Supplementary Information

The Effect of 3D Hydrogel Scaffold Properties on Osteoblast Differentiation and Mineralization Revealed by Combinatorial Screening

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Discussion of Live/Dead, Wst-1, Picogreen and Alkaline Phosphatase Assays:

Introduction: Three standard assays for measuring cell viability/number (Live/Dead, Wst-1 and Picogreen) were used. Since each has its own strengths and weaknesses, all three were performed for a more robust analysis.

Live-Dead staining is a semi-quantitative or qualitative measure of cell viability and number. The Dead stain labels nucleic acids of cells with leaky membranes (membranes rapidly breakdown when cells die). The Live stain accumulates in cells with intact membranes that have active esterase enzymes (both requisite of live cells). Note that esterase activity may vary with cell proliferation/differentiation state in addition to viability state. Also note that intact gels are required for this assay which means that diffusion of the Live-Dead stains into the gels may not be uniform. The advantage of the Live-Dead assay compared to Wst-1 and Picogreen is that Live-Dead yields images which are invaluable for general assessment of cell distribution, morphology and viability in the gels (neither Wst-1 nor Picogreen assays yield images). Live-Dead is also a more immediate indicator for loss of viability than Picogreen since esterase activity is lost before DNA degrades.

Wst-1 assays measures cellular metabolic activity (dehydrogenase) which may vary with cell proliferation/differentiation state in addition to viability state. In addition, gels and cells must be intact for this assay (all activity was lost if specimens were dounce-homogenized). Thus, diffusion of the Wst-1 reactant into the gels may not be uniform. However, Wst-1 is quantitative. Wst-1 is also a more immediate indicator for loss of viability than Picogreen since dehydrogenase activity is lost before DNA degrades.

Picogreen assay measures the amount of DNA. Note that amount of DNA may vary with cell proliferation/differentiation state in addition to with viability state. Also note that gels were homogenized for this assay to enable DNA extraction (this is the only assay of the three cell number/viability tests where gels were homogenized). Thus, non-uniform diffusion of stains or reactants into gels is not a concern. In addition, Picogreen is quantitative. However, dead cells may contribute to the Picogreen signal since it can take time for DNA to degrade.

Comparison of Live/Dead, Wst-1 and Picogreen: There is a consistent trend of decreasing cell viability/number with increasing culture time and increasing gel modulus as measured by Live-Dead, Wst-1 and Picogreen (Fig. 4). However, some subtle differences exist between the data from the three assays. For instance, the 1 d data for Picogreen shows no change in cell number with increasing modulus while Live-Dead and Wst-1 show a decrease in cell viability/number with increasing modulus for 1 d. Since DNA can take longer than 1 d to degrade, the Picogreen assay detects the presence of the dead cells after 1 d. However, esterase and dehydrogenase activity of dead cells will rapidly drop causing the Live-Dead and Wst-1 assays to detect a decrease in cell viability/number after 1 d in the high modulus gels. In sum, the three bioassays of Live-Dead, Wst-1 and Picogreen provide complimentary data that prove a more complete view of cell viability/number in the hydrogel gradients. There is a clear opportunity for the development of improved methods for quantifying cell viability/number in hydrogels.

Normalization of Alkaline Phosphatase Data: Gene expression results such as alkaline phosphatase assays are typically normalized to cell number data to yield "expression level per cell" (Franceschi et al., 1992; Choi et al., 1996; Lee et al., 2006). This makes it possible to determine i) if increased expression levels are a result of cell proliferation where "expression per cell" does not change or ii) if individual cells are expressing higher overall levels of the gene. Herein, alkaline phosphatase results were normalized to DNA in Fig. 4d. Alkaline phosphatase was normalized to DNA because the Picogreen assay has a calibration standard curve. The standard curve makes it possible to compare more directly data from assays run on different days. The alkaline phosphatase data can also be normalized to the Wst-1 metabolic assay as shown in Fig. S3. The Wst-1 metabolic assay does not have a standard calibration curve which makes comparisons of Wst-1 assay data run on different days less comparable. For these reasons, alkaline phosphatase was normalized to DNA in the main figures and was normalized to Wst-1 metabolic data in the Supplementary Information (Fig. S3). The conclusions from the alkaline phosphatase tests are the same whether the data are normalized to DNA or Wst-1, that alkaline phosphatase expression level per cell is enhanced with increasing modulus in 3D PEGDM gels (Fig. 4d, Fig. S2d, Fig. S3).

