclave and heated at 200 °C for 1 and 3 h to obtain the NT-Sr samples. Then the specimens were ultrasonically washed with 1 M HCl for 5 min to remove residual Sr(OH)2, rinsed with distilled water, and dried in air. The samples were characterized by field-emission scanning electron microscopy (FE-SEM, HITACHI S-4800), X-ray diffraction (XRD, Philips X’ Pert Pro) with Cu Kα radiation, micro Raman spectroscopy (Renishaw inVia) and X-ray photoelectron spectroscopy (XPS, ESCALAB MK-II). Water contact angle measurements were carried out by the sessile-drop method (EasyDrop Standard, KRiUSS).

2.2. Sr release determination

The NT-Sr samples were immersed in 5 ml of phosphate buffered saline (PBS) for 1 day, taken out, and then immersed again in 5 ml of fresh PBS. This process was repeated for a total of 1 month to generate solutions at different time points in order to determine the Sr release time profile. The PBS solution containing released Sr was analyzed by inductively-coupled plasma atomic emission spectrometry (ICP-AES, IRIS Advantage ER/S). The NT-Sr layers on the Ti surfaces were completely dissolved in the HF and HNO3 mixture when ultrasonically treated at room temperature for 5 min. The total amounts of Sr were determined by ICP-AES.

2.3. Protein adsorption assay

The protein adsorption assay was conducted in n-MEM containing 10% fetal calf serum (FCS). After incubation in the medium for 2 h at 37 °C, the proteins adsorbed onto the samples were detached by 1% sodium dodecyl sulfate (Solvesso) and determined using a MicroBCA protein assay kit (Pierce). The protein adsorption pattern after incubating for 2 days in n-MEM containing 10% FCS was inspected by FE-SEM.

2.4. Cell cultures, cytotoxicity, cell adhesion and proliferation

MSCs and green fluorescein sodium (GFP) labeled ones were obtained from Sprague–Dawley rats (Lab Animal Center, the Fourth Military Medical University) and GFP transgenic rats (Rat Resource & Research Center, the University of Missouri) respectively and the detailed cell isolation procedures are described in Ref. [14]. The cells were cultured in n-MEM containing 10% FCS at 37 °C with the medium changed every three days. The cells of passage 2–4 were used in the experiments. The samples were placed in 24 well plates (Costar) and the MSCs were seeded at a density of 4 × 104/well for the cell adhesion assay and 2 × 105/well for other assays unless other mentioned. For the cell adhesion assay, after incubation of 30, 60 and 120 min, the attached cells were stained by 4′,6-diamidino-2-phenylindole (DAPI, Sigma). Images were captured from several random fields by a fluorescence microscope (Leica) and the cell number in each field was counted. The activity of lactate dehydrogenase (LDH) in the culture media released by the cells was used as an index of cytotoxicity. After culturing for 3 days, the culture medium was collected (among this period the medium was not changed) and centrifuged, and the LDH activity in the supernatant was determined spectrophotometrically according to the manufacturer’s instruction (Sigma). In order to assess cell proliferation, the cells were cultured on samples for 1, 3 and 7 days and the cell numbers were assessed using the Cell Counting Kit-8 assay (CCK-8, Beyotime).

2.5. Cell morphology

The cell morphology and details of cell/biomaterial interaction were studied by FE-SEM and fluorescence image of the GFP labeled MSCs. After incubation for 2 days in n-MEM containing 10% FCS, the samples with attached cells were fixed in 3% glutaraldehyde, dehydrated in a graded ethanol series, freeze-dried, sputter coated with gold, and observed by high-resolution FE-SEM. The GFP labeled MSCs were inoculated at a density of 1 × 106/well, and after 2 h, 3 and 8 days, the images were captured by fluorescence microscopy.

2.6. Cell migration

In the wound healing assay experiments, the GFP labeled MSCs were cultured on samples at a density of 1.2 × 104/well for 18 h to reach confluence. The monolayers were wounded with a plastic pipette. After washing with PBS, the cells were incubated for another 2 days and then observed as aforementioned.

