SUPPLEMENTAL MATERIALS

Supplemental Figure 1. Ascorbic acid is not required for collagen expression by 17IIA11 cells. (A) qRT-PCR showing average ΔCT values for *Col1a1* relative to *Gapdh* in cells treated with standard osteogenic medium (AA+P_i), 10 mM Na-P_i buffer (P_i), or ascorbic acid (AA) for 24h. Data are represented as the mean values of three experiments ± SD. (B) Western blot analyses of Col1a1 in proteins isolated from undifferentiated (day 0) 17IIA11 cells and from cells grown in osteogenic medium for 9 days. Tubulin was used as a loading control. (C) Comparison of amount of hydroxyproline in cells grown in standard growth medium and cells treated with osteogenic medium (AA+P_i) or 10 mM Na-P_i buffer (P_i) for 6h and 24h. Data are represented as the mean values of three experiments ± SD. (D) Images of picrosirius red stained cells treated with osteogenic medium (AA+P_i), 10 mM Na-P_i buffer (P_i), or ascorbic acid (AA) for 24h. Top panel: bright field images (BF). Bottom panel: images taken under polarized light (PL) to observe the orientation of collagen. White arrows indicate collagen. Scale bars=100 μm.

Materials and Methods

RNA Extraction, cDNA Synthesis, and Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA (1 μg), after DNase I treatment (Life Technologies, Grand Island, NY, USA), was converted to cDNA with SuperScript III Reverse Transcriptase kit (Life Technologies, Grand Island, NY, USA). Gene expression analyses were performed using AB Biosystems 7500 Fast Real-Time PCR System and Fast SYBR Green reaction mix (Roche, Indianapolis, IN, USA). Primer sequences are as follows: Gapdh F:GCAAGAGAGGCCCTATCCCAA and R:CTCCCTAGGCCCCTCCTGTTATT and Col1a1 F:CCAGCCGCAAAGAGTCTACAT and R:GATCAAGCATACCTCGGGTTTC.

Hydroxyproline Assay

17IIA11 cells were plated at $6.1x10^6$ cells per 10 cm dish and grown for 96h until confluency. Treatment was induced by supplementing the growth medium with 10 mM Na-P_i buffer (pH 7.4) and 50 μg/ml ascorbic acid (osteogenic medium) or P_i buffer alone. After 6h or 24h, cells were washed with PBS and scraped and collected in PBS. Cells were pelleted by centrifugation at 4000 rpm for 10 min at room temperature. Pelleted cells were then processed following the manufacturer's protocol (Hydroxyproline Assay Kit, Sigma-Aldrich) starting with resuspension in 100 μl water.

Picrosirius Red Staining

Cells were plated at $8.8x10^4$ cells per 4-chamber slide and allowed to grow for 96h before treatment with 10 mM Na-P_i buffer (pH 7.4) and 50 μg/ml ascorbic acid (osteogenic medium) or P_i buffer alone. After 24h, cells were washed with PBS, fixed with 4%PFA, and stained in 0.1% picrosirius red solution (Direct Red-80, Sigma-Aldrich and saturated picric acid, Fluka/Sigma-Aldrich) for 1h. Slides were washed in water, dehydrated, mounted, and imaged under polarized light.