SUPPLEMENTARY MATERIALS

Supplementary Methods

Alkaline Phosphatase Histochemical Staining

Alkaline phosphatase staining was performed using 5-bromo-4-chloro-3-indolyl phosphate nitro blue tetrazolium (BCIP-NBT) as a substrate. This substrate was prepared by mixing 66µl NBT stock (0.5g NBT in 10ml 70% dimethyl formamide) with 10ml alkaline phosphatase reaction buffer (100mM NaCl, 5mM $MgCl_2$, 100mM Tris-Hcl, pH 9.5) and then adding 33µl BCIP stock (0.5g BCIP disodium salt in 10ml 100% dimethyl formamide). The cells were incubated in this substrate mixture for 30 min at 37^{o}C in the dark. The substrate was then aspirated and the cells were washed with dH_2O and air dried.

PCR for Gender Determination on Genomic DNA

Cells were grown in a T150 flask and cell lysis and DNA extraction were performed using a Wizard Genomic DNA Purification Kit, according to manufacturer's instructions (#A1120 Promega, Madison, WI). 200ng of DNA was amplified in a PCR reaction containing 0.5 µM Jarid primers (Jarid 1 c Forward: 5'-CTG AAG CTT TTG GCT TTG AG-3', Jarid 1 c Reverse: 5'-CCA CTG CCA AAT TCT TTG G-3') in a total volume of 50µl using REDTaq ReadyMix PCR Reaction Mix (#R2523 Sigma Aldrich). The Jarid primers amplify 2 bands in males (302 and 331 bp) but only one band in females (331 bp) [1].

References for Supplementary Methods:

1. Clapcote SJ, Roder JC. Simplex PCR assay for sex determination in mice. BioTechniques. 2005;38(5):702, 4, 6.

Supplementary Tables

Table S1 - Primers used for Taqman qPCR (numbers refer to the expression assay ID numbers, Themofisher/Lifetechnologies)

Gene symbol (alias)	TaqMan Gene Expression Assay ID
Pdpn (Podoplanin, E11/gp38)	Mm00494716_m1
Dmp1	Mm01208365_m1
Phex	Mm00448119_m1
Mepe	Mm02525159_s1
Sost	Mm00470479_m1
Fgf23	Mm00445621_m1
Tnfsf11 (RANKL)	Mm00441906_m1
Tnfrsf11b (OPG)	Mm00435454_m1
Col1a1	Mm00801666_g1
Alpl (Alkaline phosphatase liver/bone/kidney)	Mm00475834_m1
Gja1 (Connexin 43, Cx43)	Mm00439105_m1
Hif1a (HIF-1 alpha)	Mm00468869_m1
Actb (β-actin)	Mm01205647_g1

