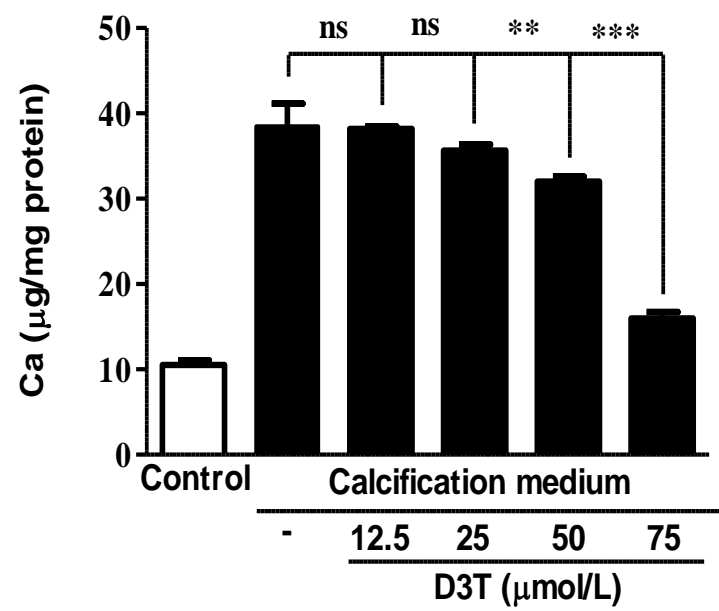
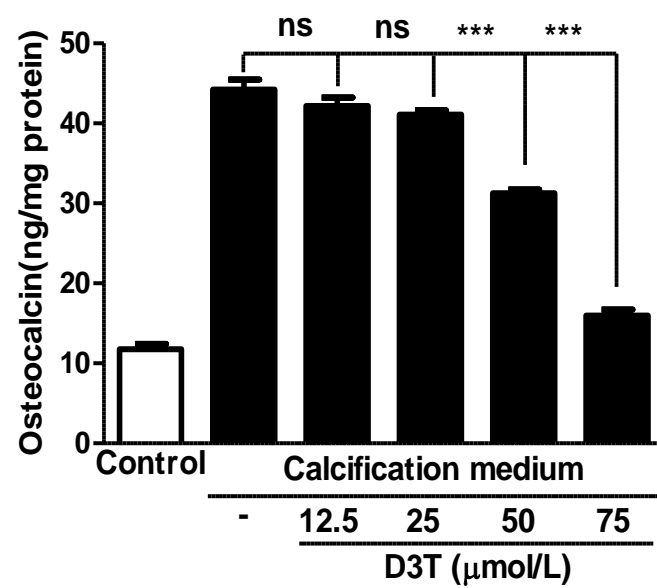
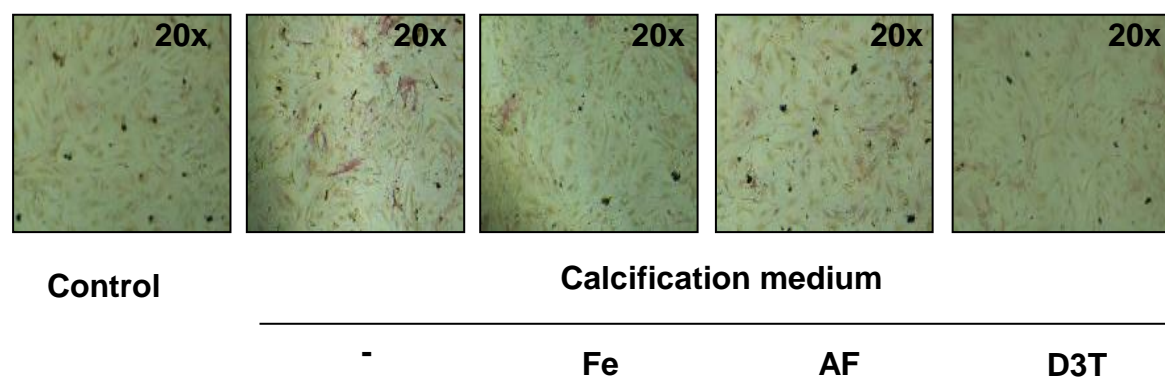
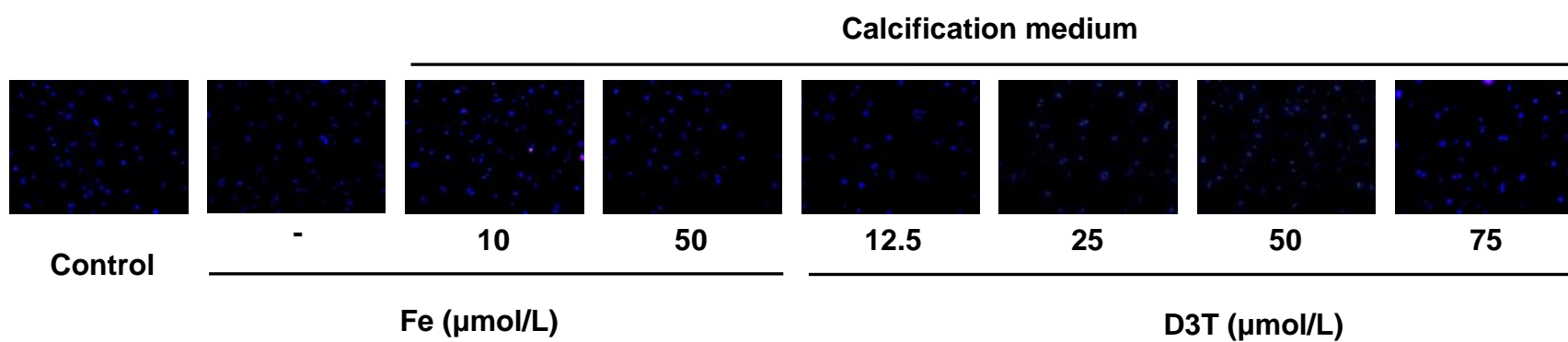
