UNIVERSITY OF
EASTERN FINLAND

Development of a reporter assay for tracing vascular calcification in smooth muscle cells

Olayinka Oluwasegun Agbabiaje
Master´s Degree Programme in
Biomedicine
University of Eastern Finland
Faculty of Health Sciences
School of Medicine
Institute of Biomedicine
05.10.2023

University of Eastern Finland, Faculty of Health Sciences, School of Medicine
Institute of Biomedicine
Master´s Degree Programme in Biomedicine
Agbabiaje, Olayinka Oluwasegun: Development of reporter assay for tracing vascular calcification in smooth muscle cells
Master of Science thesis; 50 pages, 14 appendices (14 pages)
Supervisors: Redouane Aherrahrou, Senior Researcher, PhD, Minna Kaikkonen-Määttä, Professor, AIV Institute, University of Eastern Finland, Kuopio
05.10.2023

Cardiovascular diseases, Vascular Smooth muscle cells, Calcification, Reporter genes, Fluorescence intensity (mCherry), Calcein

Abstract

Cardiovascular diseases (CVDs) continue to be a significant public health challenge. Vascular calcification (VC) is a major risk factor for the development and progression of CVDs. Smooth muscle cells (SMCs) transition into osteoblast-like cells and represent the primary contributor to the pathogenesis of calcification. Here we aimed to develop a reporter assay, to trace the activation of calcification events using five selected calcification markers (ALPL, RUNX2, ACAN, CHAD and COL2a1). Cultured human coronary smooth muscle cells (HCASMCs) were transduced with viral vector containing reporter construct driven by the above calcification marker gene promoters. Stable clones were selected using puromycin and calcification was induced with two calcifying media (inorganic phosphate (IP) and osteogenic medium (OM)) for seven (7) days. As a control we utilized media without calcification. Calcification nodules were generated and stained with calcein, dissociated with EDTA-collagenase assay into single cells and sorted by FACS. Images were generated from Incucyte Zoom. Data were analysed using Fiji/ImageJ and GraphPad (10.0.2). Calcification was successfully induced in all the stable lines, with IP treated cells showing early stronger calcification as compared to cells treated with OM. Furthermore, OM treated cells, demonstrated changes in the cell morphology and crystals rather than calcified nodules. There was no significant difference between the basal condition and IP treated cells, but there was a difference in OM-treated cells. FACS analysis demonstrated that ALPL and CHAD exhibited the highest reporter gene expression in response to OM, suggesting their superiority over the

other reporters in reflecting the calcification response of SMCs. In future, the reporter system could serve as a quick assay for effectiveness in CRISPR or drug screening for VC.

Introduction

Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels, including coronary heart disease, cerebrovascular disease, rheumatic heart disease, and other conditions (Roth et al, 2015; McAloon et al, 2016; Vaduganathan et al, 2022). According to the World Health Organization (2022), 17.9 million people worldwide are estimated to die from CVDs each year (WHO, 2022). Globally, the number of CVD deaths has increased from 12.1 million in 1990 to 20.5 million in 2021, with low- and middle-income nations accounting for four out of every five CVD fatalities (World Health Federation, 2023). In the United States (US), about 695,000 people died from heart disease in 2021, which is one in every five deaths (Vaduganathan et al, 2022). According to preliminary mortality data for 2021, heart disease and stroke continued to be the US's top two killers, respectively, even during the COVID-19 pandemic. (Ahmad, Cisewski and Anderson, 2022). Similarly, Townsend et al. (2022) reports that CVD is one of the leading causes of death in Europe, accounting for 37% of deaths within the European Union and 45% of fatalities across the continent.

Worldwide trends for disability-adjusted life years for CVD and the burden of CVD related to modifiable risk factors have also increased significantly since 1990 (Murray et al. 2020), despite improvements in managing CVD and other health outcomes. This is so because there are numerous and diverse risk factors for CVD. Tobacco use, diabetes, hypertension, high cholesterol, obesity, and unbalanced diets are some of the more prevalent risk factors. Studies have shown that CVDs are important risk factors that can influence the development of diverse pathological conditions such as metabolic syndromes (Guembe et al, 2020), diabetes (Leon and Maddox, 2015; Dal Canto et al, 2019) and kidney disease (Major et al. 2018; Jankowski et al. 2021). Therefore, these studies highlight the interconnecting relationship between the state of the heart's health and the incidence of these diseases and how these diseases influence an increased incidence of CVDs.