Figure S1

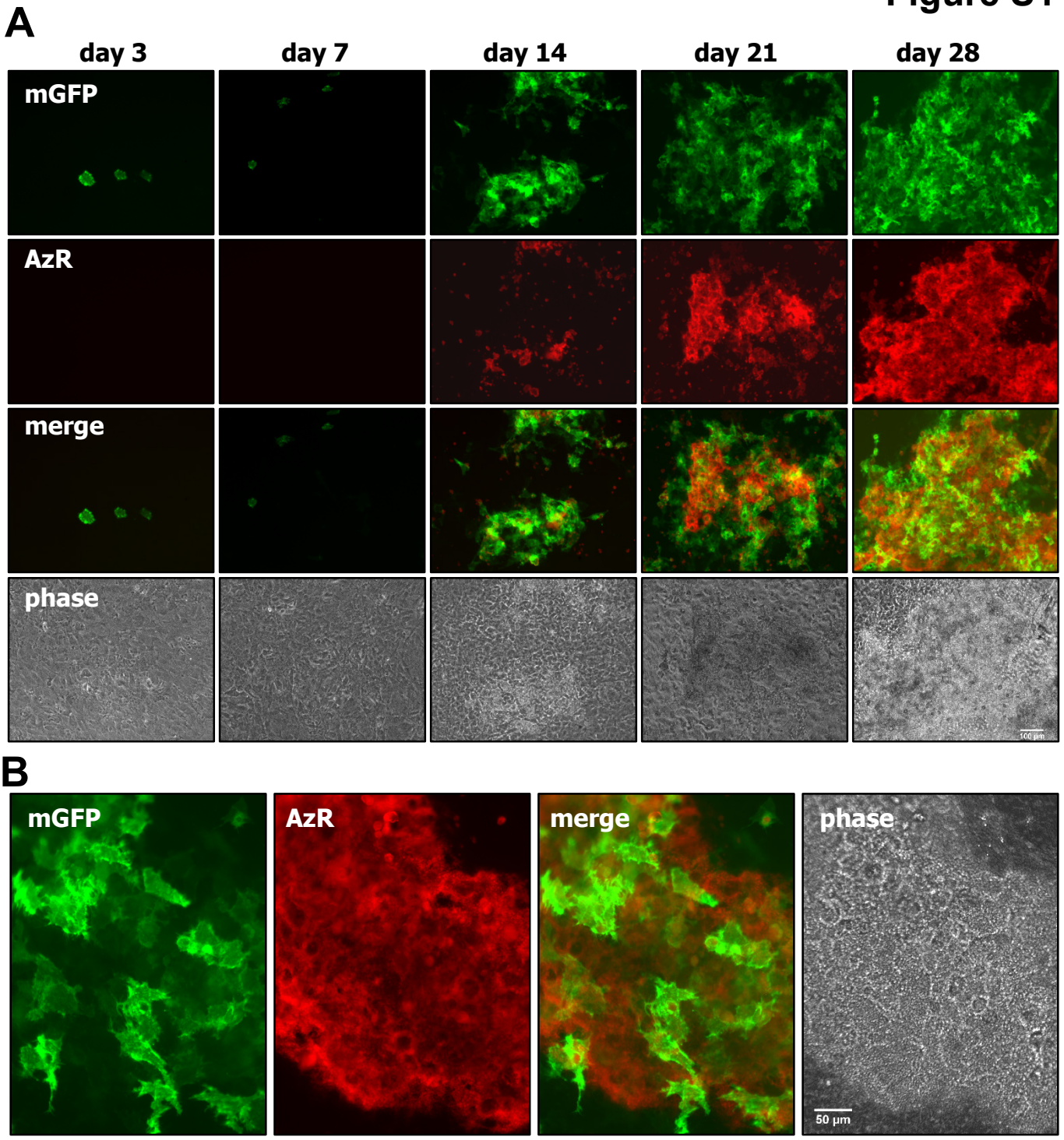

Figure S1: OmGFP10 cells form mineralized bone nodules over 7-28 days in culture. A) Time course in OmGFP10 cells showing expression of the Dmp1-mGFP reporter (green) and mineralization imaged using alizarin red fluorescence to monitor calcium deposition (red). Note the appearance of a few Dmp1-mGFP-positive cells by day 3-7 with larger foci of mGFP-positive cells by day 14, accompanied by mineral deposition. Merged images show that the Dmp1-mGFP-positive cells are associated with the mineralized foci, but extend beyond the boundary of the mineralized region. Bar = 100µm. **B)** Higher power views showing the morphology of the Dmp1-mGFP-positive OmGFP10 cells. Bar = 50µm.

Figure S2

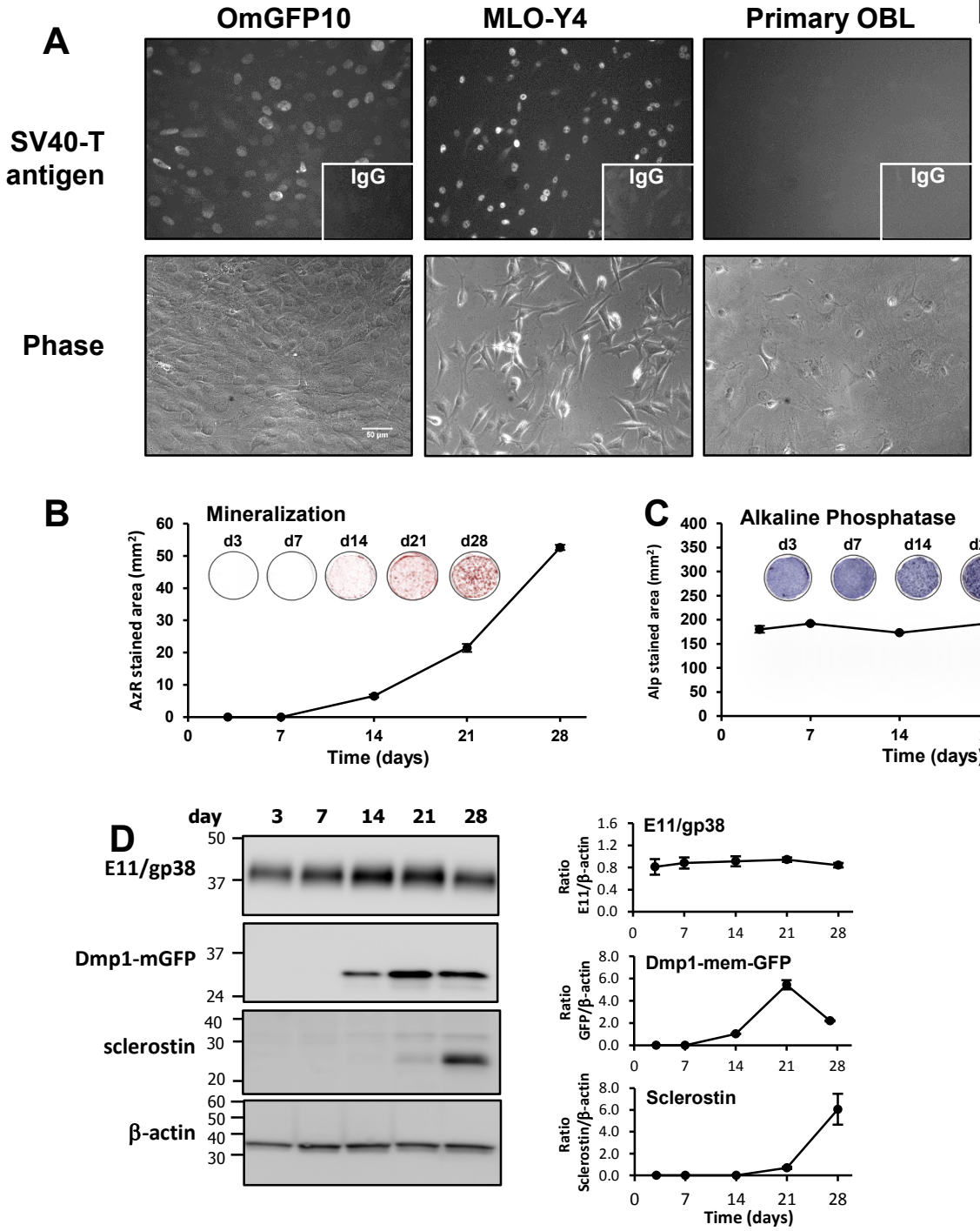

Figure S2: OmGFP10 cells mineralize over 28 days in culture and express early and late osteocyte markers. A) Immunostaining for SV40T-antigen in OmGFP10 cells compared to MLO-Y4 cells as a positive control and primary mouse osteoblasts as a negative control. Controls stained with non-immune IgG are shown in the insets and phase contrast images of the cells are shown below. Bar = 50μm. **B, C)** Quantitation of alizarin red stained area (**B**) and alkaline phosphatase stained cell area (**C**) in OmGFP10 cells over the 28-day time course (data are mean ± SEM, n= 3). **D)** Western blotting timecourse on whole cell lysates from OmGFP10 cells showing constitutive expression of E11/gp38 and showing Dmp1-mGFP expression starting at day 14, with sclerostin expression by day 21-28. β-actin was used as a control for equal loading of protein. Quantitation of the western blots is shown at right (data are mean ± SEM, n=3).