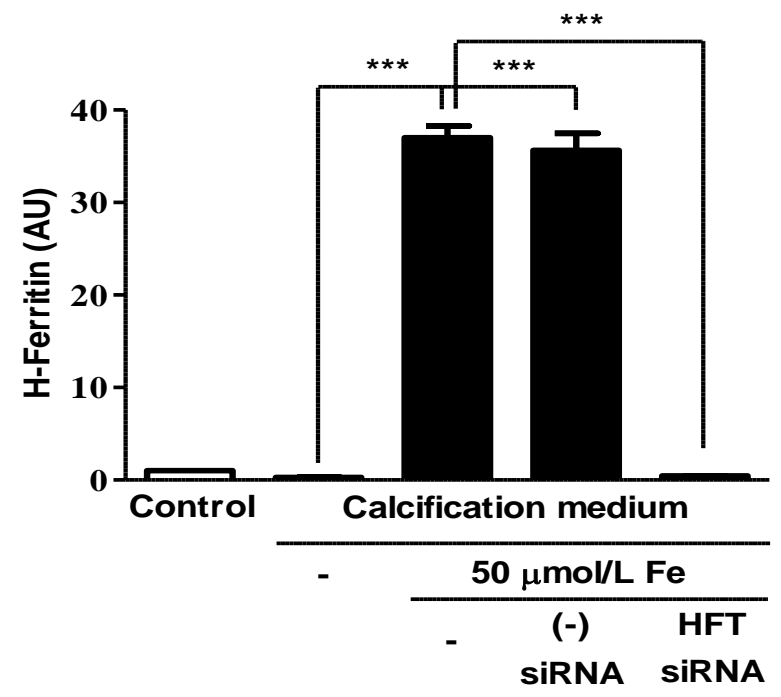
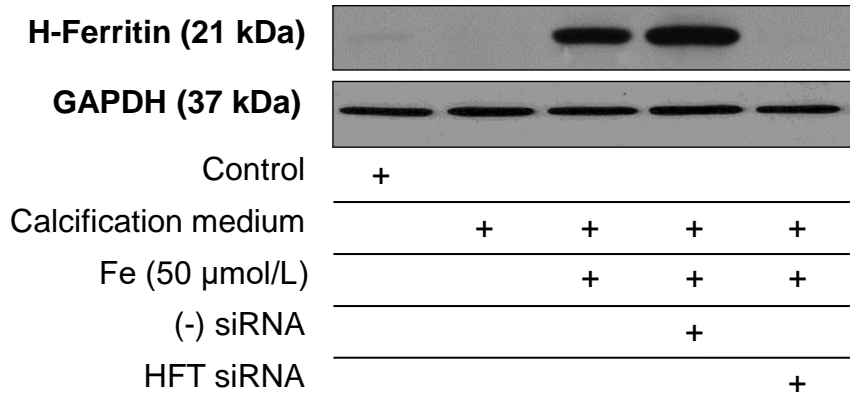
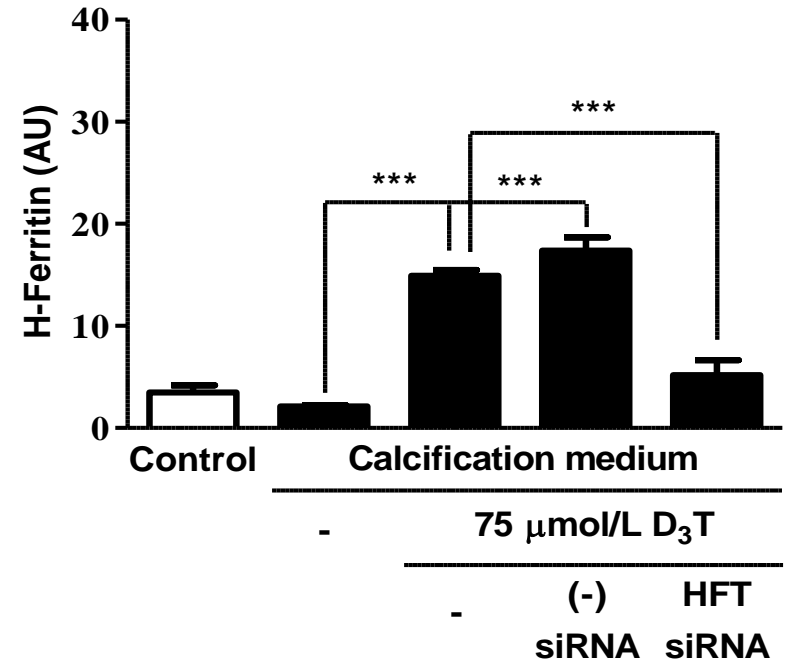
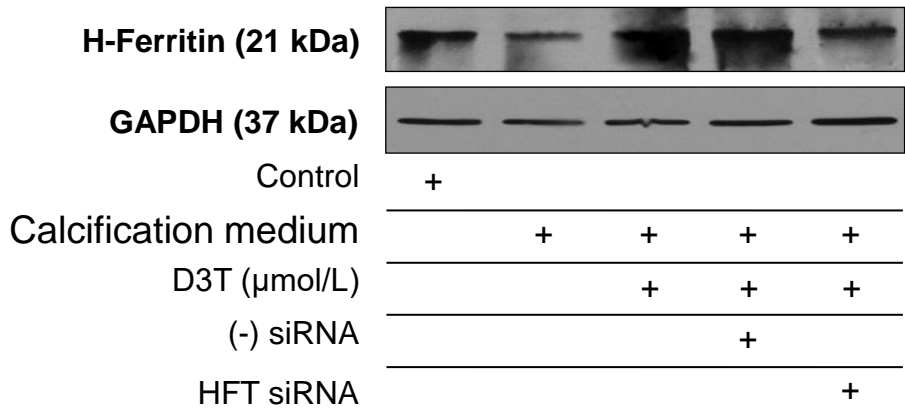