2.7. Quantitative reverse transcription polymerase chain reaction (qRT-PCR), alkaline phosphatase (ALP) and mineralized nodule staining

MSC osteogenic differentiation was assessed. The expression levels of osteogenesis related genes were measured using the qRT-PCR. The cells were seeded at a density of 2 × 104 cells/well, cultured for 2 weeks and harvested using TRIzol (Invitrogen) to extract the RNA. The obtained RNA was reverse transcribed into complementary DNA (cDNA) using PrimeScript RT reagent Kit (Takara) and the qRT-PCR analysis was performed on the Bio-Rad C1000 using SYBR Premix Ex Taq II (Takara). The primers for the target genes are listed in Table 1. The expression levels of the target genes were normalized to that of the housekeeping gene GAPDH. ALP staining was performed with the BCIP/NBT alkaline phosphatase color development kit (Beyotime) after culturing for 5 and 10 days. After culturing for 2 weeks, extra-cellular matrix (ECM) mineralization by the cells on the samples was assessed by Alizarin Red staining [33,34].
2.8. Statistical analysis

The data were collected from three separate experiments and expressed as means ± standard deviation. The one-way ANOVA and Student-Newman-Keuls post hoc tests were used to determine the level of significance and p values less than 0.05 and 0.01 were considered to be significant and highly significant, respectively.

3. Results and discussion

3.1. Synthesis and structure characterization

The NT samples produced by anodization at 10 and 40 V (NT10 and NT40) are subjected to a hydrothermal treatment for 1 and 3 h to form different Sr-containing NT samples (denoted as NT10-Sr1, NT10-Sr3, NT40-Sr1, and NT40-Sr3). SEM images indicate that NT10 and NT40 have diameters of 30 and 80 nm (Fig. 1B). In particular, NT10 contains multiple nanocues comprising bundles of 30 nm nanotubes with bundle diameters between 100 and 400 nm separated by 80 nm wide cracks. The NT10 structure is similar to the highly ordered hierarchical structure of the natural bone ECM and good biological performance is therefore expected. The top ends of 80 nm nanotube wall on NT40 are not completely smooth and have flakes. After the hydrothermal treatment, the nanotubular architecture is preserved but the tube diameters decrease with treatment time due to volume expansion from the transformation of TiO2 into SrTiO3. The flakes on top of the nanotube wall of NT40 are blunter on NT40-Sr1 but smoother on NT40-Sr3. During the hydrothermal process, the surface TiO2 dissolves and react to form SrTiO3 and the dissolution and re-recrystallization processes make the sample surface smoother.

The uneven protein distribution on NT40 and NT40-Sr can be attributed to the nonsmooth top ends of the nanotube wall with protein agglomerates covering the tubes. The proteins are distributed relatively evenly along the nanotopography. NT40 and NT40-Sr induce more protein deposition and this is in good agreement with the assay results in Fig. 4A. Unexpectedly, the adsorbed proteins are not distributed evenly but rather form pillars, especially on NT40 on which tall protein pillars with relatively small top dimensions mostly <50 nm are distributed at certain distances from each other. The uneven protein distribution on NT40 and NT40-Sr can be ascribed to the nonsmooth top ends of the nanotube wall with flakes, which provide local nucleation sites for protein aggregation. The proteins on NT40-Sr are more even because the top ends of the nanotube wall are smoother, but they are still uneven compared to NT10 and NT10-Sr. The nanotubes formed in an inorganic electrolyte have a flat top consequently inducing a uniform distribution of proteins [4]. Hence, even subtle changes in the nanotopography can lead to dramatic alteration in the protein deposition pattern. Because the immediate substrate to that cells interact is the adsorbed proteins, the notably uneven protein deposition will make the ultimate topographical cues exposed to cells quite differently from the primitive nanotopographies thereby altering the biological performance. This issue will be discussed in more details later in this paper.