The heart remains the focal point of cardiovascular diseases (Fuchs and Whelton, 2019). As a vital organ in the circulatory system, it functions as a four-chambered muscular pump that propels oxygenated blood throughout the body (Kuhn and Lynch, 2016). The right side of the heart receives oxygen-poor blood from the body and pumps it to the lungs for oxygenation. The left side receives oxygenated blood from the lungs and pumps it to the rest of the body (Lusis, 2000). Circulation of oxygenated blood to the rest of the body is executed via blood vessels called arteries (Björkegren and Lusis, 2022). Several

studies have highlighted that the leading cause of death associated with cardiovascular disease is majorly caused by atherosclerosis (Mahmoud et al, 2014; Björkegren and Lusis, 2022). Coronary artery disease (CAD), often referred to as ischemic heart disease (IHD) or coronary heart disease (CHD), remains the most prevalent form of atherosclerotic cardiovascular disease (Shao et al, 2020). It is primarily caused by the gradual build-up of atherosclerotic plaques within the coronary arteries, which supply oxygen and nutrients to the heart muscle (Bentzon et al, 2014). Frostegård (2013) reports that these plaques consist of cholesterol, fatty deposits, inflammatory cells, and cellular debris that accumulate within the arterial walls.

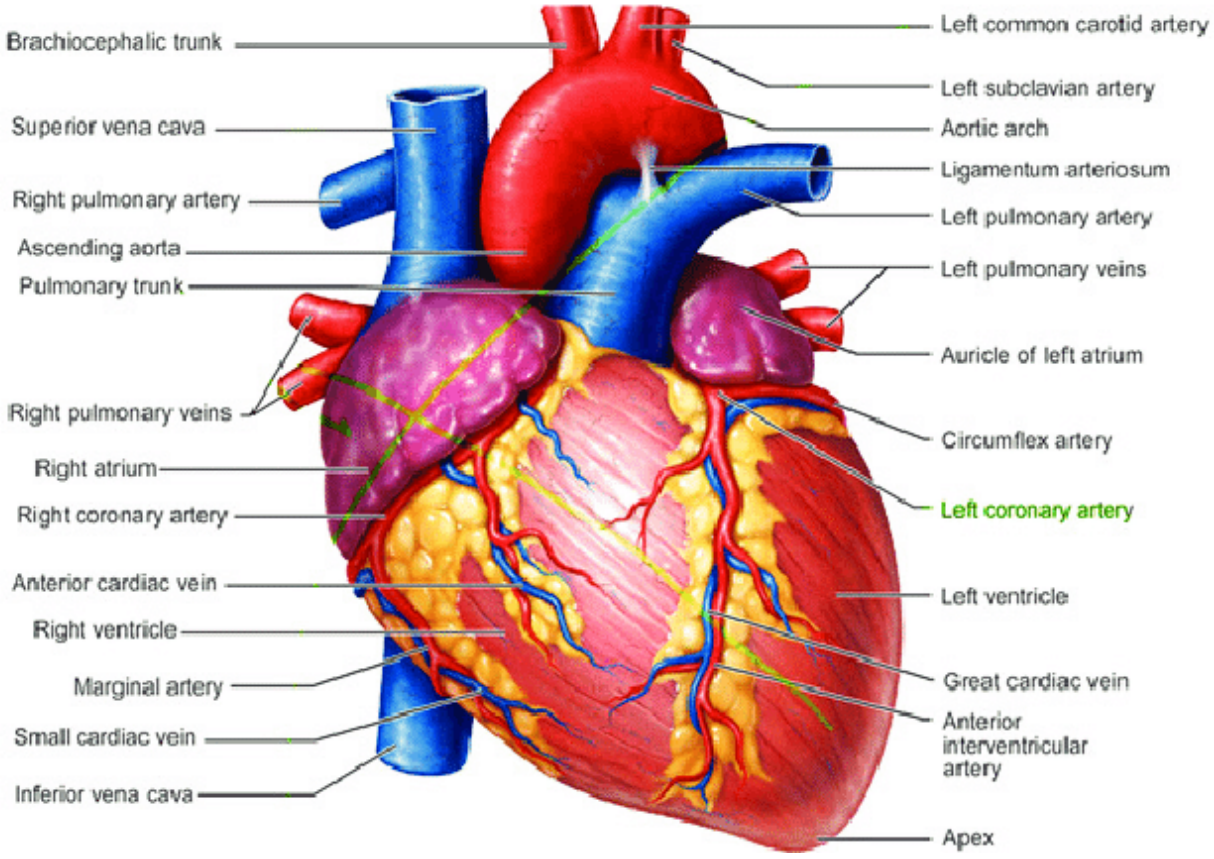


Fig. 1: Anatomy overview of human heart showing its vasculature including the coronary arteries.

Source: Kuhn and Lynch (2016).

Aside from the lifestyle risk factors of CVDs, there are pieces of evidence that the major risk factor for the development and progression of CVDs is a pathophysiological condition referred to as vascular calcification (VC) (Himmelsbach et al, 2020; Lee et al, 2021). Many diseases such as atherosclerosis, chronic kidney diseases and diabetes have been proven to have high prevalence of VC (Stabley et al,

2017; Valdivielso et al, 2019). In advanced cases, atherosclerotic plaques may advance further in development, undergoing the process of calcification, thereby contributing to the plaque stability, rigidity, and reduced flexibility (Trion and van der Laarse, 2004; Achim et al, 2022). Therefore, this transformative process has implications for the mechanical properties of the artery, reducing its ability to dilate and contract properly and further comprising the heart's ability to function.