A**B****C****D**

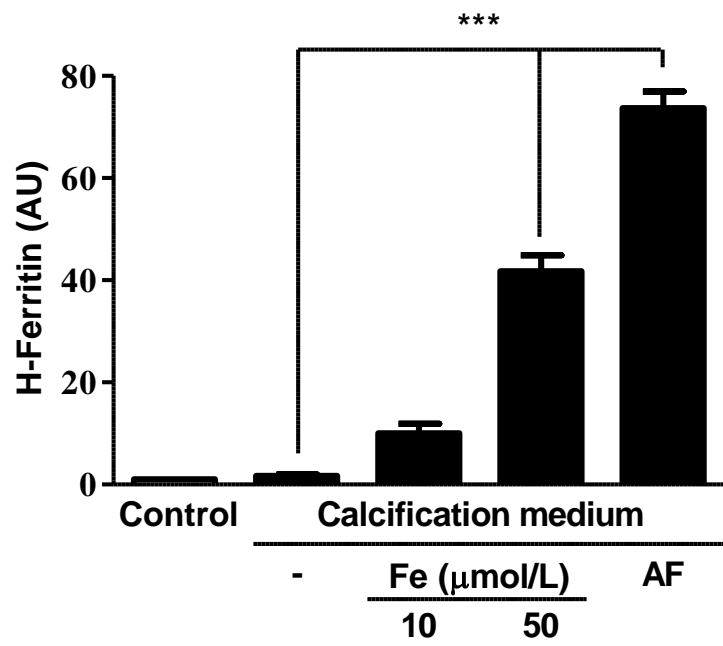
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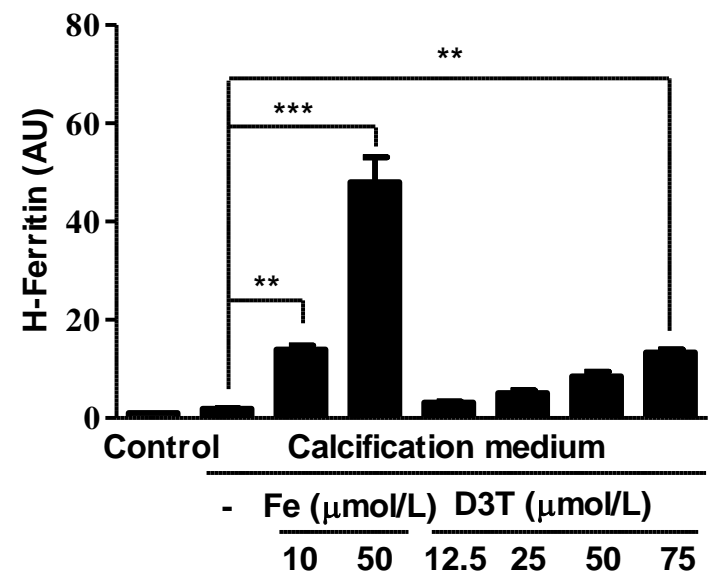
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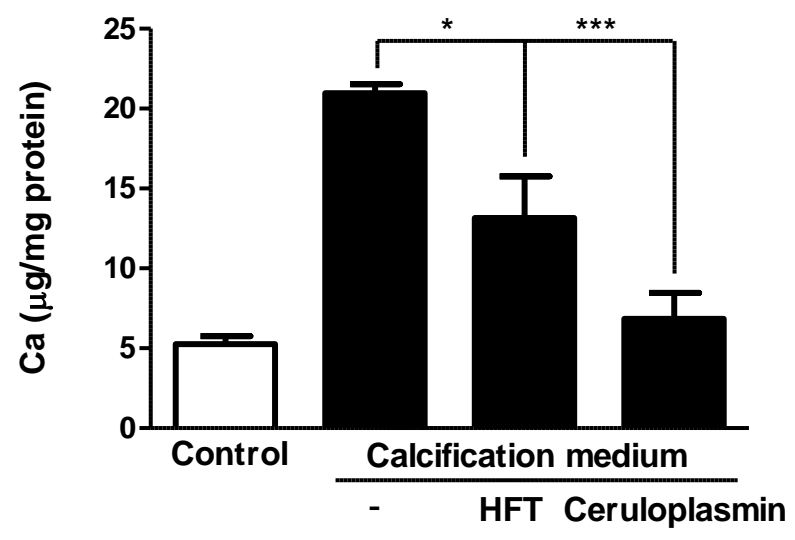
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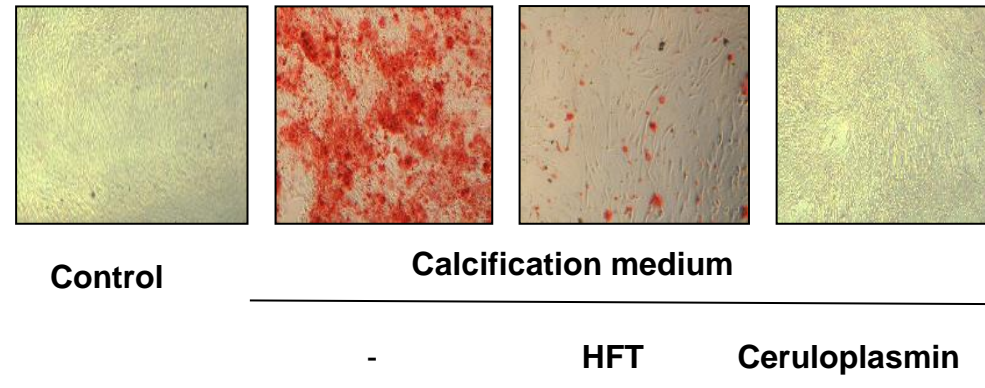
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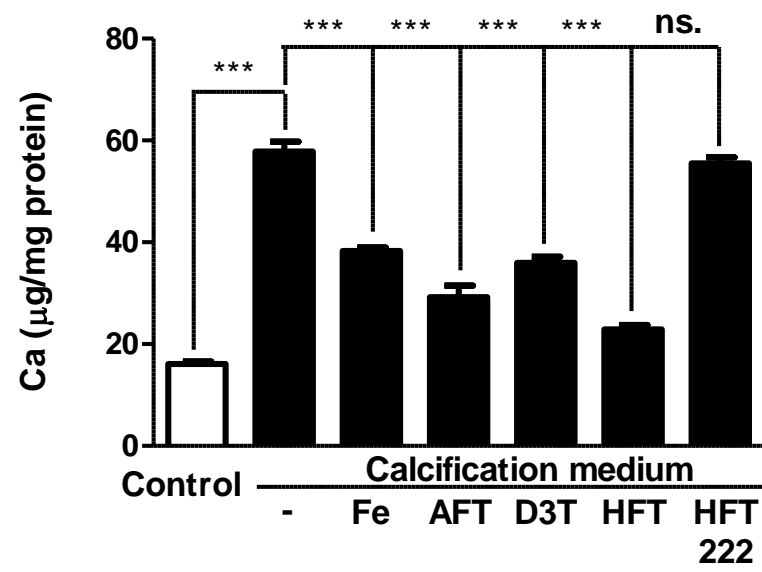
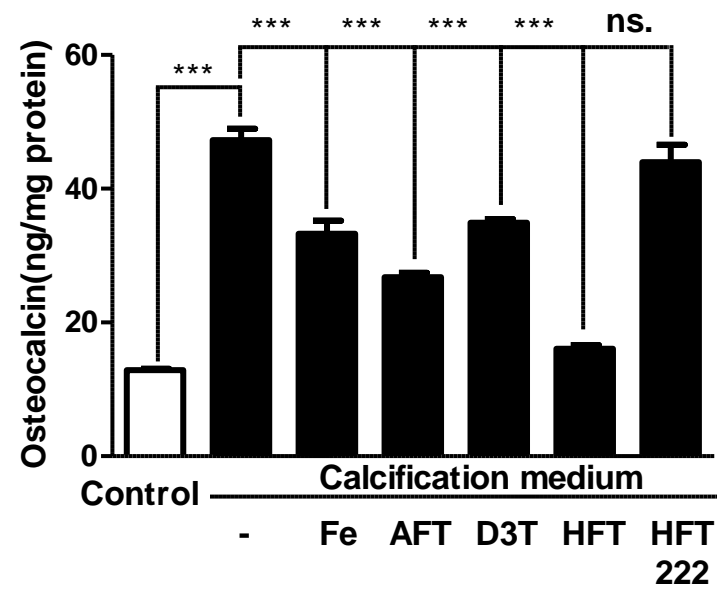
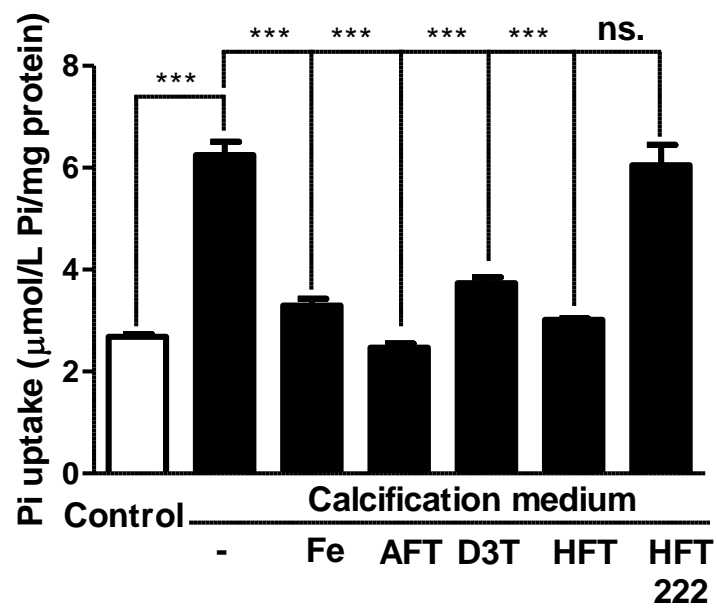


