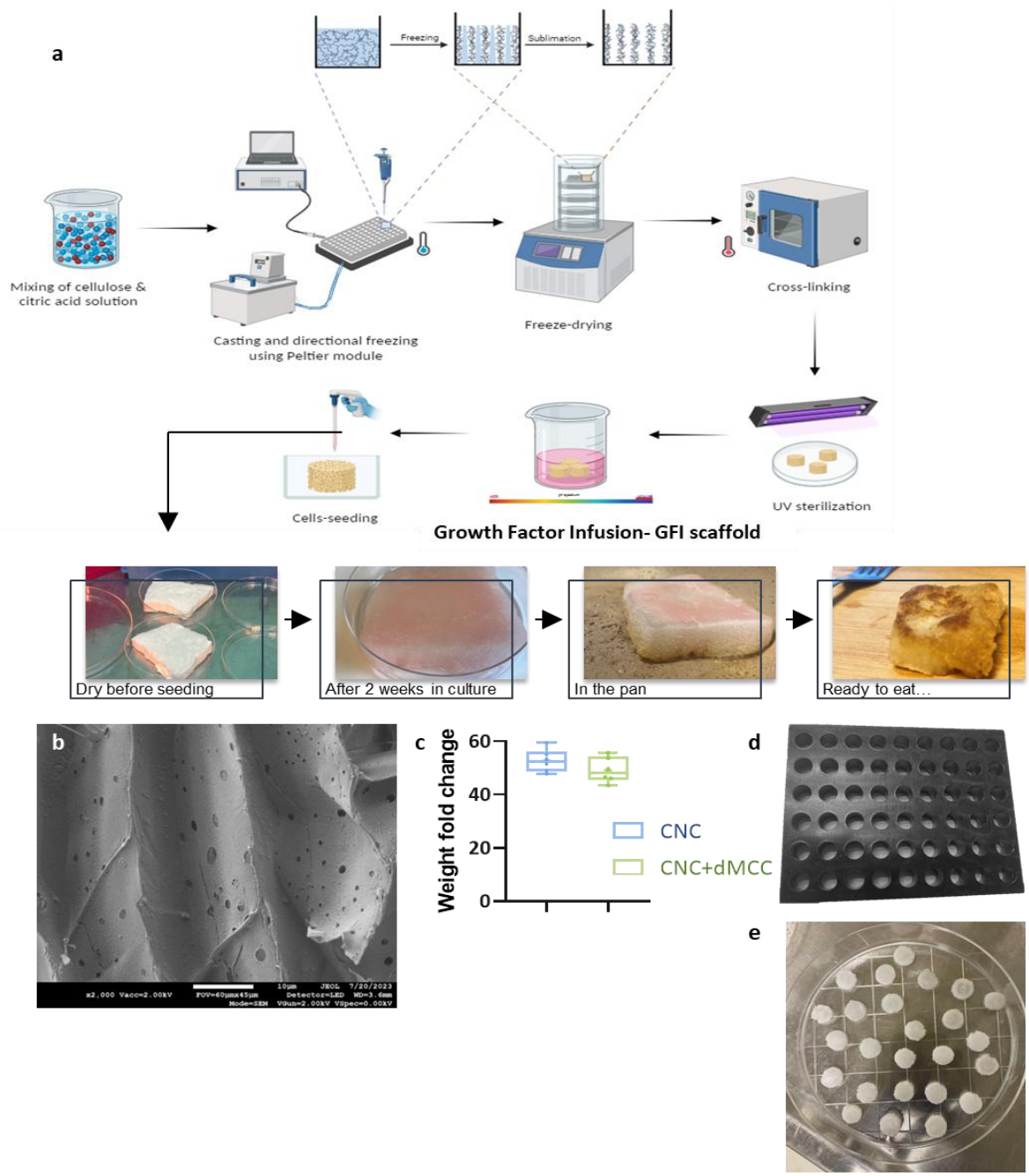
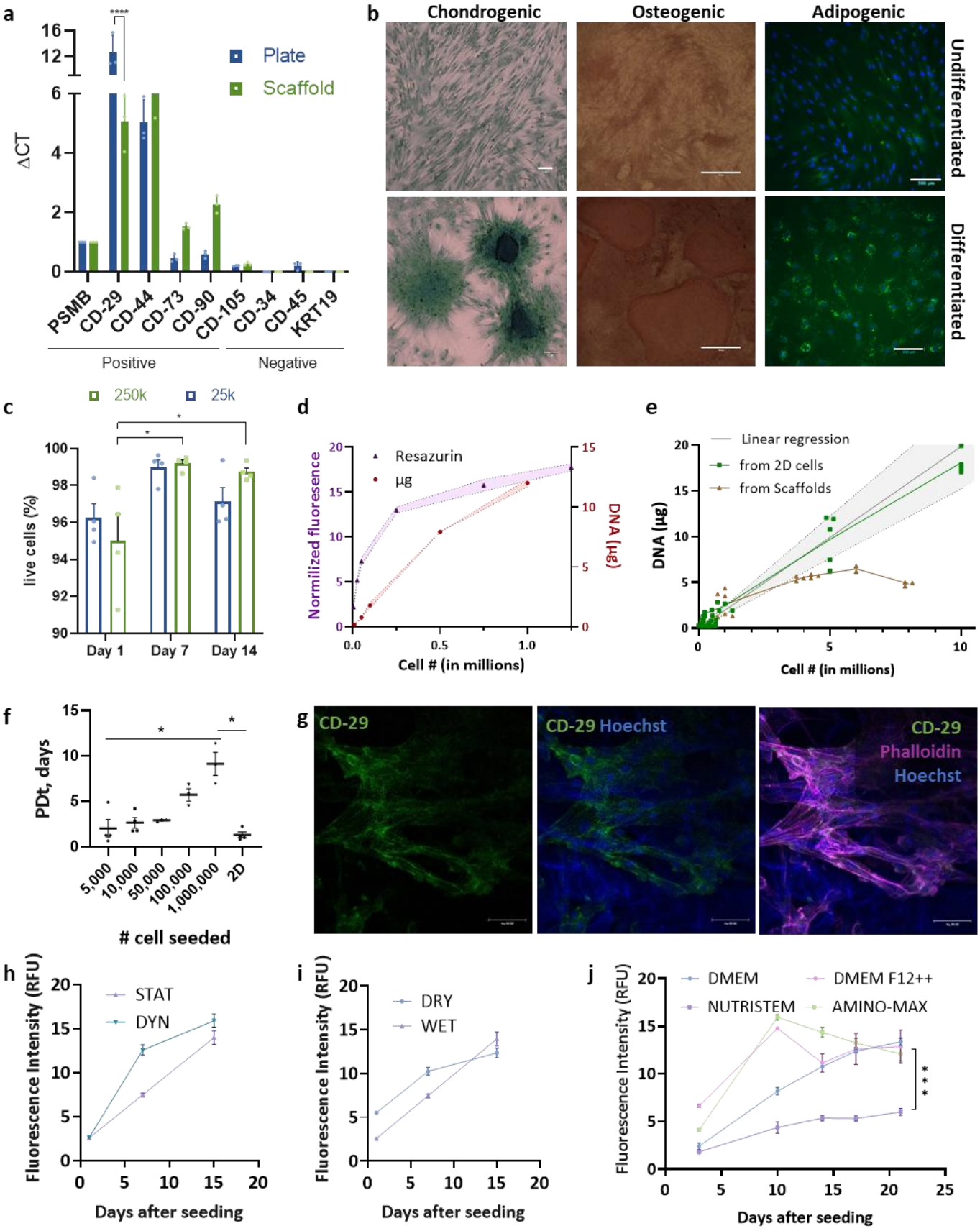
Supplementary data

Supplementary Figures.