Calcification in the context of cardiovascular diseases (CVDs) involves the accumulation and deposits of calcium and phosphate known as hydroxyapatite within tissues, particularly in the blood vessels and heart valves (Lee, Morrisett and Tung, 2012). In CVDs, calcification takes on two primary forms: vascular calcification and valvular calcification (Obisesan et al, 2022; Vieceli Dalla Sega et al, 2022). Vascular calcification (VC) occurs within the arterial walls, especially in areas affected by atherosclerosis (Wu, Rementer and Giachelli, 2013; Lee, Lee and Jeon, 2020). Valvular calcification, on the other hand, involves the deposition of calcium on heart valves, like the aortic and mitral valves (Achim et al, 2022). The accumulation of calcium on valve leaflets leads to their stiffening and reduced capacity to open and close as needed. This condition, known as valvular stenosis, obstructs blood flow and places additional strain on the heart (Björkegren and Lusis, 2022).

VC occurs in both intimal and medial layers of the blood vessel (arteries) (Durham et al, 2018) Fig.1. Intimal calcification (IC) occurs in atherosclerotic plaque formed in the lumen of the blood vessel, consisting of fibrous cap and other cell types contributing to stability, rupture of plaque and restriction of blood flow (Lanzer et al, 2021). As shown in Figure 4, medial calcification (MC) has been discovered to be caused by alteration of the VSMCs microenvironment giving rise to diverse cell type such as the osteoblast-like, foam cells which promote mineralization (deposit and accumulation of calcium and phosphate) in extracellular matrix (Durham et al, 2018). Arterial blockage and atherosclerotic plaque rupture are both associated with intimal calcification (Karwowski et al, 2012). Contrarily, medial calcification is associated with vascular stiffness, systolic hypertension, and increased pulse wave velocity, all of which contribute to heart failure and diastolic dysfunction (Chow and Rabkin, 2015; St. Hilaire, 2022). VC is an active process similar to bone formation process (Espinosa-Diez *et al*., 2021). The pathogenesis of VC has been reported to be primarily driven by vascular smooth muscle cells (VSMCs) (Durham *et al*., 2018).