Figure S3

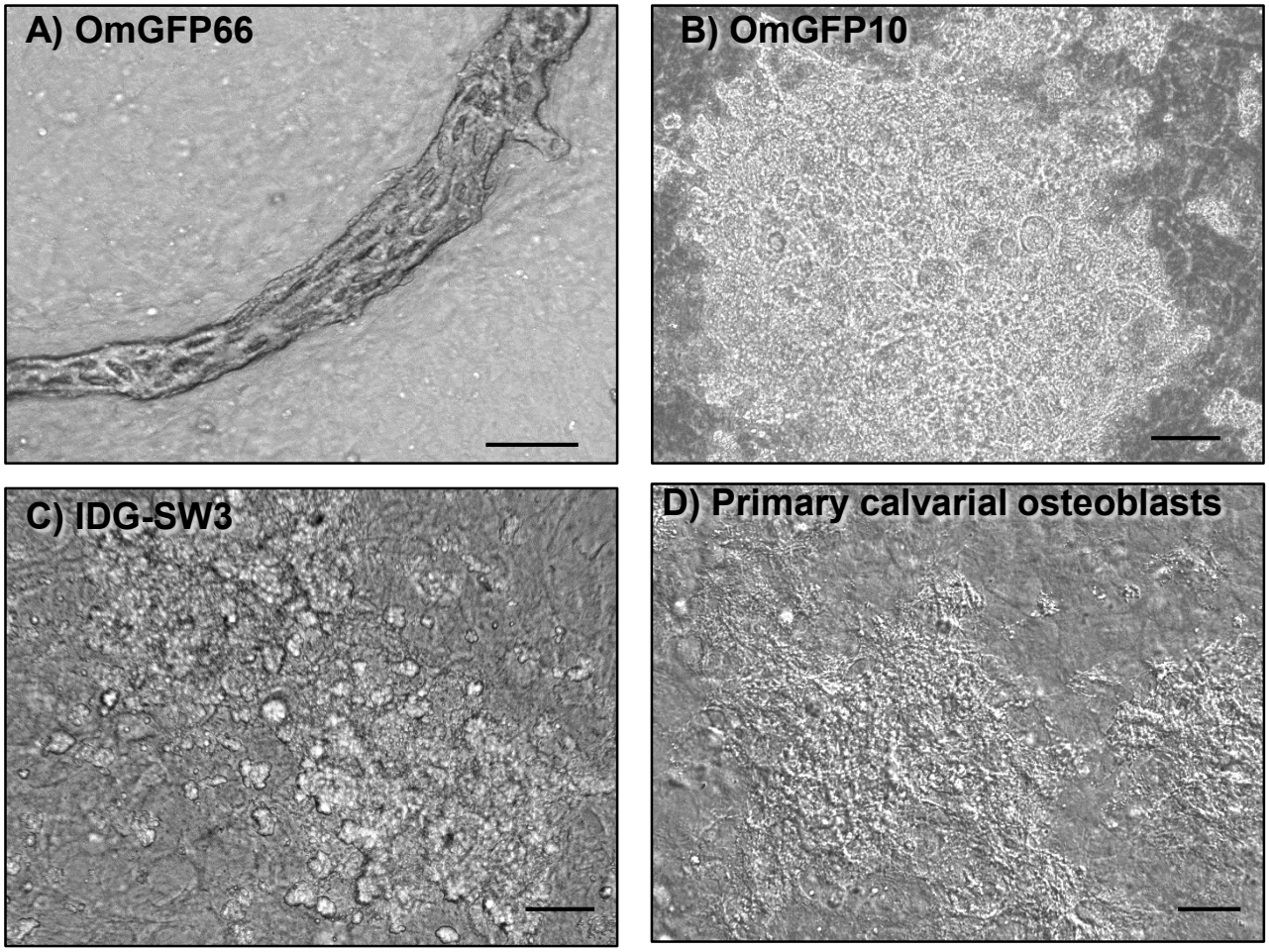

Figure S3: Comparison of highly organized OmGFP66 bone-like structures to mineralized bone nodules formed in other osteogenic cell lines and primary osteoblasts. A) OmGFP66 cells showing highly structured mineral with well defined lacunae, compared with **B)** OmGFP10 cells, **C)** IDG-SW3 cells and **D)** primary calvarial osteoblasts, all of which show a more disorganized and less structured mineral deposition. Bar = 40μm.