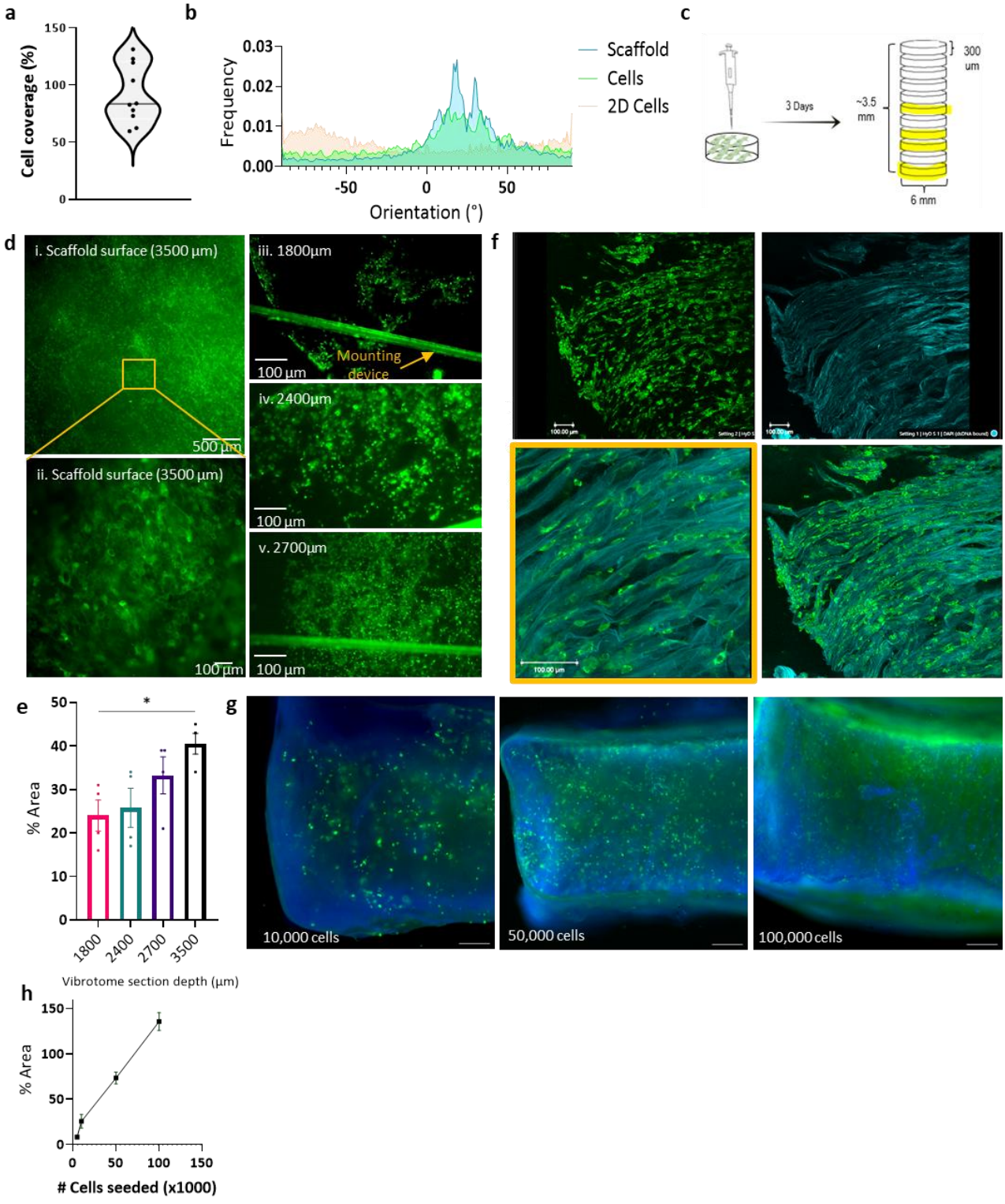



**Supplementary Fig. 1: fabrication and characterization of cellulose GFI-Scaffolds: production process, micromorphology, and water absorption. a** graphical representation of the scaffold fabrication process. thermometers indicate steps performed at low (blue) or high (red) temperatures. Directional freezing and sublimation are depicted above, with water (blue) and cellulose (black). BioRender© was used to create the figure. **b** cryo-SEM image of a fragmented scaffold showing micro-pores within channel walls (scale bar = 10µm). **c** scaffold absorbance over time. "weight fold change" calculated as wet weight divided by dry weight. n=3. **d** polyoxymethylene mold capable of casting 70 scaffolds at a time. **e** the CNC scaffolds on 15 cm plate.