3.2. Sr release

The Sr release kinetics is assessed by immersing the NT-Sr in 5 mL of PBS for as long as 1 month, and as shown in Fig. 3A, the released Sr amounts at various time slots follow the order of NT40-Sr3 > NT40-Sr1 > NT10-Sr3 > NT10-Sr1. There are two possible mechanisms by which Sr is released from the NT-Sr samples, namely dissolution of strontium titanate and ion exchange between Sr ions in strontium titanate and hydrogen ions in the surrounding solution. Generally, NT-Sr shows an initial Sr release burst but four days later, the released Sr amounts are relatively constant and in fact exhibit a slight decline. The average Sr amounts released from NT10-Sr1, NT10-Sr3, NT40-Sr1, and NT40-Sr3 daily are 0.025, 0.039, 0.042, and 0.053 ppm, respectively, with the exception of the burst release at day 1. The total Sr contents leached from the four samples are 45.48, 54.95, 88.20, and 118.00 µg based on the 1 cm² coatings, respectively (Fig. 3B). Theoretically, Sr may be delivered from the NT-Sr for a period longer than 1 year.

3.3. Protein adsorption

The proteins adsorbed on the NT and NT-Sr samples are investigated because they play a crucial role in conveying the biological effects of the topographical cue [35]. In comparison with the pristine Ti control, the NT samples adsorb more proteins and NT40 absorbs even more (Fig. 4A). The adsorbed protein amount depends mainly on the nanotopography irrespective of Sr incorporation, even though Sr incorporation alters the nanotopography to some degree. Besides the amount, the species, conformation, and orientation of the adsorbed proteins have been reported to influence the cell/biomaterials interaction [35]. The details of protein adsorption are examined by FE-SEM and the results are displayed in Fig. 5. On NT10 and NT10-Sr, there are abundant proteins on the top ends of the nanotube bundles along the nanotube walls or protein aggregates covering the tubes. The proteins are distributed relatively evenly along the nanotopography. NT40 and NT40-Sr induce more protein deposition and this is in good agreement with the assay results in Fig. 4A. Unexpectedly, the adsorbed proteins are not distributed evenly but rather form pillars, especially on NT40 on which tall protein pillars with relatively small top dimensions mostly <50 nm are distributed at certain distances from each other. The uneven protein distribution on NT40 and NT40-Sr can be ascribed to the nonsmooth top ends of the nanotube wall with flakes, which provide local nucleation sites for protein aggregation. The proteins on NT40-Sr are more even because the top ends of the nanotube wall are smoother, but they are still uneven compared to NT10 and NT10-Sr. The nanotubes formed in an inorganic electrolyte have a flat top consequently inducing a uniform distribution of proteins [4]. Hence, even subtle changes in the nanotopography can lead to dramatic alteration in the protein deposition pattern. Because the immediate substrate to that cells interact is the adsorbed proteins, the notably uneven protein deposition will make the ultimate topographical cues exposed to cells quite differently from the primitive nanotopographies thereby altering the biological performance. This issue will be discussed in more details later in this paper.

3.4. Cytotoxicity, cell adhesion, and proliferation

Initial cell adhesion is the key step for the ensuing cell proliferation and differentiation on biomaterials [36]. As shown in Fig. 4B and Fig. 5A, the big or small NT samples do not induce obvious difference in the initial adherent cell number and this is consistent with our previous observation from NT formed in an inorganic electrolyte [4,37]. The available evidence demonstrates that the NT samples have a negligible effect on the initial adherent cell number. After Sr incorporation, NT10-Sr1, NT10-Sr3, and NT40-Sr1 still show no obvious difference in the initial adherent cell number in contrast with the Sr free counterparts, whereas NT40-Sr3 induces
a larger adherent cell number at 1 h. Panzavolta et al. show that Sr incorporation has a slight positive effect on the early adherent cell number [38], while Park et al. find that Sr incorporated Ti–6Al–4V fabricated by a hydrothermal process lead to no difference in the initial attached cell numbers [30]. Hence, Sr incorporation has no obvious influence on the initial adherent cell number. The variation shown in different reports may be explained by the different Sr amounts and release kinetics.

Since Sr overdose may lead to cytotoxicity [31], the LDH released by cells when co-cultured with the samples is evaluated as an indication of cytotoxicity. As shown in Fig. 4C, all the NT and NT-Sr samples exhibit no cytotoxicity compared to the Ti control and Sr

Fig. 1. (A) Schematic showing that the NT-Sr coating, combining the effect of Sr and the nanomorphology of NT, dramatically promotes MSC spreading and induces MSC selective differentiation toward osteoblasts. (B) FE-SEM images of NT and NT-Sr.
amounts released from all the NT-Sr samples are safe. The LDH release from NT10-Sr is slightly smaller than that from the Ti control, indicating even better cytocompatibility on NT10-Sr. The good cytocompatibility is further verified by the good attachment, spread and proliferation of cells after incubation for 8 days as shown by the FE-SEM and fluorescence pictures in Figs. 6 and 7A.