Figure S4

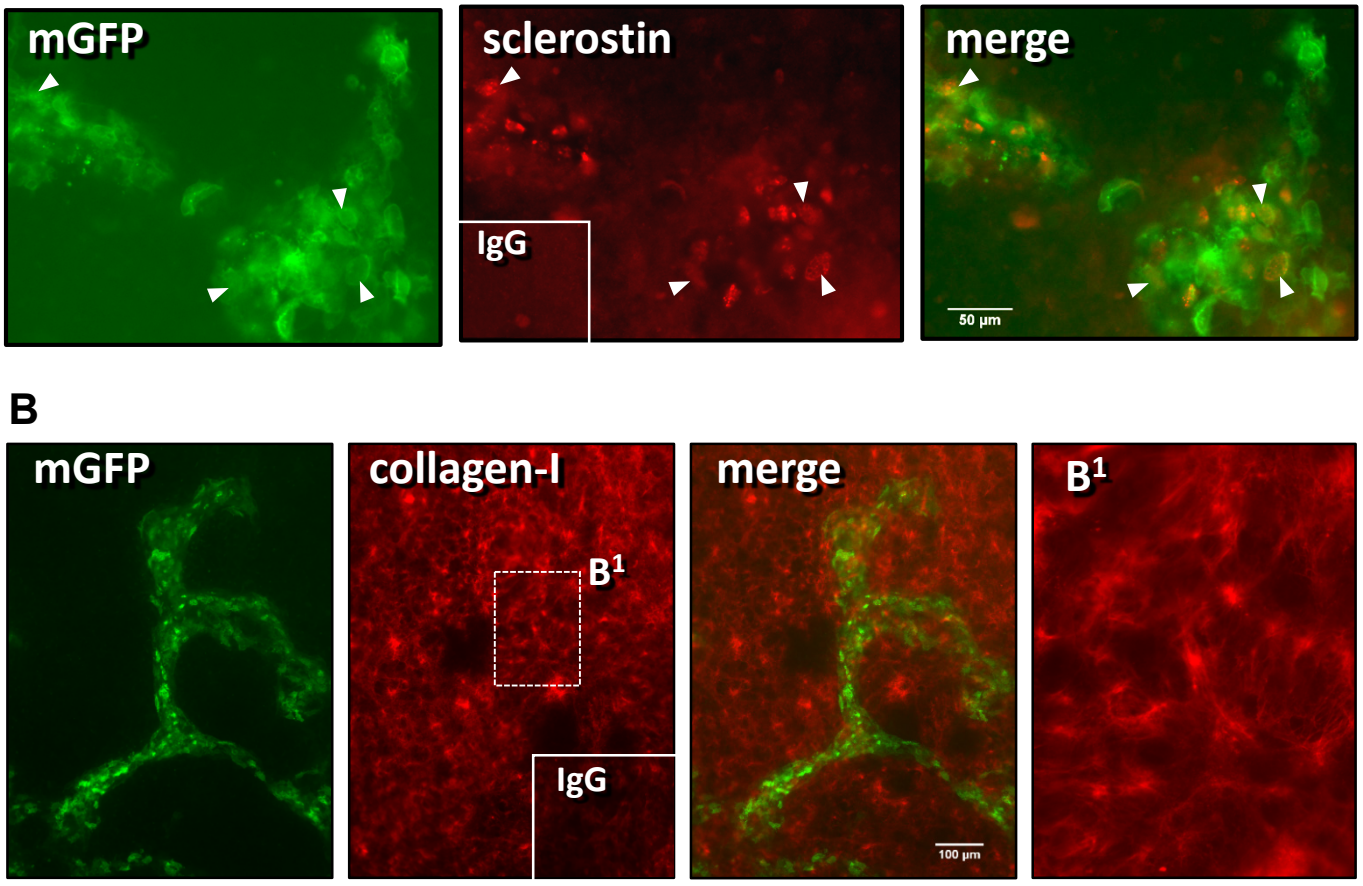

Figure S4: OmGFP66 cells express sclerostin and type I collagen. **A)** Widefield epifluorescence images of sclerostin immunostaining in day 21 OmGFP66 cell cultures. The control stained with non-immune IgG is shown in the inset. Note that several of the more deeply embedded cells within the bone-like structure are positive for both sclerostin and Dmp1-mGFP (arrowheads), bar = 50µm. **B)** Widefield epifluorescence images of type I collagen immunostaining in day 21 OmGFP66 cell cultures. The control stained with non-immune IgG is shown in the inset. Note that type I collagen is expressed by the monolayer cells and is also present in the bone-like structures demarcated by Dmp1-mGFP positive cells. B^1 shows an enlargement of the boxed area, showing the fibrillar nature of the collagen immunostaining. Bar = 100µm.

.