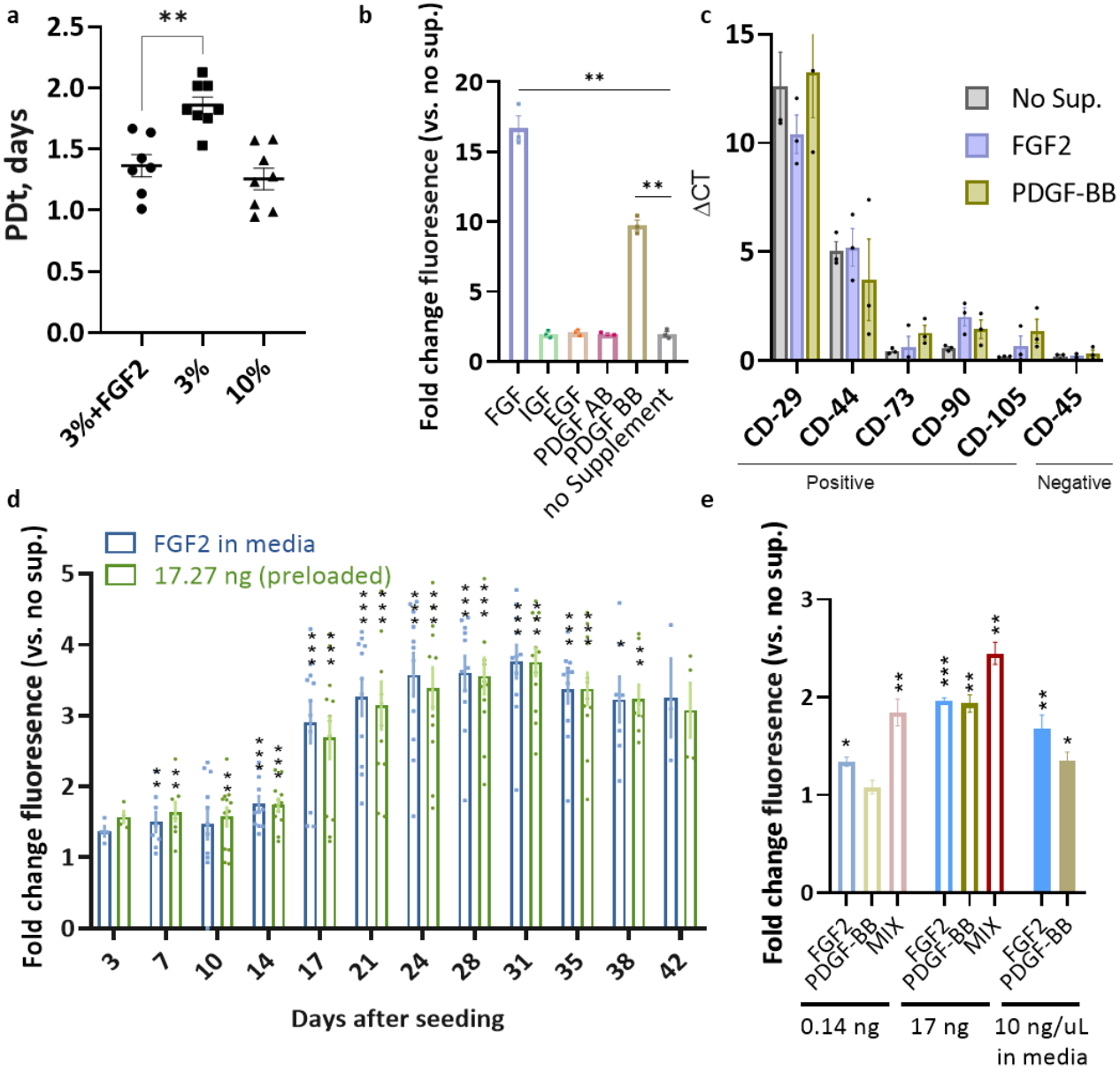

**Supplementary Fig. 2. Characterization of 2D and 3D grown bMSCs. a** Positive and negative MSCs markers on tissue culture plates (blue) compared to those grown on a 1% CNC scaffold (green). Expression measured by RT–qPCR, normalized to PSMB control gene (n = 3), data are the mean ± s.e.m., P value was calculated using Two-way ANOVA Test, ****p<0.0001. **b** bMSCs

differentiated to chondrocytes (Alcian blue staining), osteoblasts (Alizarin red staining) and adipocytes (BODIPY 493/503 and DAPI staining). Chondrocytes and osteoblast staining were observed using EVOS FL Auto microscope (scale bar = 100 μm). Adipocyte staining was observed using a fluorescent microscope (scale bar = 50 μm). **c** Viability of bMSCs cultured on scaffold for 1, 7 and 14 days, initially seeded in two different quantities: 25,000 & 250,000, mean ± s.e.m. are shown (n = 3). A two-way ANOVA was performed with Šidák's post-hoc multiple comparison, *p-value<0.05. **d** Cellular metabolic activity (left Y axis) and extracted genomic DNA (ng, right Y axis) from scaffolds seeded with various cell numbers 24 hours earlier (n=3). **e** Extracted DNA from scaffolds seeded at different densities (violet) vs. cells grown in 2d and counted (blue) allowed us to calculate linear regression (black) to analyze the relationship between cell # and DNA concentration. The solid line represents the line of best fit, and the dotted lines show the 95% confidence interval. The regression results include $R^2$=0.993 with p-value <0.0001. Based on that, the maximal cell number on scaffold was calculated as 6.23 million cells (n=3). **f** based on the DNA concentration, population doubling time of