Investigating the interplay between environmental conditioning and nanotopographical cueing on the response of human MG63 osteoblastic cells to titanium nanotubes

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Supplementary Information

Nanotubes Fabrication

In accordance with the procedures outlined in previous work from our group[36], we carried out a comparison between a 2-step (biphasic) and 3-step (triphasic) anodization process to assess whether the degree of bifurcation can be reduced, thereby generating more consistent nanotubular surfaces. To this end, we considered two sets of parameters, which differed in the initial voltage used and the number of anodization steps. In particular, with the biphasic (-B) procedure, NT30-B samples were fabricated using a constant voltage of 30 V. The initial anodization step lasted for one hour, followed by a second step of five minutes. Conversely, NT60-B samples were subjected to a constant voltage of 60 V, where the first anodization step lasted for 30 minutes and the subsequent step for five minutes. For the triphasic (-T) procedure, the initial anodization step was repeated, resulting in two instances of oxide layer stripping, designated as NT30-T and NT60-T. The experimental parameters adopted for this comparison are summarized in Table 1S.

Table S1. Voltage and time parameters for anodization of NT30-B, NT30-T, NT60-B and NT60-T substrates.

	Condition	NT30-B	NT30-T	NT60-B	NT60-T
Step 1	Voltage (V)	30	30	60	60
	Time (Min)	60	60	30	30
Step 2	Voltage (V)	30	30	60	60
	Time (Min)	5	60	5	30
Step 3	Voltage (V)	/	30	/	60
	Time (Min)	/	5	/	5

By leveraging the hexagonal packing of the nanotubes, a ratio was formulated between the NND and the average distance of the first six NNDs (N1:N6 ratio). This ratio was then used to determine the degree of bifurcation and level of order. In addition, Voronoi tessellation[36,85,86] was applied to the SEM micrographs to create an experimental planar tiling, which served as a qualitative image to compare the change in the degree of order between the bi- and tri-phasic anodization. As evidenced by the N1:N6 ratio, the triphasic samples displayed higher order when compared to their biphasic counterparts (Figure S1a). The distribution plots, illustrated in Figure S1b, highlighted the bifurcation levels of the biphasic surfaces through two distinct clusters of N1:N6 ratios.

Notably, the triphasic treatment effectively eliminated the second distribution cluster, thereby augmenting the order and consistency of the triphasic surfaces, as it is also visible from qualitative Voronoi tessellation of SEM images (Figure S1c).

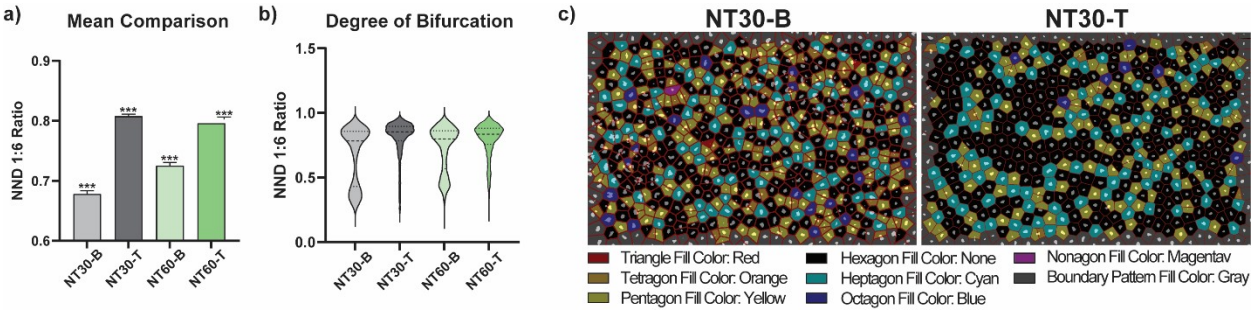

Figure S1. Comparison of NND 1:6 ratios of (a) mean values and (b) distribution plots indicating divergence in degree or order in biphasic samples. Qualitative Voronoi tessellation (c) provides visual representation of this increase in the order of topographical features.

Preconditioning

A noticeable decrease in PrestoBlue intensity (Figure S2a) was observed post-preconditioning in high glucose (HG), in comparison to cells cultured under standard conditions, i.e. pre-conditioned in low glucose (LG) and cultured in low glucose. Interestingly, this effect appears reversible, as metabolic rates for cells grown in LG but seeded in HG (LinH), and cells preconditioned in HG but seeded in LG (HinL), situate between the readings for LG and HG samples. By day 14, ALP activity (Figure S2b) was substantially elevated compared to LG conditions. LinH samples trended toward the HG samples, while HinL was indistinguishable from the LG samples. The mineralization process was notably influenced under the two different regimes, with a significant uptick in cell mineralization for cells grown in LG versus those in high, via Alizarin assay and staining (Figure S2 c-d). Similar inhibitory effects of HG on behaviour were observed in quantitative results of scratch assays (Figure S2 e-f), with the highest rates of wound closure are present in LG environments, with the greatest reduction in migratory behaviour existing in the HG regimes.