Cell proliferation is assessed by CCK-8 and as shown in Fig. 4D, the cells on all the samples proliferate with time. Cell proliferation on NT is obviously lower than that on the Ti control, and NT10 and NT40 induce similar levels of cell proliferation. NT10-Sr and NT40-Sr show better cell proliferation than NT10 and NT40, except at day 7, NT10-Sr, NT10 and NT40 generate similar cell proliferation. Cell proliferation on NT40-Sr is similar to that on the control, whereas that on NT10-Sr is still lower than the control. The reduced cell proliferation on NT10, NT40, and NT10-Sr cannot originate from cytotoxicity which can be ruled out based on the LDH assay and so can be partly attributed to the extraordinary extension and intimate attachment of cells on them, especially on NT10-Sr, because
detachment from the substrate is needed for cells to undergo division and excessively strong adhesion will hinder this process and cell proliferation. Another potential reason is the osteogenic differentiation tendency of MSCs for the reciprocal relationship between cell proliferation and differentiation [39]. On NT40, the compromised cell proliferation may be ascribed to the protein nanopillars which compromise cell focal adhesion and proliferation. Previous reports have demonstrated the dose dependent effects of Sr incorporation in cell proliferation [29,40] and our results are consistent. The enhanced cell proliferation by NT10-Sr in contrast to NT10 should be mainly related to Sr, but the higher cell proliferation on NT40-Sr than NT40 is the combined effects of Sr and smoothing of the nanotube wall. Even though the cells on NT40-Sr also show the osteogenic differentiation tendency, the larger amount of released Sr still within the safe dosage range, relatively loose cell attachment to the substrate, and smaller cell spread area give rise to relatively high cell proliferation compared to NT10-Sr.

![Graphs and images showing protein adsorption and initial adherent MSC number over time, LDH amount released, and cell proliferation over culture days.](image)

**Fig. 4.** (A) Protein adsorption to the samples after 2 h of immersion in α-MEM containing 10% FCS, (B) Initial adherent MSC number at 0.5, 1 and 2 h measured by counting cells displayed with DAPI, (C) LDH amount released by cells during the first 3 days of incubation and (D) cell proliferation measured by CCK-8 after 1, 3 and 7 days of culture. *, **p < 0.05 or 0.01 vs Ti, #, ##p < 0.05 or 0.01 vs NT10, and %, %p < 0.05 or 0.01 vs NT40.

**Fig. 5.** Protein adsorption pattern after 2 days of incubation in α-MEM containing 10% FCS.
3.5. Cell morphology

The cell morphology is closely related to the cell fate [41]. At 2 h, the cells do not spread completely and there is no discernible difference in the cell morphology (Fig. S4). However, after complete spreading, dramatic differences in the cell shape can be observed (Fig. 6). The MSCs on the flat Ti spread poorly into a spindle shape indicative of undifferentiated quiescent cells. On the other hand, those on NT10 are more extended exhibiting a typical polygonal osteoblastic shape, but cell spreading is not promoted appreciably on NT40. Longer incubation reveals that the effects on the cell morphology are long lasting, as shown in Fig. 7A and Fig. S4. At first glance, the results appear to contradict our previous observation that both NT samples (30 and 80 nm) fabricated in an inorganic electrolyte show notable promoting effects on MSC adhesion and spread [4]. Regarding the distribution of the adsorbed proteins, the present results actually are intrinsically consistent with our previous observation and they can be explained by cell/nano-topography interactions. Stable cell adhesion relies on the formation of focal adhesion involving clustering of ligated integrins and