cells on scaffolds was calculated and found to be similar to that of cells in 2D when initial seeding numbers are low (n=3-4), data points represent mean ± s.e.m., statistical analyses were performed using nonparametric Mann-Whitney test, *p-<0.05. **g** Representative images of MSCs on 1% CNC scaffold, after 1 week of growth. Cells were stained with Anti-CD-29 antibody (green), phalloidin (magenta) and Hoechst33342 (blue), and imaged using a confocal microscope (scale bar = 500 μm). **h** Cellular Metabolic activity of bMSCs seeded on dry or wet 1.5% scaffolds, measured by resazurin over 15 days. Changes in fluorescence were normalized to values of scaffolds without cells. (n=3-5). **i** Cellular metabolic activity of bMSCs grown on 1.5% scaffolds in static plates (STAT) and plates placed on orbital shaker (DYN), measured by resazurin over 15 days. Changes in fluorescence were normalized to values of scaffolds without cells. (n=3-5) **j** Cellular Metabolic activity of bMSCs grown on scaffolds in 4 different media, measured by resazurin over 21 days. Changes in fluorescence were normalized to values of scaffolds without cells. (n=2-3). The data points represent mean ± s.e.m. A two-way ANOVA was performed with post-hoc Tukey's multiple comparison test. The asterisk indicates significance between the marked day and the previous day, where the color of the asterisk denotes the trend being referred to, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

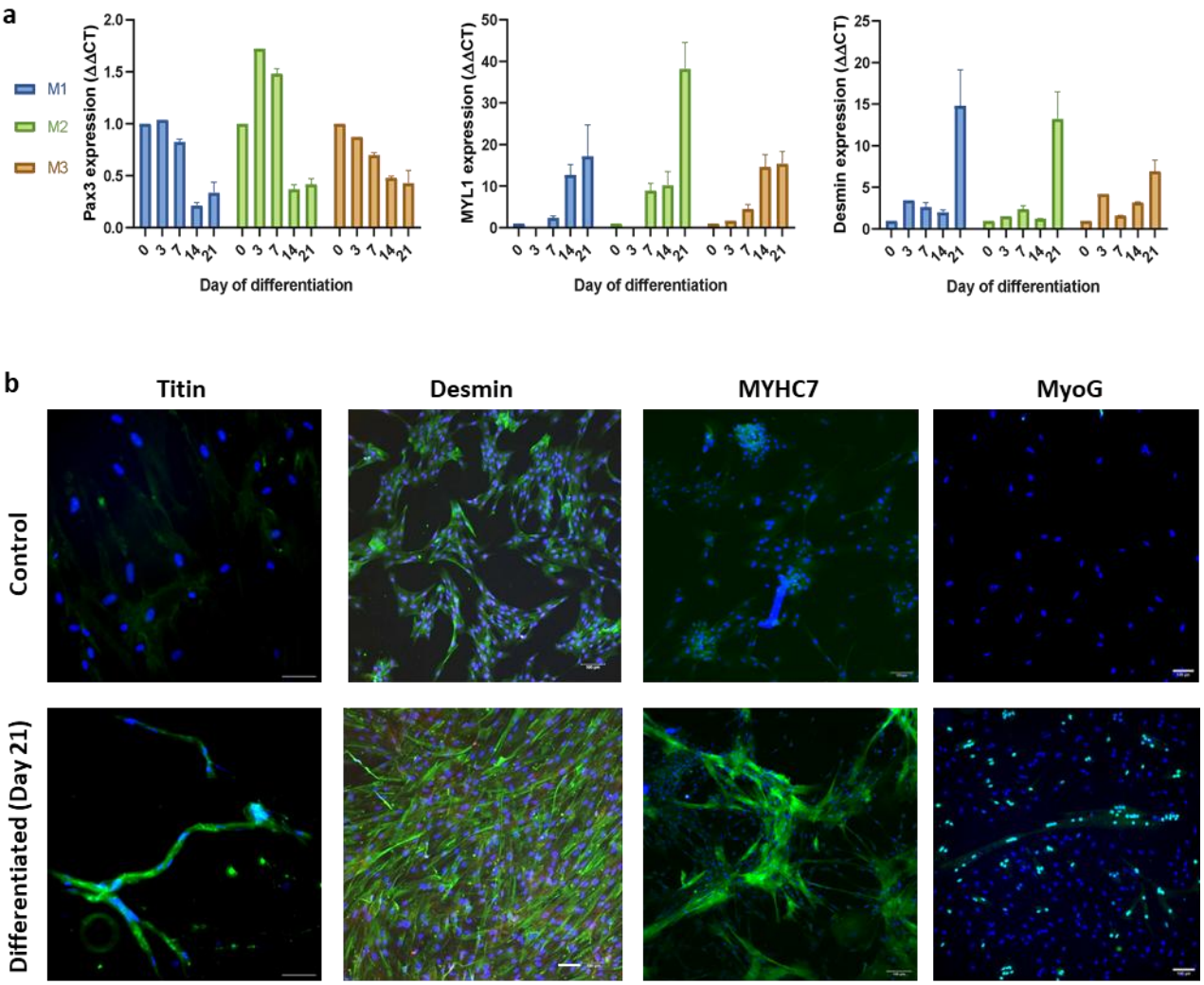