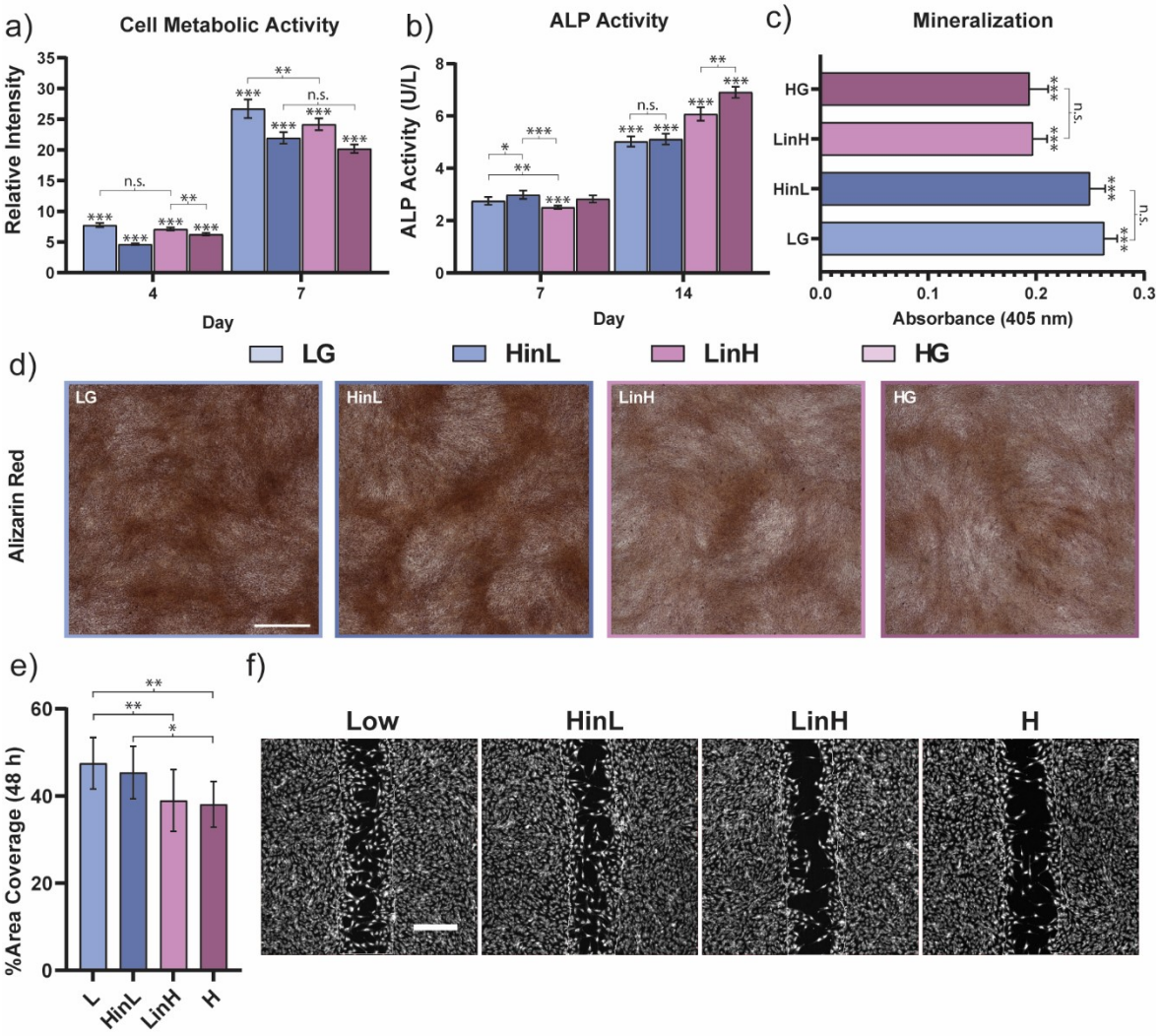

Figure S2. Preconditioned cells were analyzed for changes in (a) metabolic activity (PrestoBlue), (b) ALP Activity and (c) degree of mineralization via Alizarin Red Staining. (d) Widefield images of MG63 cells stained with Alizarin Red Staining. Migration assay analysis for (e) preconditioned MG63 cells grown in culture plates, showing total coverage after 48 h. (f) Representative widefield images of live cells subjected to scratch assay and stained with CellTracker Red CMPTX fluorescent dye and allowed to migrate for 48 h.