Supplementary References

Franceschi RT, Iyer BS. Relationship Between Collagen Synthesis and Expression of the Osteoblast Phenotype in MC3T3-El Cells. J Bone Miner Res 1992;2(2):235-46.

Choi J-Y, Lee B-H, Song K-B, Park R-W, Kim I-S, Sohn K-Y, Jo J-S, Ryoo H-M. Expression Patterns of Bone-Related Proteins During Osteoblastic Differentiation in MC3T3-El Cells. J Cell Biochem 1996;61(4):609-18.

Lee DH, Park BJ, Min-sub Lee, Lee JW, Kim JK, Yang H-C, Park J-C. Chemotactic Migration of Human Mesenchymal Stem Cells and MC3T3-E1 Osteoblast-Like Cells Induced by COS-7 Cell Line Expressing rhBMP-7. Tiss Eng 2006;12(6)1577-86.

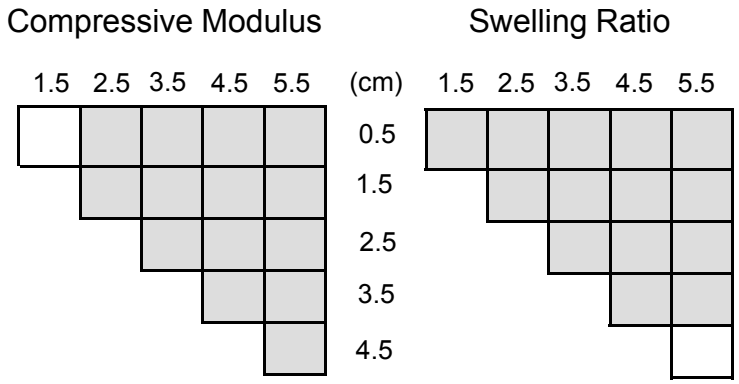

Figure S1. Statistical analysis of compressive modulus and swelling ratio of the hydrogels along the gradients presented in Fig. 3a. The numbers in the margins of the figure indicate position in the gradient scaffolds. Grey squares indicate that differences are statistically significant ($p < 0.05$, 1-way ANOVA with Tukey's), whereas white squares indicate no statistical significance ($p > 0.05$).