**Supplementary Figure 3: Cellular penetration, coverage, and directional growth on CNC scaffolds**. **a** Quantification of bMSCs coverage after 7 days of growth on 1% CNC scaffolds. Maximum intensity projections were generated from obtained 200 µm z-stacks. Coverage was determined as the ratio between the area of stained bMSCs (green) and the area of the scaffold (blue/cyan). The data points represent individual values, average coverage is 83.26% (n = 9). **b** Histogram depicting cell and scaffold orientation frequency in image f of this figure, quantified using the ImageJ Directionality Plug-In, compared to orientation of 2D-cultutred cells. **c** Schematic representation of the cell seeding and sectioning procedure, indicating the specific

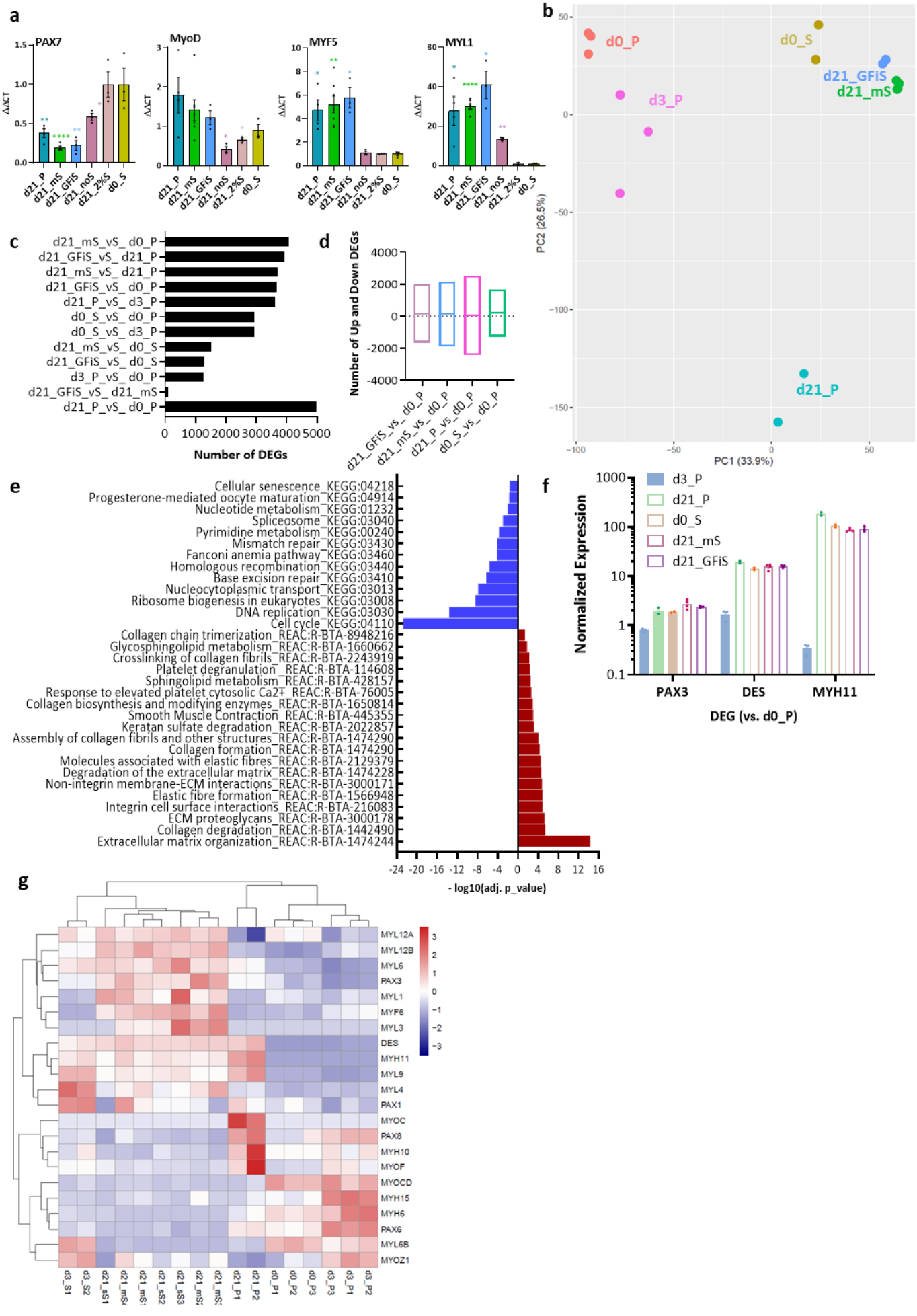
levels from which the sections were obtained. **d** Representative images captured with a fluorescent microscope, depicting horizontal 300 µm vibratome sections of a 1% CNC scaffold seeded with actin-GFP fibroblasts (green) 3 days before sectioning. Images showing scaffold surface (i, ii), (scale bar = 500 & 100 µm, respectively); and inner sections (iii-v) (scale bar = 100 µm). **e** cell coverage area relative to scaffold fiber area in vibratome sections (n=4), data points represent mean ± s.e.m., statistical analyses were performed using nonparametric Mann-Whitney test, *p-<0.05. **f** Representative confocal 3D projection, revealing the inner part of a 1% CNC scaffold cut vertically in the middle, along the cellulose fibers (cyan), and murine GFP-Actin NIH3T3 (green) (scale bar = 100 µm). **g** Representative images of scaffolds (blue) seeded with 10,000, 50,000 and 100,000 bMSCs (green) captured with a fluorescent microscope 1 day after seeding (scale bar = 500 µm). **h** Quantification of cell coverage area relative to scaffold fiber area, one day after seeding with varying cell numbers (n=3-4), data points represent mean ± s.e.m.