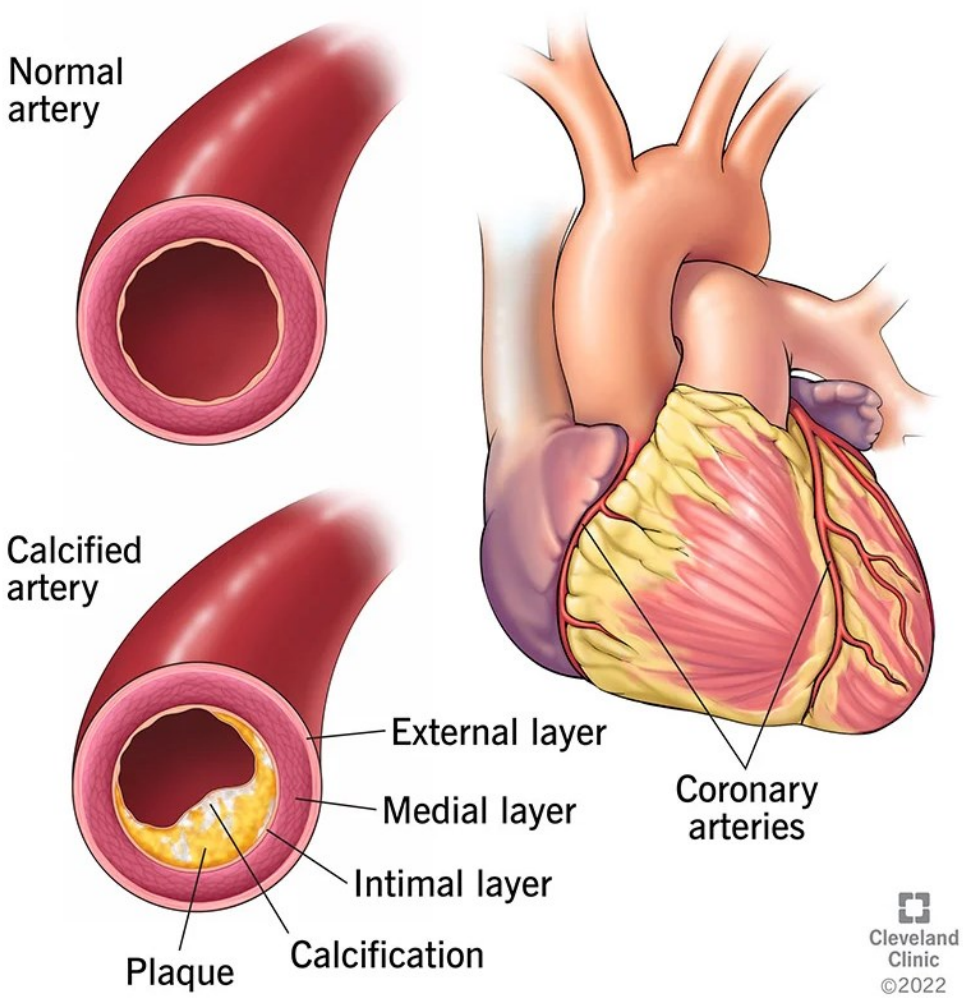


Fig.2: Coronary Artery Calcification

In the blood vessel there are diverse cell types which include VSMCs, pericytes, fibroblasts, endothelial cells contributing to the normal physiological function of the vessel (Jiang et al, 2021). VSMCs are integral components of blood vessel playing various crucial role in many physiological processes. They are non-striated, contractile and non-voluntary cells which are not terminally differentiated and show phenotypic plasticity. They are known to be the most abundant cell type in the blood vessel that aids proper flow of blood around the body by contraction and relaxation (Durham et al, 2018). These cells possess unique contractile properties, enabling them to regulate blood vessel tone, blood pressure, and blood flow (Anwar et al, 2012; Cao et al, 2022).