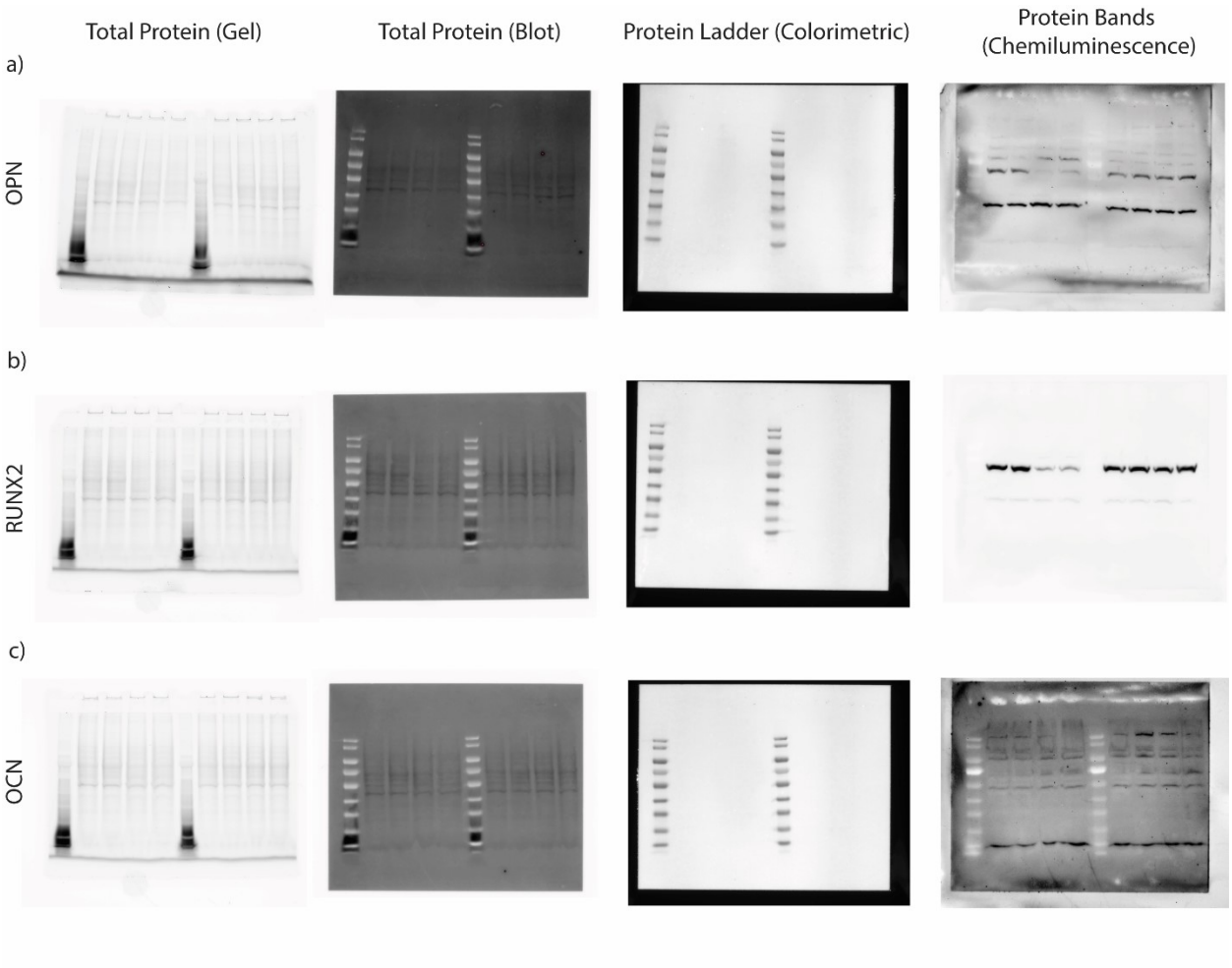

Figure S3. Uncropped and unprocessed images of full gel and blot for Western blot (left to right) total protein (gel), total protein (blot), protein ladder (PL) (colorimetric), protein bands (chemiluminescence) for (a) OPN (~35 kDa), (b) RUNX2 (~60 kDa), and (c) OCN (~15 kDa). Band order for all images (left to right): PL, Ti (LG), NT1 (LG), NT2 (LG), HC (LG), PL, Ti (HG), NT1 (HG), NT2 (HG), HC (HG).