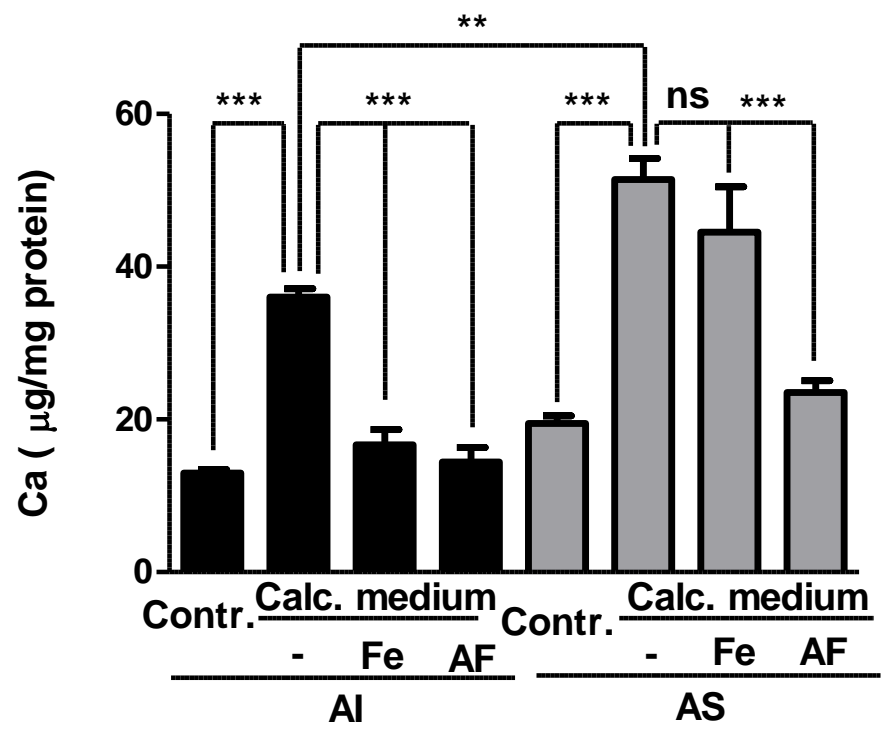
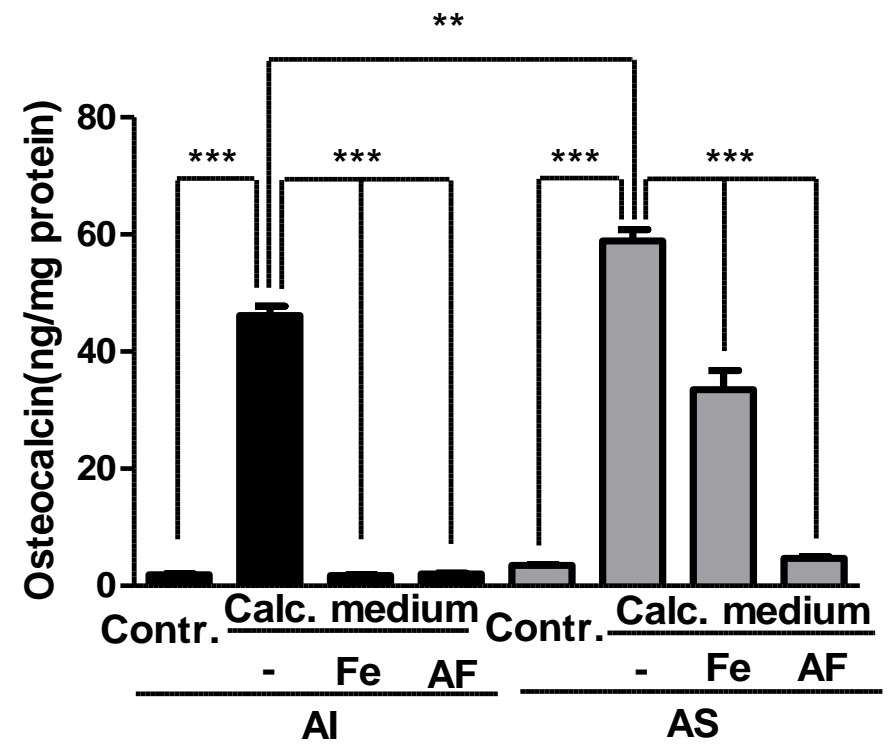
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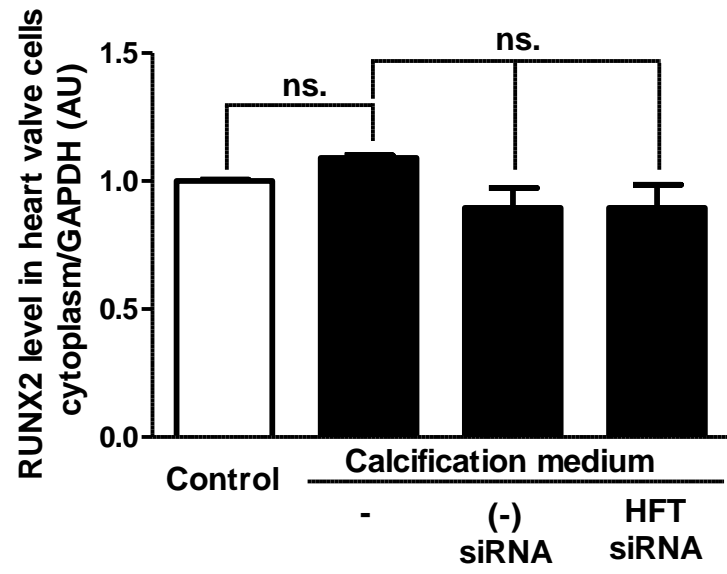