Figure S5

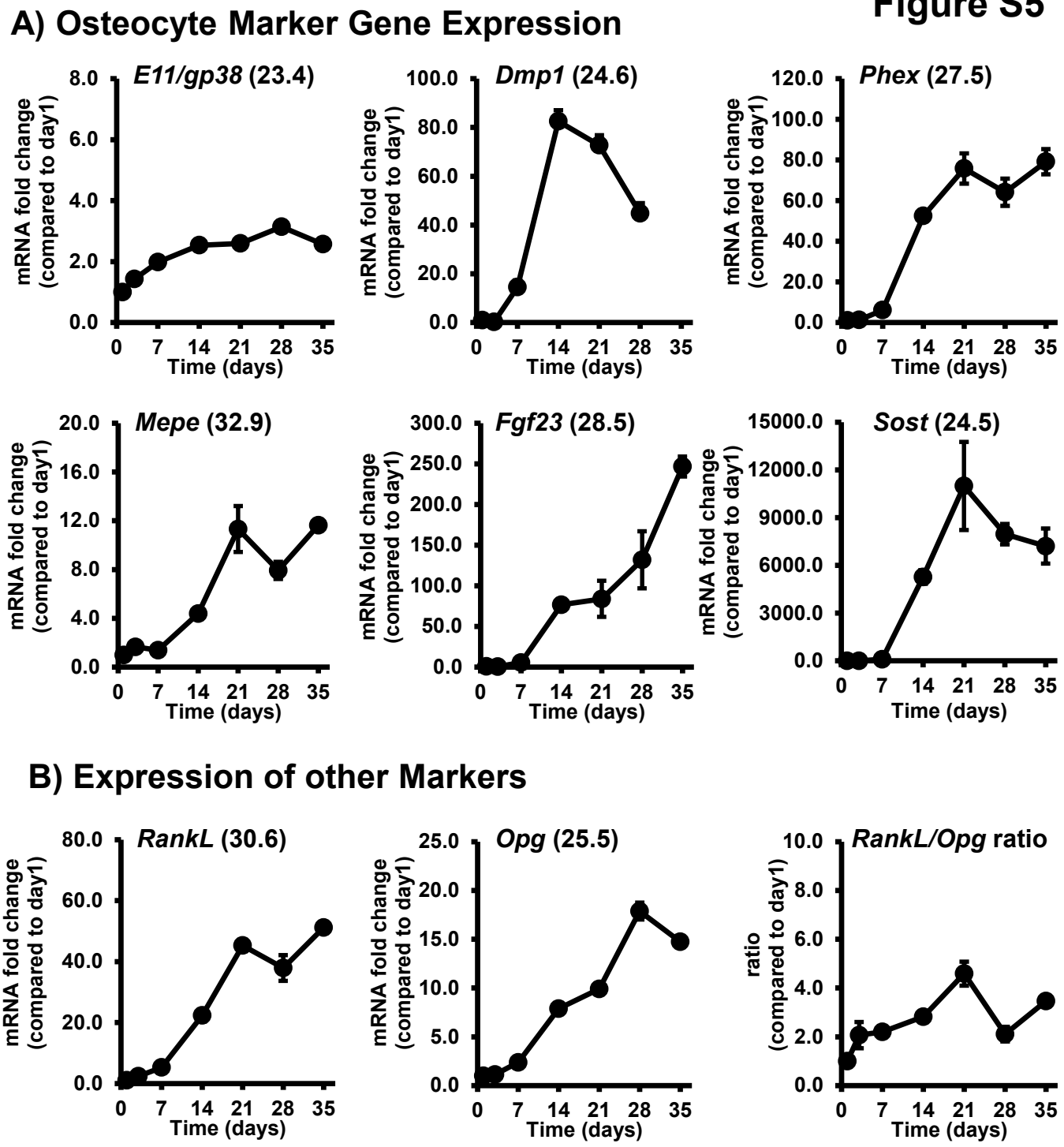

Figure S5: Timecourse of expression of osteocyte markers by OmGFP10 Cells. qPCR analysis of the timecourse of expression of **A**) osteocyte marker genes, *E11/gp38, Dmp1, Phex, Mepe, Fgf23* and *Sost* by OmGFP10 cells and **B**) other markers, *RankL* and *Opg*. Data were normalized to β-actin as a housekeeping control and are presented as the fold change compared to day 1 (mean ± SEM, n=3). Numbers in parentheses are the Ct values for the highest level of expression of each gene.

Figure S6

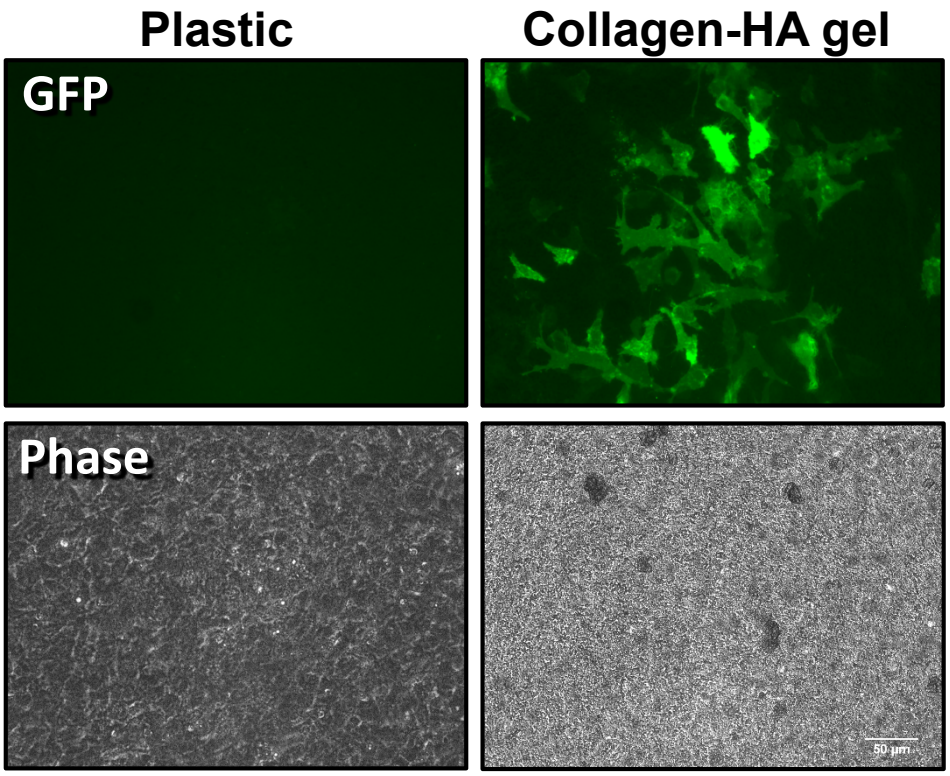

Figure S6: A collagen-hydroxyapatite gel can induce expression of Dmp1-mGFP in OmGFP66 cells. OmGFP66 cells were grown in culture dishes coated with a collagen-HA gel for 5 days. Note the strong induction of Dmp1-mGFP fluorescence in the cells cultured on collagen-HA compared to control cells cultured on plastic. Phase contrast images are shown below. Bar = 50µm.