Fig. 6. SEM views of cells after 2 days of culture on different samples: (a) Ti, (b) NT10, (c) NT10-Sr1, (d) NT10-Sr3, (e) NT-40, (f) NT40-Sr1 and (g) NT40-Sr3.
requiring a distance smaller than 50–70 nm between two neighboring integrins. A distance beyond that will impede integrin clustering and focal adhesion assembly [4]. With regard to NT10, although focal adhesion will be interrupted by the 80 nm cracks and constrained to the top area of the nanotube bundles, the abundant and relatively evenly distributed nanoscale proteins on the bundles allow the formation of high quality focal adhesion to foster good cell adhesion, as clearly indicated in Fig. 6b3. However, for NT40, the relatively sparsely distributed nanoscale protein pillars, to some extent like the quasi-aligned nanowire arrays previously reported by us [42], do not favor cell adhesion, because the focal adhesion will mostly and maybe even totally be constrained to the small top end of the protein pillars, thus resulting in compromised cell adhesion and spreading. The cell mobility assay results in Fig. 7B further demonstrate the impairing effect of NT40 on cell functions. The cell mobility on NT40 is severely impaired and it becomes better on NT40-Sr possibly due to the more even protein distribution. Our study demonstrates that the protein deposition pattern can be influenced by the nanotopography which in turn can affect the biological performance of biomaterials.

Incorporation of Sr into the NT promotes cell spreading as shown in Figs. 6, 7A, and Fig. S4. Further improvement in cell spreading on NT10-Sr compared to NT10 is attributed to Sr incorporation. With regard to the better cell spreading on NT40-Sr relative to NT40, besides Sr incorporation, smoothing of the nanotube wall is also a factor. As shown in Fig. 6f3 and g3, the more evenly distributed proteins on NT40-Sr, especially on NT40-Sr3, leads to closer cell adhesion. Nonetheless, the cell spreading on NT40-Sr is still worse than that on NT10-Sr. In addition, an interesting phenomenon is that the cells on NT40-Sr have a thin cell body but that on NT10-Sr is thicker, as shown in Fig. 6. The abnormally thin cell body on NT40-Sr is due to two factors, the Sr incorporation and the uneven protein distribution. Sr incorporation enables a larger spread area, but the unevenly deposited proteins give rise to smaller and fewer focal adhesions allowing fewer skeleton fibers to be linked to them. As a result, a thin cell body occurs. On the contrary, NT10-Sr allows formation of more and bigger focal adhesions that translate into more skeleton fibers anchored to them and eventually a thicker cell body. Sr incorporation into biomaterials has been previously observed to promote spreading of osteoprecursor cells and osteoblasts [18,31,43]. Our study definitively confirms the robust effect of Sr incorporation on MSC adhesion and spreading, while complete understanding of the underlying mechanism still needs further studies.

3.6. Osteogenic differentiation

It has been reported that well spread MSCs are inclined to undergoing osteogenesis [41], and thus the effects of NT-Sr on inducing fast and good spreading of MSCs bode well for the osteogenic ability. The surrogate makers for osteogenic differentiation of MSCs cultured on the samples are studied in the absence of OS. The gene expression level analysis results in Fig. 8 show that NT10-Sr3 and NT40-Sr significantly promote the expressions of osteogenesis related genes including the runt-related transcription factor 2 (RUNX2, a key transcript factor for bone formation), ALP (an early marker for osteogenic differentiation), osteocalcin (OCN, a late marker for osteogenic differentiation), type 1 collagen (Col-1, main content of bone ECM), and bone morphogenic protein 2 (BMP-2), demonstrating excellent osteogenic activity. In contrast, NT10-Sr1 promotes the expression of ALP, Col-1, and BMP-2 to a much lesser degree. The

Fig. 7. Fluorescence images showing: (A) morphology of GFP labeled MSCs after 8 days of culture and (B) cell migration on different samples.

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topographical cue modulates the cell shape and focal adhesion and alters the indirect mechanotransduction, namely the integrin dependent signal pathways such as MAPK and the direct one in which gene expressions originate from cell nuclei distorted by cytoskeletal tension \[4,44,45]\). Sr has been found to modulate the Wnt/β-catenin and MAPK pathways in MSCs to initiate osteogenic differentiation \[19,20]\). Although NT10-Sr3 and NT10-Sr1 have similar topography and cell spreading area, there is a dramatic difference in the gene expression of the MSCs cultured on these two samples. NT10-Sr3 shows striking effects but NT10-Sr1 displays a weak effect. The observation is in line with the Sr release trend shown in Fig. 3. In addition, the NT40-Sr samples that exhibit relatively smaller cell spreading induce similar high gene expression than NT10-Sr3 as a result of the large Sr release. The present results indicate that the released Sr has a stronger effect on initiating MSC osteogenic differentiation than the topography and cell area. Actually, the strong effect of Sr to induce MSC osteogenic differentiation has been reported by Choudhary et al. \[21\] and previous studies indicate that the osteogenesis inducing effect of topography may be limited \[4,46\].