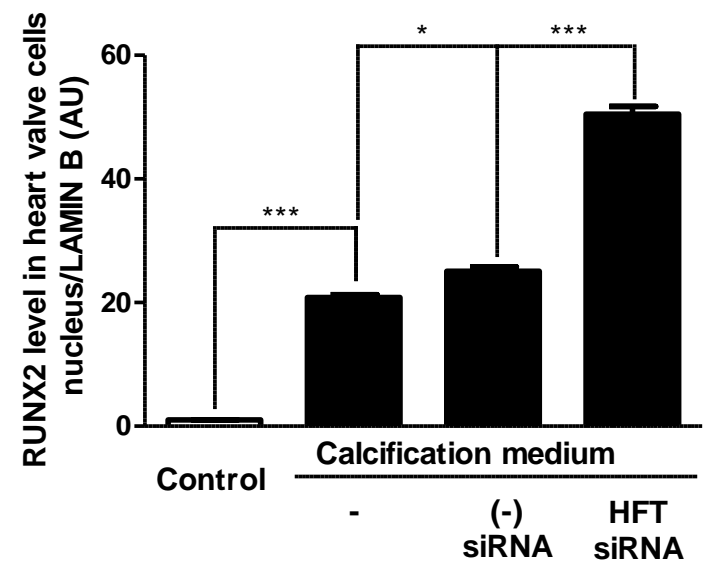
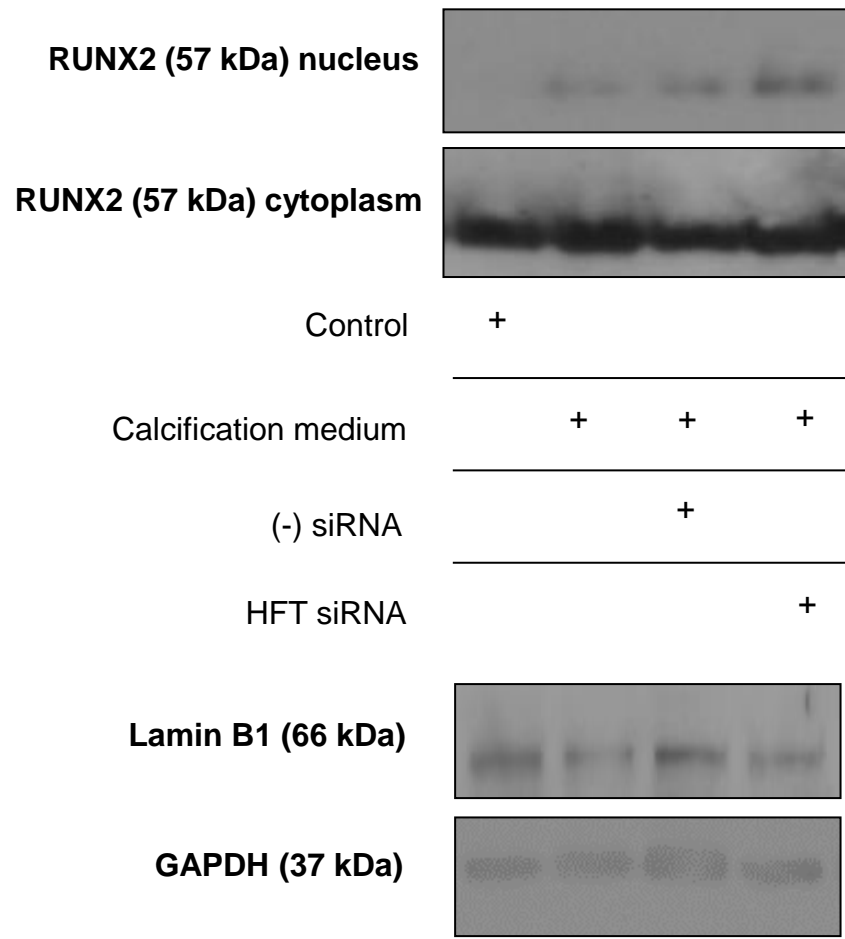
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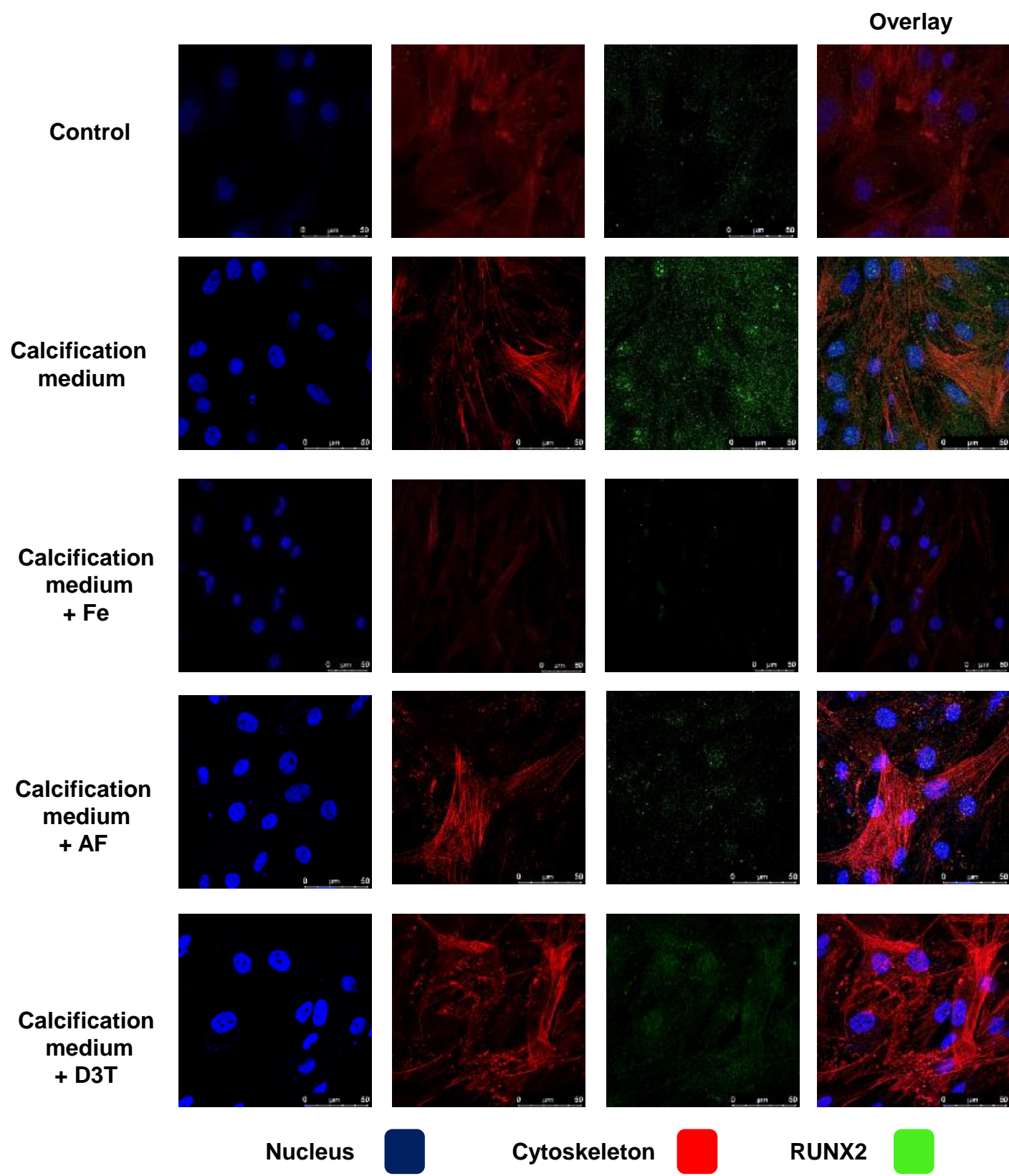
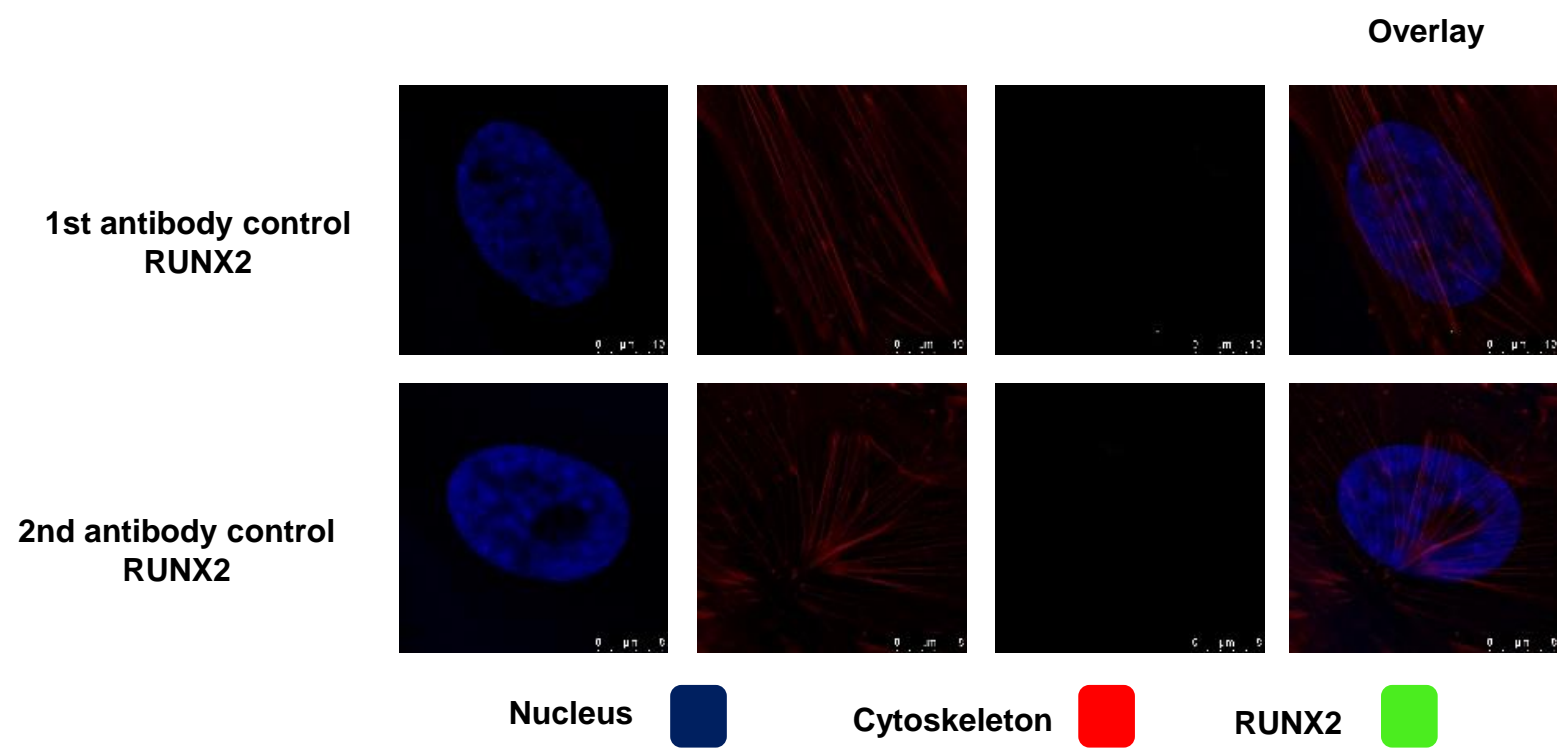