In normal physiological state, VSMCs are contractile, not dividing or migrating however, when altered due to specific stimuli such as response to injury or pathogenesis such as mineral imbalance,

atherosclerosis etc. in their microenvironment, they undergo phenotypic switch; hence, the cells are said to obtain a synthetic state (which can be plastic and known as differentiated cells) (Schurgers et al, 2018). The characteristic features observed during phenotypic switch include accelerated cell proliferation, migration and extracellular matrix (Schurgers et al, 2018). Furthermore, phenotypic switching of VSMCs has been established with the onset of atherosclerosis and vascular calcification (Tang et al, 2022). In addition, several studies have identified genes that are differentially expressed these alternative states of VSMC phenotypes such as SM α-actin, smoothelin, calponin acting as markers of contractile SMCs and, Runt-related transcription factor 2 (RUNX2), Alkaline phosphatase (ALPL), SOX9, Aggrecan (ACAN), CHAD, osteopontin, Col2A1, Osterix (OSX), as markers of synthethic osteogenic converting SMCs (Alves et al, 2014; Durham et al, 2018). When VSMCs undergo phenotypic switch, they have been discovered to switch towards osteogenic environment (Durham et al, 2018). Although, phenotypic switching process is important for the process of vascular repair in SMCs due to their plasticity. However, the VSMC are susceptible to changing to diverse cell types such as osteoblast-like cells, foam cell-like cells etc. (Pustlauk et al, 2020).

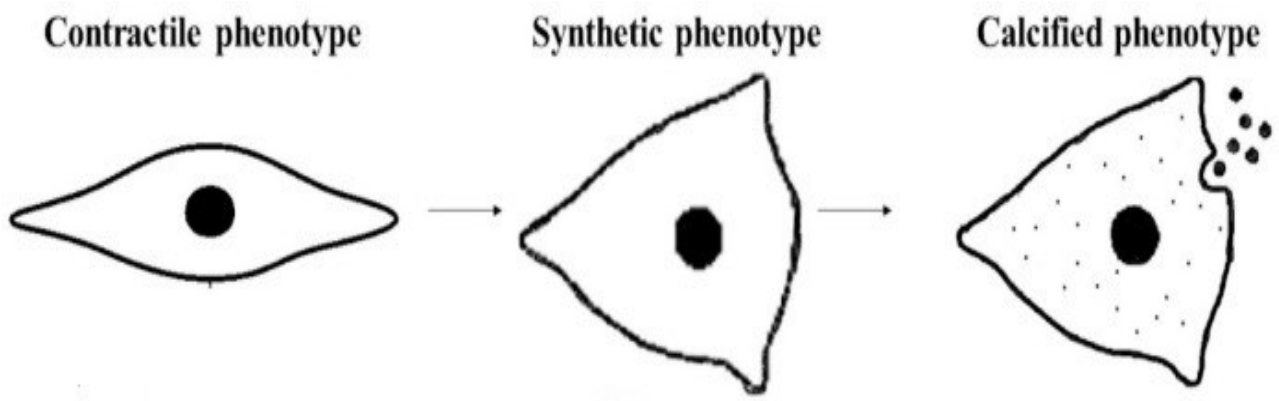


Fig. 3: Cell morphology changes occurring during VSMCs switch from contractile to calcified phenotype. The calcified phenotype shows released matrix vesicles which contributes to nucleation of calcium and phosphate deposits and accumulation.

Source: Ceccherini et al, 2022

A review on VC pathogenesis has proven that in the aging population and individuals with pathological conditions such as chronic renal disease, obesity, there is high expression of osteochondrogenic gene markers RUNX2, ALPL, Collagen type 1 and 2 etc (Durham *et al*., 2018). Many other factors which include

cytokines (such as Tissue-nonspecific alkaline phosphatase (TNAP)) is associated with the expression of the osteochondrogenic genes hence increasing the risk of VC (Savinov et al, 2015). TNAP acts by hydrolysing pyrophosphate (PPi), thereby enabling deposited calcium and phosphate forming crystals in the extracellular matrix to increase association with collagen matrix (Kawtharany et al, 2022). Furthermore, overexpression of Tissue-nonspecific alkaline phosphatase (TNAP) in the vasculature has been established and frequently observed in VC (Sheen *et al*., 2015; Savinov *et al*., 2015). In addition, TNF (Tumor necrosis factor alpha) increases the development and progression of calcification in vascular cell cultures (Lai et al, 2012; Lim et al, 2016). When active, the transcription factor RUNX2, which is involved in phenotypic switch of VSMCs to osteoblast-like phenotype, orchestrates the expression of downstream genes (such as ALPL, Collagen Type 1, Osterix) that are critical in inducing mineralization (Komori et al, 2010; Long, 2012; Durham et al, 2018). The alteration in the genes associated with the activity of VSMCs are established to contribute to the components of atherosclerotic plaque.