Figure S7

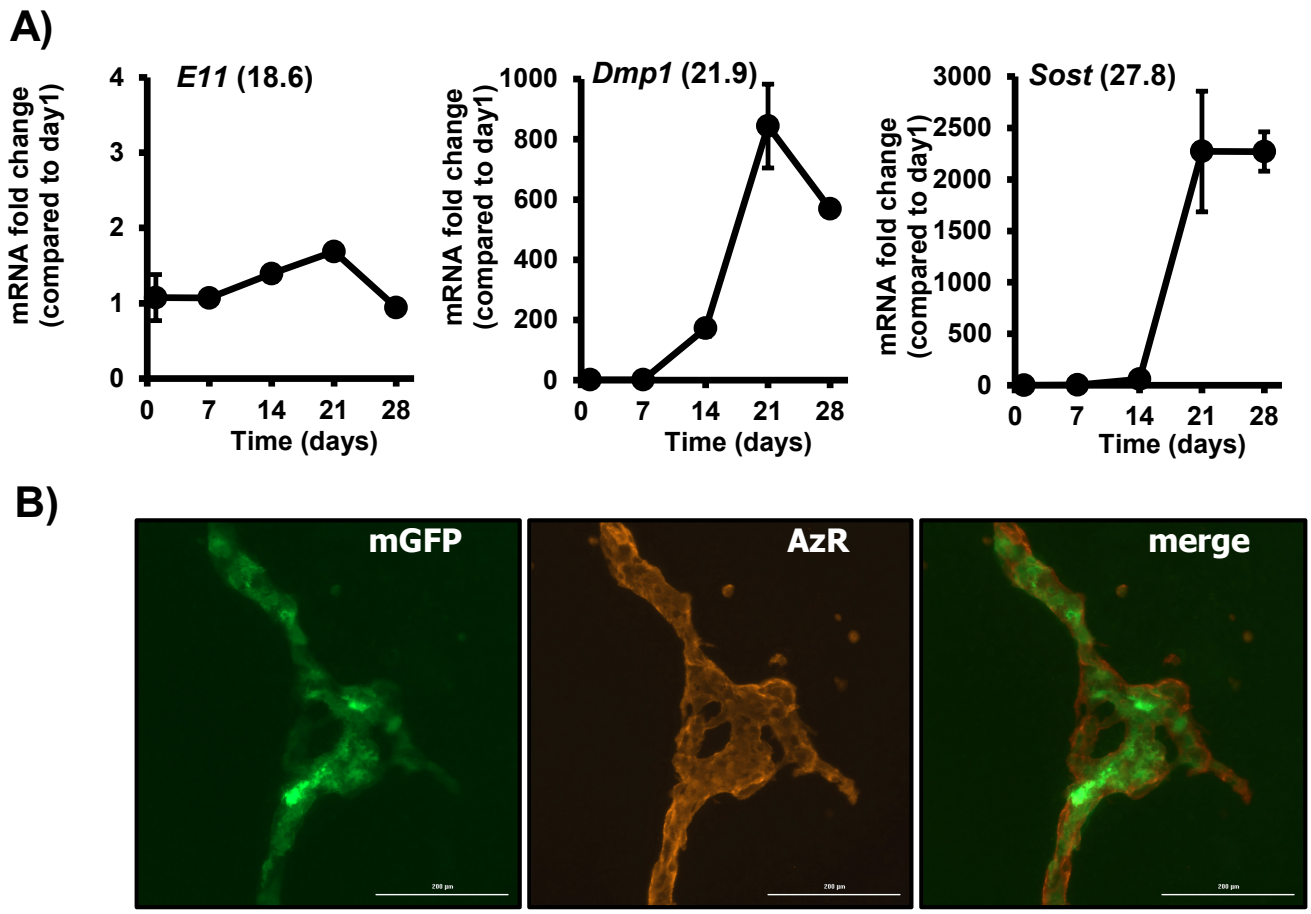

Figure S7: Reproducibility of OmGFP66 phenotype – OmGFP66 cells were grown independently in the laboratory of Dr. Matt Prideaux, Indiana University. **A)** Analysis of the timecourse of expression of osteocyte marker genes, *E11/gp38, Dmp1* and *Sost* showed similar results to our laboratory (figure 4A in main manuscript). Data were normalized to β-actin and are presented as the fold change compared to day 1 (mean ± SEM, n=3). Numbers in parentheses are the Ct values for the highest level of expression of each gene. **B)** Fluorescence images of Dmp1-mGFP, alizarin red and merged image in day 21 OmGFP66 cells showing reproducible formation of bone-like structures containing mGFP-positive osteocytes. Bar = 200μm.