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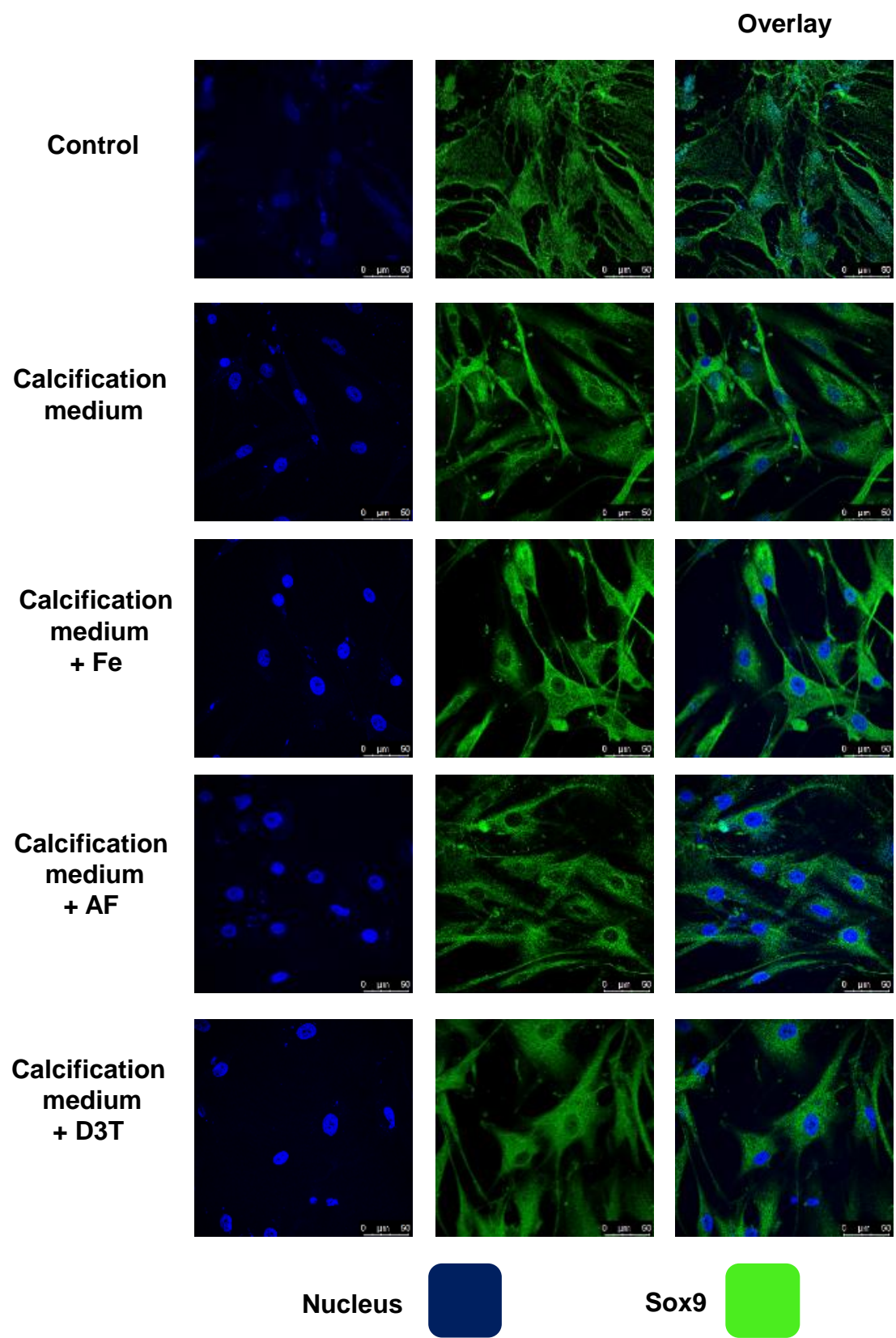
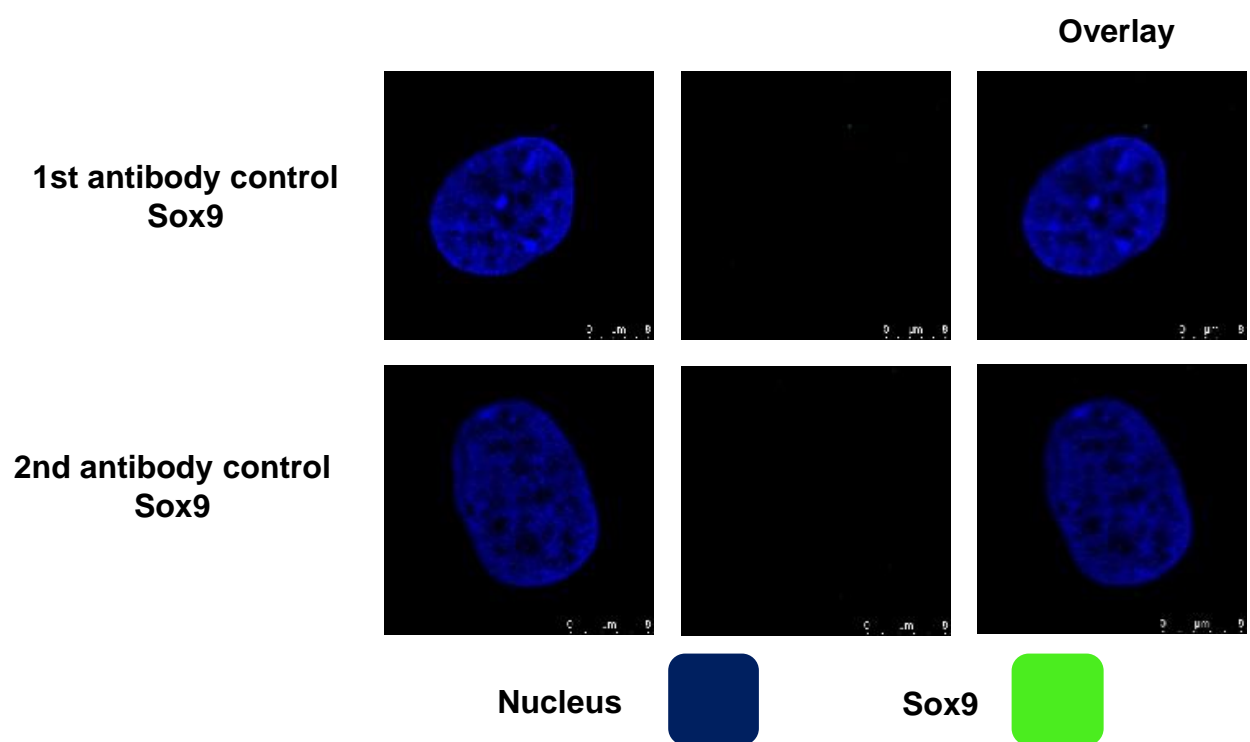
Isolated aortic valve without calcification (AI)