**Supplementary Fig. 4: significantly enhanced cell proliferation with FGF2, in 2d and on scaffolds. a** bMSCs were seeded on plates in media containing 3% or 10% FBS, with or without the addition of 10ng/ml FGF2 in the media. Cells were counted every 3 days for 2 weeks (n = 7-8). Statistical analysis performed using nonparametric Mann-Whitney test (*p<0.05). **b** bMSCs were grown on plates in media containing 3% FBS, with the addition of 10ng/ml FGF2, IGF1, EGF, PDGF AB, PDGF BB, and no supplement, resazurin was measured after 14 days. Fold change of cell growth vs. PBS only is shown (n=4). shown are the mean ± s.e.m. One sample t-test was performed (**p<0.01). **c** Positive and negative MSCs markers in cells grown without or with GFs for 14 days. Expression measured by RT–qPCR, normalized to PSMB control gene (n = 3), shown are the mean ± s.e.m **d** Fold change of cell growth on scaffolds over 42 days, preloaded or media-supplemented FGF2 vs. no supplement (n=4). **e** Fold change of cell growth on scaffolds after 14 days, preloaded with FGF2, PDGF-BB or both vs. no supplement (n=4). For d and e, shown are the mean ± s.e.m., one sample t-test was performed (*p<0.05, **p<0.01, ***p<0.001) .




**Supplementary Fig. 5: 2D Optimization of myogenic and adipogenic protocols for bMSC**. **a** RT-qPCR analysis of Pax3, MYL1 and Desmin in bMSCs cultured in M1, M2 and M3 media for 21 days (n = 1-2). data are the mean ± s.e.m. **b** Immunofluorescent staining of bMSCs after 21 days of differentiation. Cells were stained with anti-Titin/ Desmin/ MHC7/ MyoG (green), anti-pol2 (red) and DAPI (blue) and imaged using a fluorescent microscope (scale bar = 200 µm & 100 µm).