Figure S8

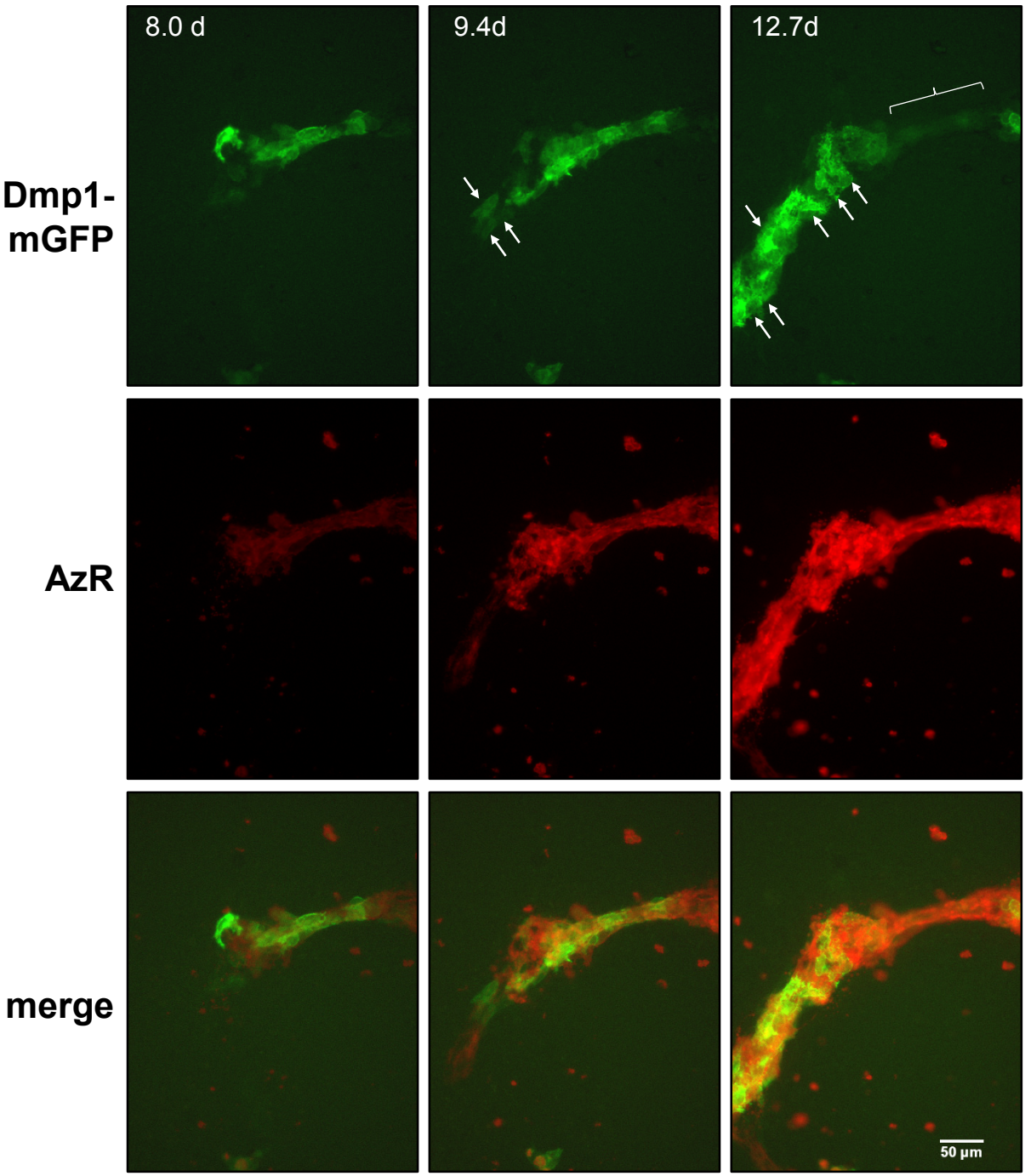

Figure S8 : Widefield epifluorescence timelapse imaging of OmGFP66 mineralized bone structures. Still frames from a timelapse movie of a day 8 OmGFP66 bone-like structure using widefield epifluorescence imaging. Dmp1-mGFP is shown in green and mineral deposition is vitally stained with alizarin red (AzR). Note that at day 8 there are small condensed ridges of Dmp1-mGFP positive cells that are closely associated with bone mineral. By 9.4 days, additional mineral has been deposited as the bone grows and there are more Dmp1-mGFP positive cells (arrows). By 12.7 days, the bone structure has continued its growth and more cells in the new bone have switched on the Dmp1-mGFP transgene (arrows). Many have also adopted an osteocyte-like dendritic morphology. Some of the Dmp1-mGFP cells appear to show motile properties before they adopt their osteocyte-like morphology. This is best appreciated by viewing the accompanying movie (**supplementary movie 1**). In the oldest region of the bone, by 12.7 days many of the cells have switched off expression of Dmp1-mGFP (see bracketed area), suggesting maturation to a late osteocyte phenotype. Bar = 50µm.