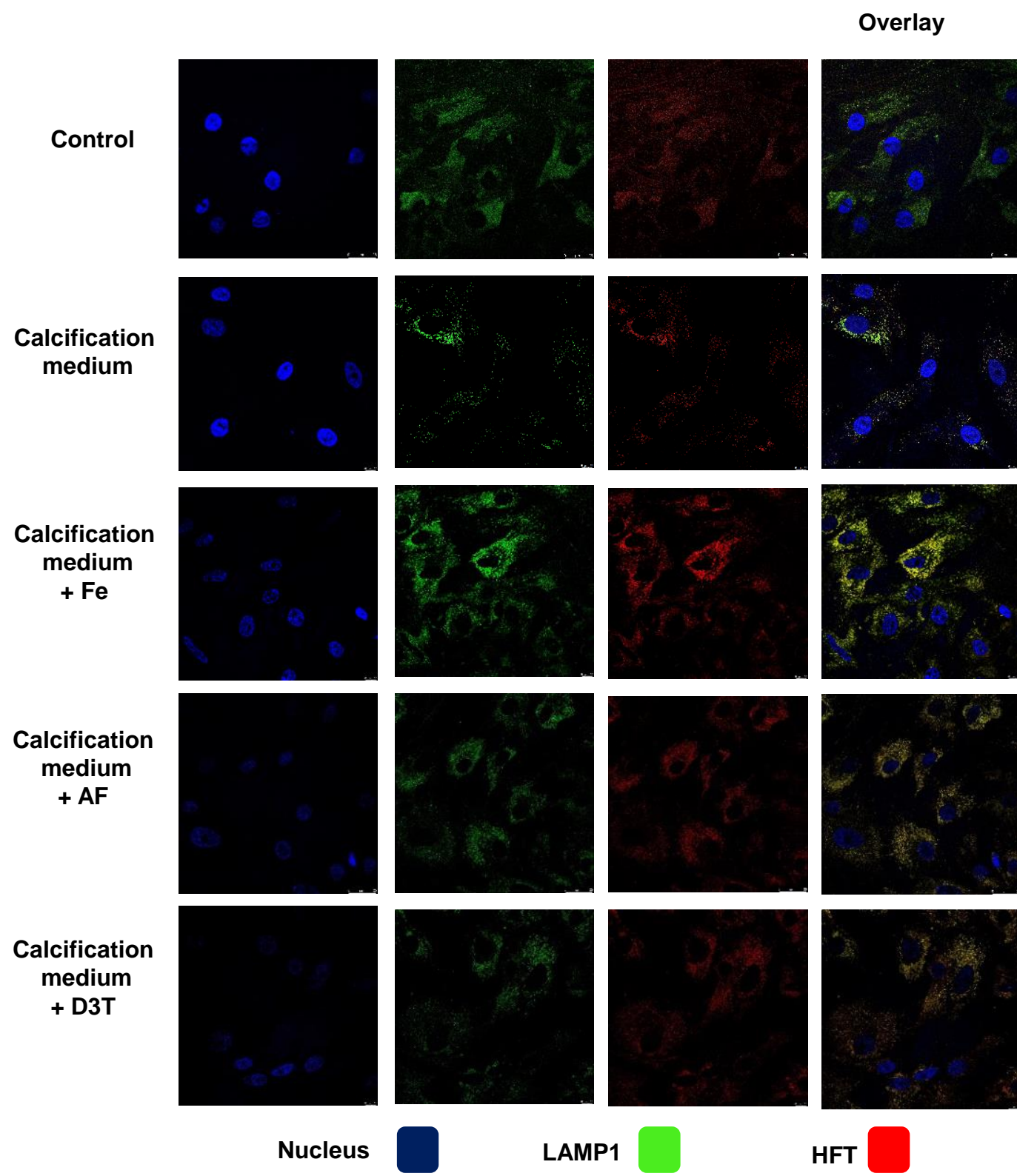
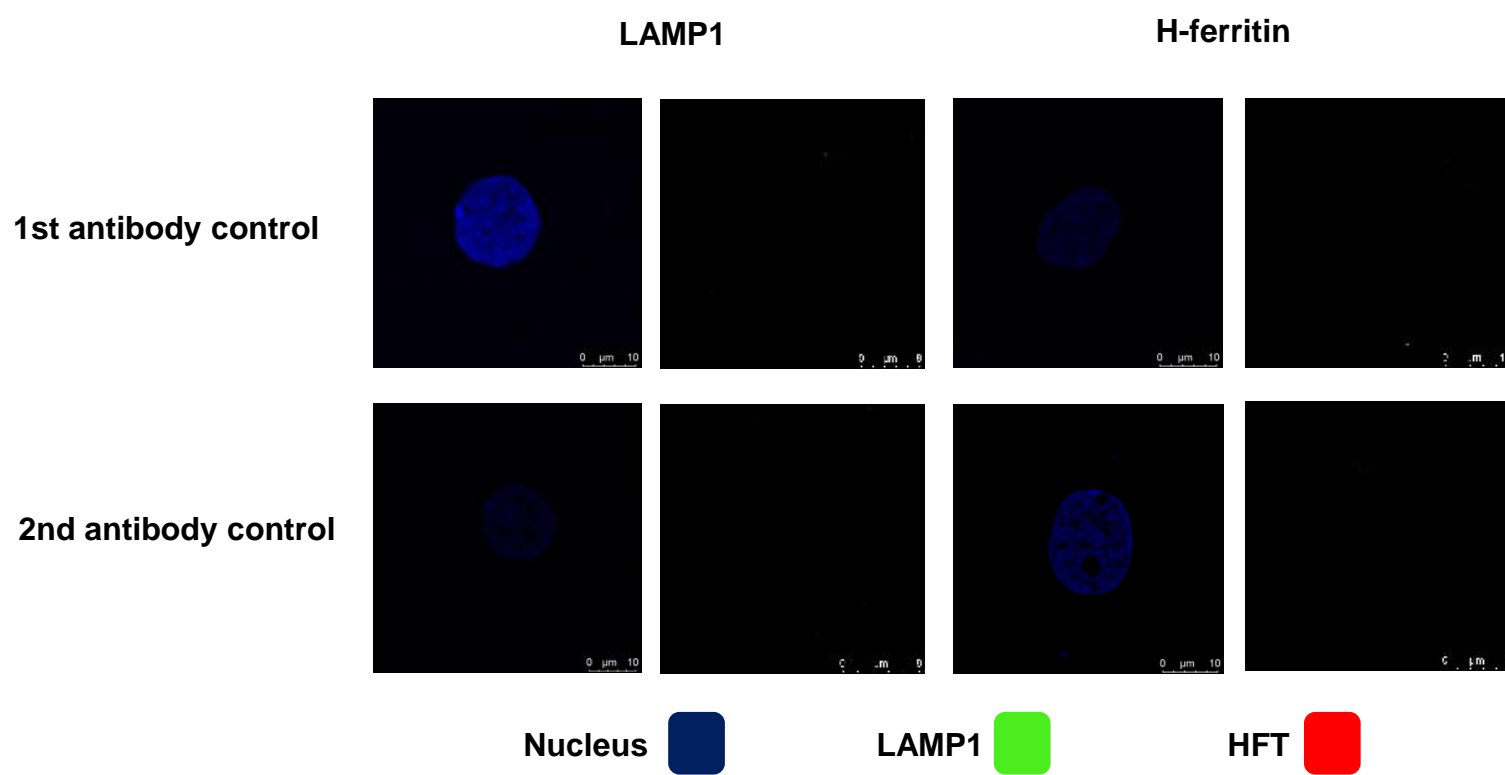