Fig. 8. Relative expressions of RUNX2, ALP, OCN, Col-1 and BMP-2 by MSCs cultured on different substrates for 2 weeks, all values normalized to GAPDH. *, **p < 0.05 or 0.01 vs Ti, #, ##p < 0.05 or 0.01 vs NT10, and %, %%p < 0.05 or 0.01 vs NT40.
To further verify the Sr-induced enhanced osteogenic activity, the production of ALP and ECM mineralized nodules is investigated (Fig. 9). As early as 5 days during incubation, the cells already produce a certain amount of ALP, as shown in Fig. S5. After culturing for 10 days, there are large nodular ALP positive areas on NT10 and NT10-Sr. The NT10-Sr samples induce more ALP than NT10 and Ti control, especially NT10-Sr3 although the amounts of ALP on NT40 and NT40-Sr are much less. After incubation for 2 weeks, the mineralized nodules are clearly observed from NT10 and bigger ones appear on the NT10-Sr samples, but no discernible mineralized nodule is found on NT40, NT40-Sr, or flat Ti. It is quite unexpected that the NT40-Sr samples do not show high ALP production and fail to induce mineralized nodules. These results are different from the gene expression analysis results and may be attributed to the uneven protein distribution. Our results indicate that the nanotopography also plays an important role in the biological performance of NT-Sr, even though Sr induces stronger osteogenic signaling. The structure of NT10 allows high-quality focal adhesion formation to support cell spreading and induces big nodular ALP products and mineralized nodules on account of the suitable multiple nanoscale structure. It can be further enhanced by Sr release, especially NT10-Sr3. Kaur [47] has also demonstrated the synergistic effects of nanotopography provided by tobacco mosaic virus and phosphate and on osteogenic differentiation of MSCs. On the contrary, NT40, due to the protein nanopillar topography, triggers unfavorable signaling in cell functions, shows small ALP production, and fails to induce mineralized nodules. Further Sr loading and release (NT40-Sr) cannot retrieve the cell functions. On account of the uneven protein distribution, the cells on NT40-Sr have an abnormally thin body and the protein protrusions seem to break into the cell body thus impairing cell functions and potentially inducing apoptosis. In brief, on NT40-Sr1 the protein nanopillars generate unfavorable signaling and thus bad results, but on NT10-Sr3, the multiple nanoscale structure that facilitates good cell spreading and proper Sr release synergistically give rise to good osteogenic activity.

4. Conclusion

NT-Sr coatings with different tube diameters and Sr release rates have been fabricated. Long-lasting and controllable Sr release is observed from the NT-Sr samples with no cytotoxicity. In particular, sample NT10-Sr3 shows the best effects in inducing MSC osteogenic differentiation due to the proper Sr release amount and suitable multiple nanoscale structure with even protein adsorption favoring cell functions. The NT-Sr gives rise to good osteogenic activity without the need to apply foreign complex biomolecules. The materials are easy to fabricate and have good stability that facilitating large-scale industrial production, storage, transport, sterilization and clinical use. They are very attractive to bone implants and clinical applications.

Acknowledgments

This work was supported by National Natural Science Foundation of China Nos. 50902104 and 31200716, Hong Kong Research Grants Council (RGC) General Research Funds (GRF) No. 112510, and the Opening Project of State Key Laboratory of High Performance Ceramics and Superfine Microstructure (SKL201109SIC). Lingzhou Zhao also thanks the grants from The Fourth Military Medical University and School of Stomatology, The Fourth Military Medical University.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2012.09.041.

References

[1] Tasso R, Fais F, Reverberi D, Tortelli F, Cancedda R. The recruitment of two consecutive and different waves of host stem/progenitor cells during the