a
PAX7
MyoD
MYF5
MYL1
ΔΔCT
d21_P
d21_mS
d21_GFiS
d21_noS
d21_2%S
d0_S
b
d0_P
d0_S
d21_GFiS
d21_mS
d3_P
d21_P
PC2 (26.5%)
PC1 (33.9%)
c
d21_mS_vS_ d0_P
d21_GFiS_vS_ d21_P
d21_mS_vS_ d21_P
d21_GFiS_vS_ d0_P
d21_P_vS_ d3_P
d0_S_vS_ d0_P
d0_S_vS_ d3_P
d21_mS_vS_ d0_S
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d3_P_vS_ d0_P
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d21_P_vS_ d0_P
Number of DEGs
d
Number of Up and Down DEGs
d21_GFiS_vs_ d0_P
d21_mS_vs_ d0_P
d21_P_vs_ d0_P
d0_S_vs_ d0_P
e
Cellular senescence_KEGG:04218
Progesterone-mediated oocyte maturation_KEGG:04914
Nucleotide metabolism_KEGG:01232
Spliceosome_KEGG:03040
Pyrimidine metabolism_KEGG:00240
Mismatch repair_KEGG:03430
Fanconi anemia pathway_KEGG:03460
Homologous recombination_KEGG:03440
Base excision repair_KEGG:03410
Nucleocytoplasmic transport_KEGG:03013
Ribosome biogenesis in eukaryotes_KEGG:03008
DNA replication_KEGG:03030
Cell cycle_KEGG:04110
Collagen chain trimerization_REAC:R-BTA-8948216
Glycosphingolipid metabolism_REAC:R-BTA-1660662
Crosslinking of collagen fibrils_REAC:R-BTA-2243919
Platelet degranulation_REAC:R-BTA-114608
Sphingolipid metabolism_REAC:R-BTA-428157
Response to elevated platelet cytosolic Ca2+_REAC:R-BTA-76005
Collagen biosynthesis and modifying enzymes_REAC:R-BTA-1650814
Smooth Muscle Contraction_REAC:R-BTA-445355
Keratan sulfate degradation_REAC:R-BTA-2022857
Assembly of collagen fibrils and other structures_REAC:R-BTA-1474290
Collagen formation_REAC:R-BTA-1474290
Molecules associated with elastic fibres_REAC:R-BTA-2129379
Degradation of the extracellular matrix_REAC:R-BTA-1474228
Non-integrin membrane-ECM interactions_REAC:R-BTA-3000171
Elastic fibre formation_REAC:R-BTA-1566948
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ECM proteoglycans_REAC:R-BTA-3000178
Collagen degradation_REAC:R-BTA-1442490
Extracellular matrix organization_REAC:R-BTA-1474244
- log10(adj. p_value)
f
d3_P
d21_P
d0_S
d21_mS
d21_GFiS
Normalized Expression
PAX3
DES
MYH11
DEG (vs. d0_P)
g
MYL12A
MYL12B
MYL6
PAX3
MYL1
MYF6
MYL3
DES
MYH11
MYL9
MYL4
PAX1
MYOC
PAX8
MYH10
MYOF
MYOCD
MYH15
MYH6
PAX6
MYL6B
MYOZ1

**Supplementary Figure 6: RNAseq analysis of muscle differentiation on scaffold. a** RT-qPCR analysis of MYF5, MYL1, PAX3, and MYOD expression in bMSCs cultured for 21 days plate (d21_P) or on CNC scaffolds in Myogenic Medium 2 (MM2), with growth factors supplied either in the medium (d21_mS) or delivered through one-time soaking of the scaffold with GFs (d21_GFiS) (n = 3-5). Additional controls were cells grown on scaffolds with MM2 without the GF (d21_noS) or with normal (non-myogenic) growth medium with 2% serum (d21_2%S). Expression levels were measured by RT–qPCR, normalized to PSMB and to control- growth medium with 10% serum (d0_S). Data presented as mean ± s.e.m., and p-values were calculated relative to the hypothetical value of 1 (d0_S) using one-sample t-test, with *p-value<0.05, **p<0.01, ****p<0.0001. **b** Principal component analysis (PCA) of global gene expression (RNA-seq) showing distinct separation of gene transcripts among the groups: control of non-differentiated cells on a a plastic plate (d0_P, red), day 21 of differentiation on a plastic plate (d21_P, turquoise), day 21 of differentiation on a scaffold with growth factors in media (d21_mS, green), day 21 of differentiation on a scaffold with growth factors (d21_GFiS, blue), day 3 of differentiation (d3_P, pink), and control of non-differentiated cells on a scaffold (d0_S, olive). **c** Total number of differentially expressed genes (DEGs) in each type of differentiation**. d** Number of upregulated and downregulated DEGs in all types of differentiation compared to control d0_P. **e** Significantly (p_adj < 0.05) upregulated and downregulated KEGG/Reactome pathways in d0_S versus d0_P. **f** Expression levels of Pax3, Desmin, and Myh11 genes normalized to GAPDH and to d0_P across different types of differentiations. Empty bars represent DEGs, while filled bars correspond to non-DEGs (n=2-4). **g** Heatmap of specific DEGs related to myogenesis across different types of differentiations. Colors represent scaled, normalized DESeq expression values, with blue indicating below-average expression, white indicating average expression, and red indicating above-average expression relative to the mean expression across all samples.