Aortic valve with stenosis and massive calcification (AS)



A**B**

A**B**

A**B**

1 **Supplementary Figure I. D3T attenuates calcification process of the valvular**
 2 **interstitial cell**

3 Upregulation of ferritin levels by iron and D3T in human valvular interstitial cells
 4 decreased calcium deposition and osteocalcin levels. VIC was cultured in growth
 5 medium or calcification medium and supplemented with 10, 50 $\mu\text{mol/L}$ iron (Ammonium
 6 iron (III) citrate), 2 mg/mL apo-ferritin or 12.5, 25, 50, 75 $\mu\text{mol/L}$ D3T for five days and
 7 cell viability was determined using fluorescence staining assay. A) Calcium content of
 8 the cells and B) osteocalcin levels were measured and normalized by cellular protein
 9 content. C) Alkaline phosphatase staining of VIC. The samples magnifications are 20x.
 10 Scale bar: 50 μm . D) Cell viability staining of VIC was shown. Data were analyzed by
 11 One Way ANOVA, Bonferroni's Multiple Comparison Test and show average \pm SEM of
 12 the three independent assays performed in triplicate. Not significant (ns), **P < 0.001,
 13 ***P < 0.0001.

14
 15 **Supplementary Figure II. H-ferritin induced by iron and D3T mediates the**
 16 **inhibition of phosphate provoked osteoblastic transformation**

17 VIC at 60% confluency was transfected with siRNA specific to H-ferritin or negative
 18 control siRNA 24 hours before the experiment. Cells were cultured in growth medium
 19 or calcification medium in the absence or presence of iron (Ammonium iron (III) citrate)
 20 or 75 $\mu\text{mol/L}$ D3T for five days. A) and B) H-ferritin western blot shows the efficacy of
 21 H-ferritin knock-down by siRNA. Densitometry of the band intensities for H-ferritin was
 22 normalized to GAPDH. C) and D) Densitometry of H-ferritin western blot of Figure 1C.
 23 Results were analyzed by Bonferroni's Multiple Comparison test (Supplementary
 24 Figure 2A-B) and One Way ANOVA, Bonferroni's Multiple Comparison Test
 25 (Supplementary Figure 2C-D) and are presented as mean values \pm SEM of at least
 26 three independent experiments each performed in triplicate. ***P < 0.0001.

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 28 **Supplementary Figure III. Ferroxidase activity of ceruloplasmin and H-ferritin is**
 29 **crucial in inhibiting differentiation of VIC to osteoblasts**

30 VIC was cultured in growth medium or calcification medium in the presence or absence
 31 of 375 $\mu\text{mol/L}$ ceruloplasmin and 1 mg/mL H-ferritin. A) Calcium content of the cells
 32 was measured and B) representative Alizarin Red S is shown. Data were analyzed by
 33 One Way ANOVA, Bonferroni's Multiple Comparison Test and samples were derived
 34 from four separate experiments performed in triplicates and shown as mean \pm SEM
 35 ***P < 0.0001.

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 37 **Supplementary Figure IV. Inhibition of calcification of VIC derived from healthy**
 38 **aortic valve by Fe, AFT,D3T, HFT and HFT222**

39 VIC cells were cultured in growth medium alone or calcification medium in the
 40 presence or absence of 50 $\mu\text{mol/L}$ iron (Ammonium iron(III) citrate), 2 mg/mL apo-
 41 ferritin, 75 $\mu\text{mol/L}$ D3T, 1mg/mL H-Ferritin and 1mg/mL H-Ferritin 222 (mutant H-
 42 Ferritin without ferroxidase activity). A) Calcium deposition, B) osteocalcin level and C)
 43 phosphate uptake were measured in VIC. Graphs analyzed by One Way ANOVA,
 44 Bonferroni's Multiple Comparison Test and show mean \pm SEM of three independent
 45 experiments. Not significant (ns), *** P< 0.0001.

46 **Supplementary Figure V. Calcification potential of AI and AS derived VIC**

47 VIC was isolated from AI or AS tissues and cultured in growth or calcification medium
 48 and supplemented with iron (Ammonium iron (III) citrate), 2 mg/mL apo-ferritin or 75
 49 $\mu\text{mol/L}$ D3T for five days. A) Calcium and B) osteocalcin level were measured. C)
 50 Macroscopic images of AI and AS valves are shown. Data was analyzed by One Way
 51 ANOVA, Bonferroni's Multiple Comparison Test s and shown average \pm SEM of the
 52 three independent assays performed in triplicate. Not significant (ns), **P < 0.001, ***P
 53 < 0.0001.

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Supplementary Figure VI. Silencing of H-ferritin facilitates nuclear translocation of RUNX2 in VIC

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Cells were transfected with siRNA against H-Ferritin. Next day cells were washed and maintained in calcification media supplemented with 1mg/mL of H-ferritin (for 2 days.) A). RUNX2 content in the nuclear and cytoplasmic fractions is shown. Data were analyzed by One Way ANOVA, Bonferroni's Multiple Comparison Test. Graph shows mean \pm SEM of five independent experiments. Ns.: not significant; *P < 0.05; ***P < 0.001.

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Supplementary Figure VII. RUNX2 high volume pictures

VIC was cultured in growth medium or calcification medium in the presence or absence of iron (Ammonium iron (III) citrate), 2 mg/mL apo-ferritin or 75 μ mol/L D3T. A) Immunofluorescence staining of VIC for RUNX2 is shown. Images were obtained employing immunofluorescence-confocal microscope. B) Antibody controls of immunofluorescence staining were shown. Representative staining is shown from at least three independent experiments.

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Supplementary Figure VIII. Sox9 high volume pictures

VIC was cultured in growth medium or calcification medium in the presence or absence of iron (Ammonium iron (III) citrate), 2 mg/mL apo-ferritin or 75 μ mol/L D3T. A) Immunofluorescence staining of VIC for Sox9 is shown. Images were obtained employing immunofluorescence-confocal microscope. B) Antibody controls of immunofluorescence staining are shown. Representative staining is shown from at least three independent experiments.

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Supplementary Figure IX. LAMP1-H-ferritin double immunostaining high volume pictures

VIC was cultured in growth medium or calcification medium in the presence or absence of iron (Ammonium iron (III) citrate), 2 mg/mL apo-ferritin or 75 μ mol/L D3T. A) Double immunofluorescence staining of VIC for LAMP1 and H-ferritin are shown. Images were obtained employing immunofluorescence-confocal microscope. B) Antibody controls of immunofluorescence staining are shown. Representative staining is shown from at least three independent experiments.