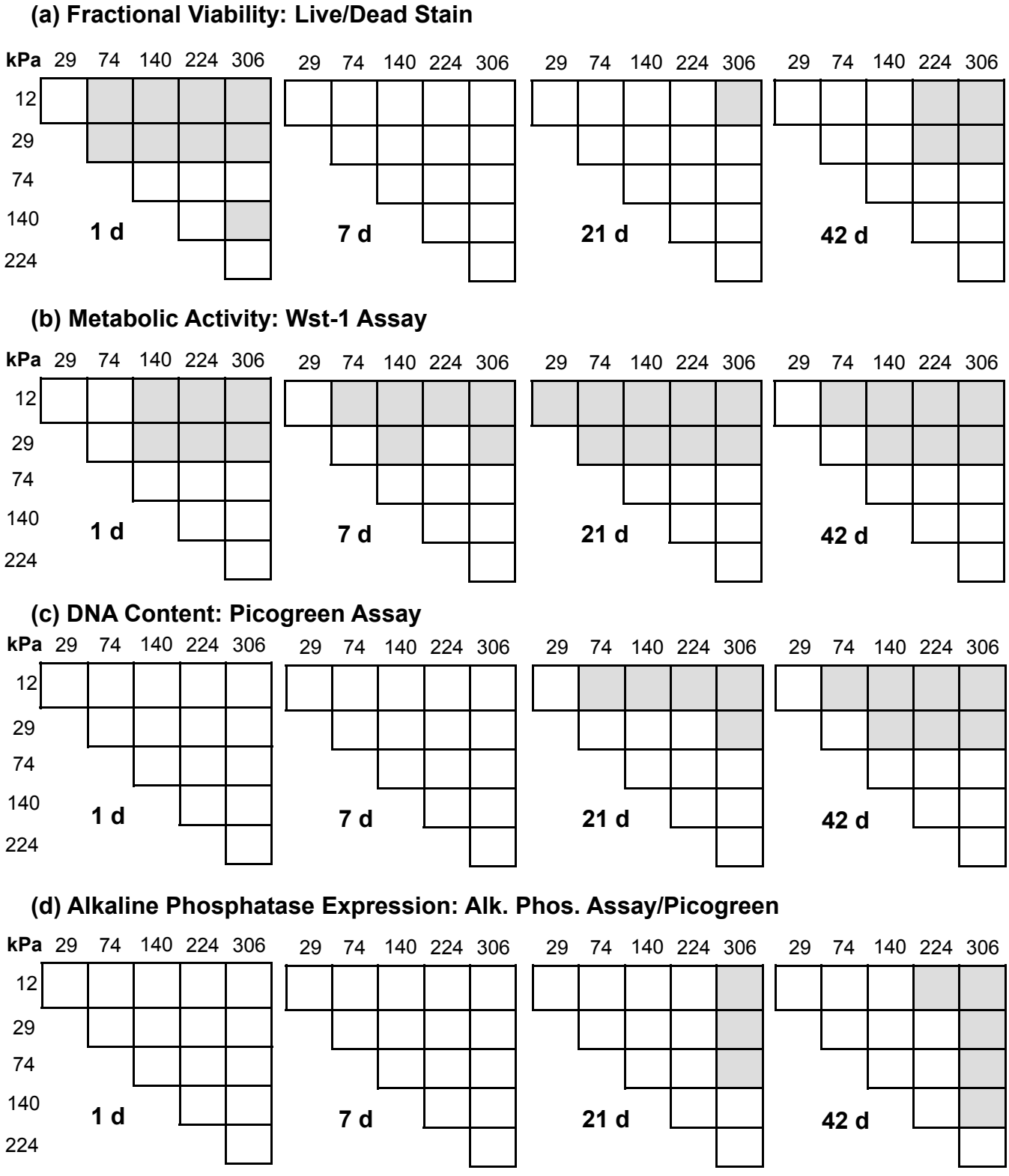

Figure S2. Statistical analyses (1-way ANOVA with Tukey's) of (a) fractional viability, (b) metabolic activity, (c) DNA content, and (d) alkaline phosphatase expression measurements presented in Fig. 4. Numbers in the margins indicate modulus from the gradient scaffolds. Grey squares indicate statistically significant differences ($p < 0.05$), whereas white squares indicate no statistical significance ($p > 0.05$).