Figure S9

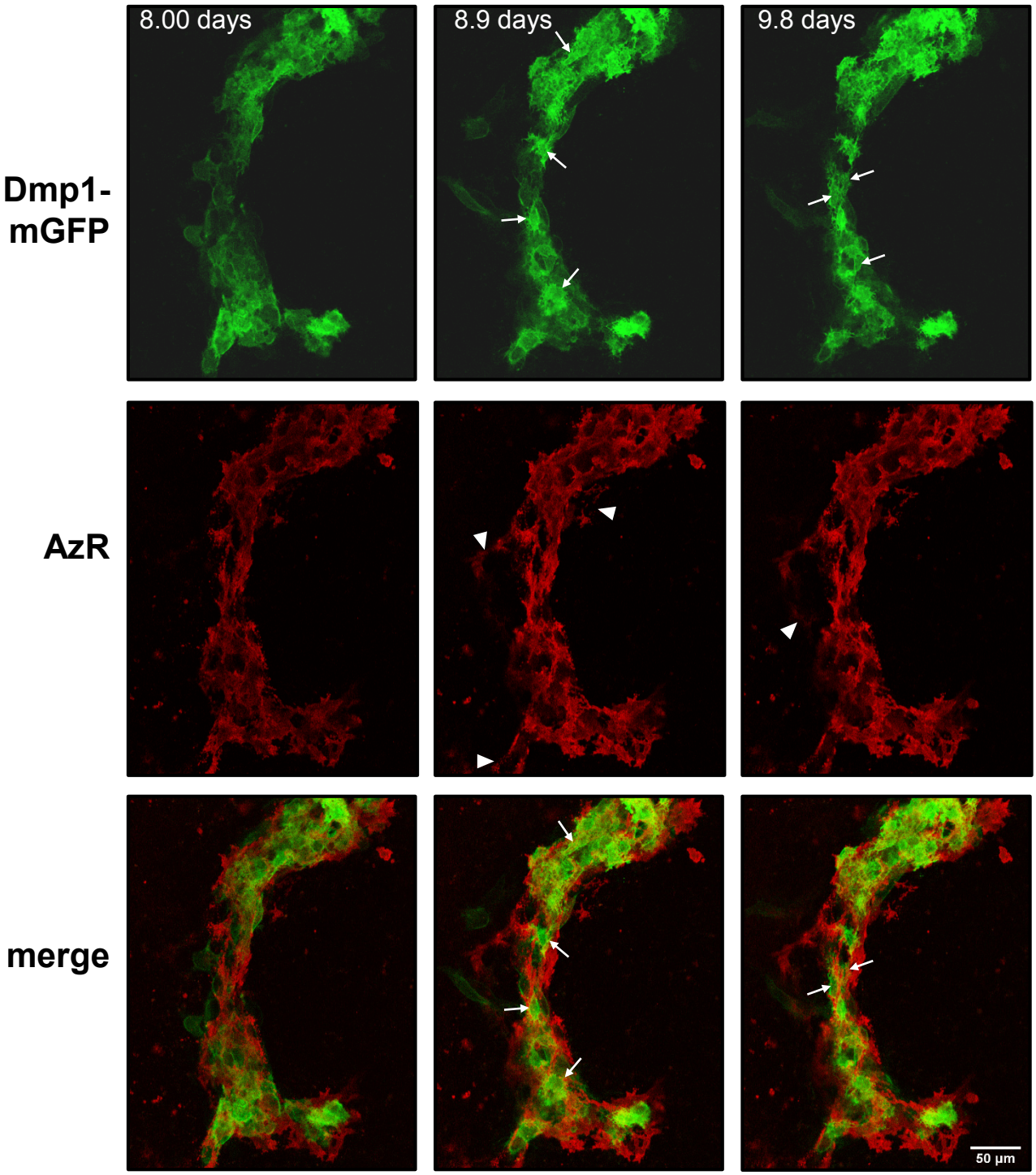

Figure S9: Confocal timelapse imaging of OmGFP66 bone-like structure. Still frames from a timelapse movie of a day 8 OmGFP66 bone stucture using confocal imaging (single Z-plane). Dmp1-mGFP is shown in green and mineral deposition is vitally stained with alizarin red (AzR). Note that at day 8 there is a condensed ridge of Dmp1-mGFP positive cells closely associated with the bone mineral. Many of these cells can be seen to be motile when viewing the accompanying movie (**supplementary movie 2**). By 8.9 days several of the cells have embedded, increased their intensity of Dmp1-mGFP expression and adopted an osteocyte-like morphology and spacing (arrows). Additional cells appear to switch on Dmp1-mGFP expression in situ within their lacunae by 9.8 days (arrows). A small amount of mineral is added to the forming bone during the 1.8 day imaging period (arrowheads). Please view **supplementary movie 2** to appreciate the dynamic motion of the Dmp1-mGFP positive cells during this maturation process. Bar= 50µm.

Legends for Supplementary Movies

Movie 1: Widefield epifluorescence timelapse movie in a day 8 OmGFP66 bone-like structure [*From left:* FIRST PANEL: Dmp1-mGFP; SECOND PANEL: alizarin red (vital stain for mineral); THIRD PANEL: merge of Dmp1-mGFP/alizarin red; FOURTH PANEL: merge of Dmp1-mGFP/alizarin red/differential interference contrast (DIC)]. Note that at the start of the movie (day 8) there are small condensed ridges of Dmp1-mGFP positive cells that are closely associated with bone mineral. By 9.5-10 days, additional mineral is deposited as the bone structure grows downwards and to the left and there are more Dmp1-mGFP positive cells. By 12.7 days, the bone has continued its growth and more cells in the new bone have switched on the Dmp1-mGFP transgene. Many have also adopted an osteocyte-like dendritic morphology. Some of the Dmp1-mGFP cells appear to show motile properties before they adopt their osteocyte-like morphology. In the oldest region of the bone, by 12.7 days many of the cells have switched off expression of Dmp1-mGFP suggesting maturation to a mature osteocyte phenotype (bracketed region in first panel). Bar = 50μm.

Movie 2: Single z-plane confocal timelapse movie of a day 8 OmGFP66 bone-like structure. [*From left:* FIRST PANEL: Dmp1-mGFP; SECOND PANEL: alizarin red (vital stain for mineral); THIRD PANEL: merge of Dmp1-mGFP/alizarin red; FOURTH PANEL: merge of Dmp1-mGFP/alizarin red/differential interference contrast (DIC)]. Note that at day 8 there is a condensed ridge of Dmp1-mGFP positive cells closely associated with bone mineral. Many of these cells are motile. By 9 days several of the cells have embedded, increased their intensity of Dmp1-mGFP expression and adopted an osteocyte-like morphology and spacing. Additional cells appear to switch on Dmp1-mGFP expression in situ within their lacunae by 9-10 days. A small amount of mineral is added to the forming nodule during the 1.8 day imaging period (arrowheads). Bar = 50μm.