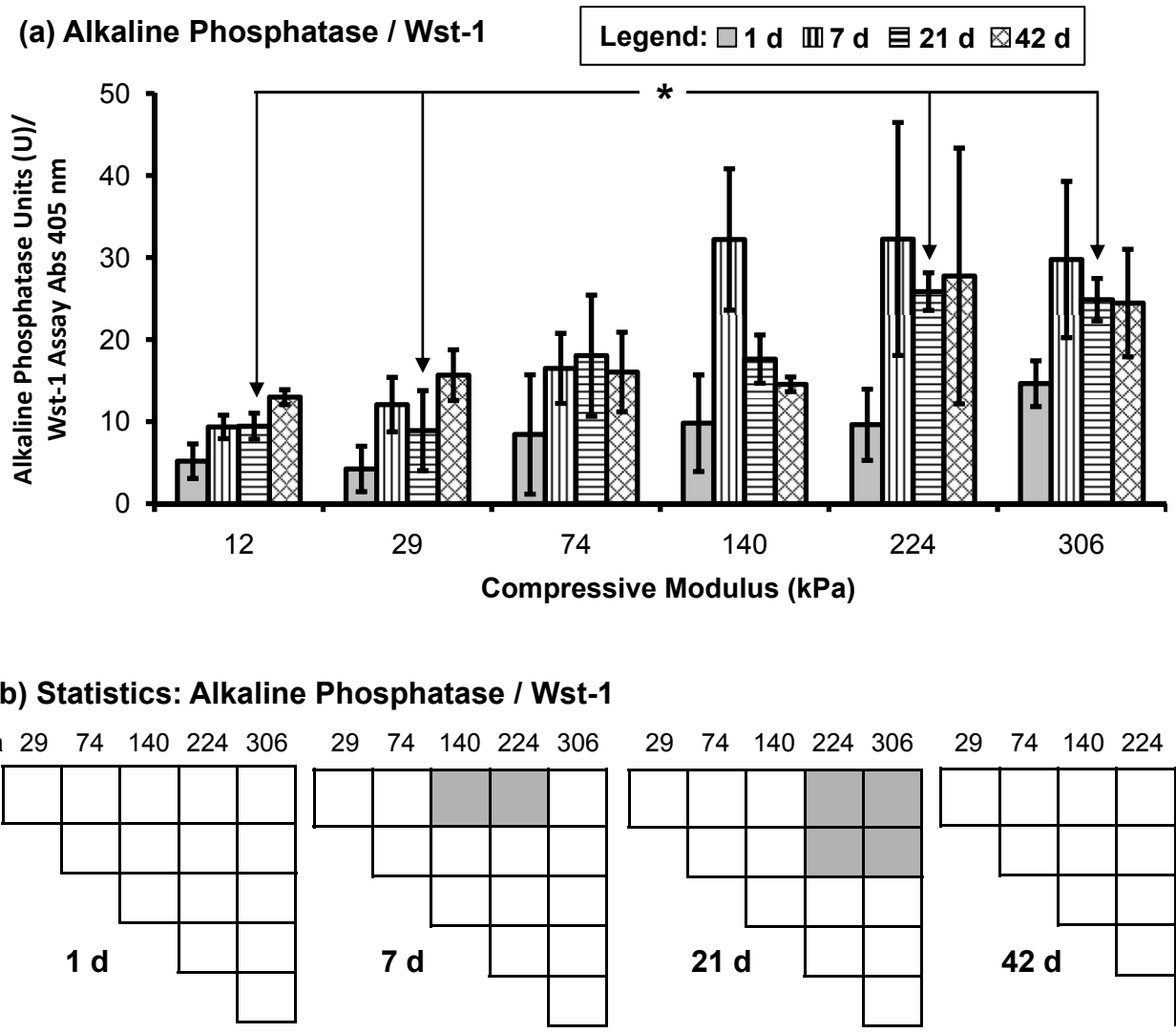

Figure S3. Alkaline phosphatase activity normalized to Wst-1 activity. (a) Alkaline phosphatase activity normalized to Wst-1 activity in the modulus gradients determined at 1 d (solid gray), 7 d (vertical lines), 21 d (horizontal lines) and 42 d (cross-hatched). All measurements were normalized to the mass of the gel slices to account for differences in the size of the gel slice. Error bars are standard deviation and n is 3 for all data points. Statistically significant differences ($p < 0.05$) for 21 d results are indicated by an asterisk (ANOVA with Tukey's) (if an asterisk is encountered when following the line between two data points, then the data points are significantly different). (b) Complete statistical analyses (1-way ANOVA with Tukey's) of alkaline phosphatase data normalized to Wst-1 activity shown in Panel (a). Numbers in the margins indicate modulus from the gradient scaffolds. Grey squares indicate statistically significant differences ($p < 0.05$), whereas white squares indicate no statistical significance ($p > 0.05$).

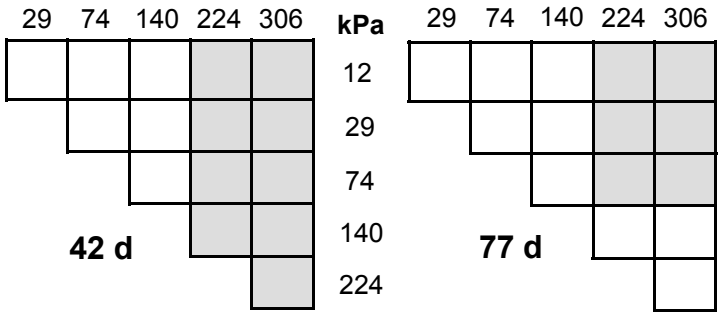

Figure S4. Statistical analysis of volume fraction of mineral deposits in the hydrogels gradients presented in Fig. 6b,c,e. The numbers in the margins of the figure indicate position in the gradient scaffolds. Grey squares indicate statistically significant differences ($p < 0.05$, ANOVA with Tukey's), whereas white squares indicate no statistical significance ($p > 0.05$).