The enzyme alkaline phosphatase (ALP) is homodimeric which catalyses dephosphorylation in alkaline pH conditions (Shaban et al, 2022). ALP is also an osteoblast functional phenotypic marker which reduces the synthesis of inorganic pyrophosphate, a strong inhibitor of vascular calcification (Liu et al, 2018). Several ectonucleotidases, notably ALPL and ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), are expressed in valve interstitial cells (VICs) in response to a procalcifying signal. The latter generates pyrophosphate, a powerful mineralization inhibitor (Côté et al, 2012). ALPL, on the other hand, hydrolyses a wide range of phosphate-rich compounds, including pyrophosphate, to produce inorganic phosphate. As a result, high levels of ALPL and ENPP1 expression contribute to the release of significant amounts of inorganic phosphate (*Côté* et al, *2012*). An increased inorganic phosphate ratio to pyrophosphate stimulates tissue mineralization (Chignon et al, 2020). Zhu et al, 2011 studied VSMC calcification using both an *in vitro* model and ex vivo model of medial calcification, they reported that calcified VSMCs showed increased key osteogenic marker (alkaline phosphatase, phosphate transporter (PiT)) upon induction of calcification (Zhu et al, 2011).

Runt-related transcription factor 2 (Runx2), also known as polyoma-enhancer binding protein 2A (Pebp2A), is a crucial regulator of osteoblastic development and maturation of chondrocyte (Wu et al, 2014; Jiang and Qian, 2023). RUNX2 is mainly associated with bone and cartilage development and remodelling, however, it has been proven to play a significant role in VC. Its expression in VSMCs, similar

to bone cells, acts as an early, unequivocal marker of osteoblastic differentiation, the first stage in vascular calcification (Sun et al, 2012). A growing number of research on diverse factors that contribute to VC, including increasing inorganic phosphate concentration, have been demonstrated to stimulate RUNX2 expression (Lin et al, 2016; Kim et al, 2020). The VSMC-specific deficiency of RUNX2 expression inhibits vascular calcification. Therefore, RUNX2 can serve as an early and definitive marker of osteoblastic differentiation, bone formation, and initiation of vascular calcification (Liu et al, 2018). Several studies have shown RUNX2 as a major factor in phenotypic switching of SMCs and suppression of normal SMCs phenotype, a feature of established VC pathogenesis. Studies have implicated the increased expression of RUNX2 in calcified arterial tissues of chronic kidney disease (CKD) patients. In the development of calcification with atherogenic lesions, hydrogen peroxide triggers the phenotypic switch of VSMC due to increased RUNX2 expression and transactivation through Akt signalling (Lee, 2020). Interaction between RUNX2 and downstream transcription factor genes such as osterix (also known as SP7) have been reported to drive osteogenic phenotype during osteogenesis and VC (Durham et al, 2018).

Excessive collagen build-up in tissue has been linked to a number of clinical disorders, including organ fibrosis, malignancies, bone disease, and VC. Type II collagen alpha 1 chain (Col2a1) encodes the alpha-1 chain of type II collagen, a fibrillar collagen found in cartilage and vascular tissue linings. Col2a1 interacts with RUNX2 to drive SMCs towards the osteogenic phenotype (Durham et al, 2018). Col2a1 expressed in transdifferentiated VSMCs is a characteristic marker of chondrocyte phenotype in vascular calcification (Augstein et al, 2018). In dialysis patients, the phenotypic switch of VSMCs to osteoblast-like phenotype generating VC was observed in the medial layer of the blood vessel (Dube et al, 2021). Previous studies on tracing of SMC lineage in atherosclerosis led to the discovery of three definite clusters of SMC derived cells which include fibromyocytes, pro-calcific chondromyocytes and mature SMC (Alencar et al, 2020; Pan et al, 2020; Chen et al, 2022). In plaques, there was high expression of Col2a1, ACAN and SOX9 in pro-calcific chondromyocytes, hence, the cells were suggested to contribute to increased VC (Chen et al, 2022).

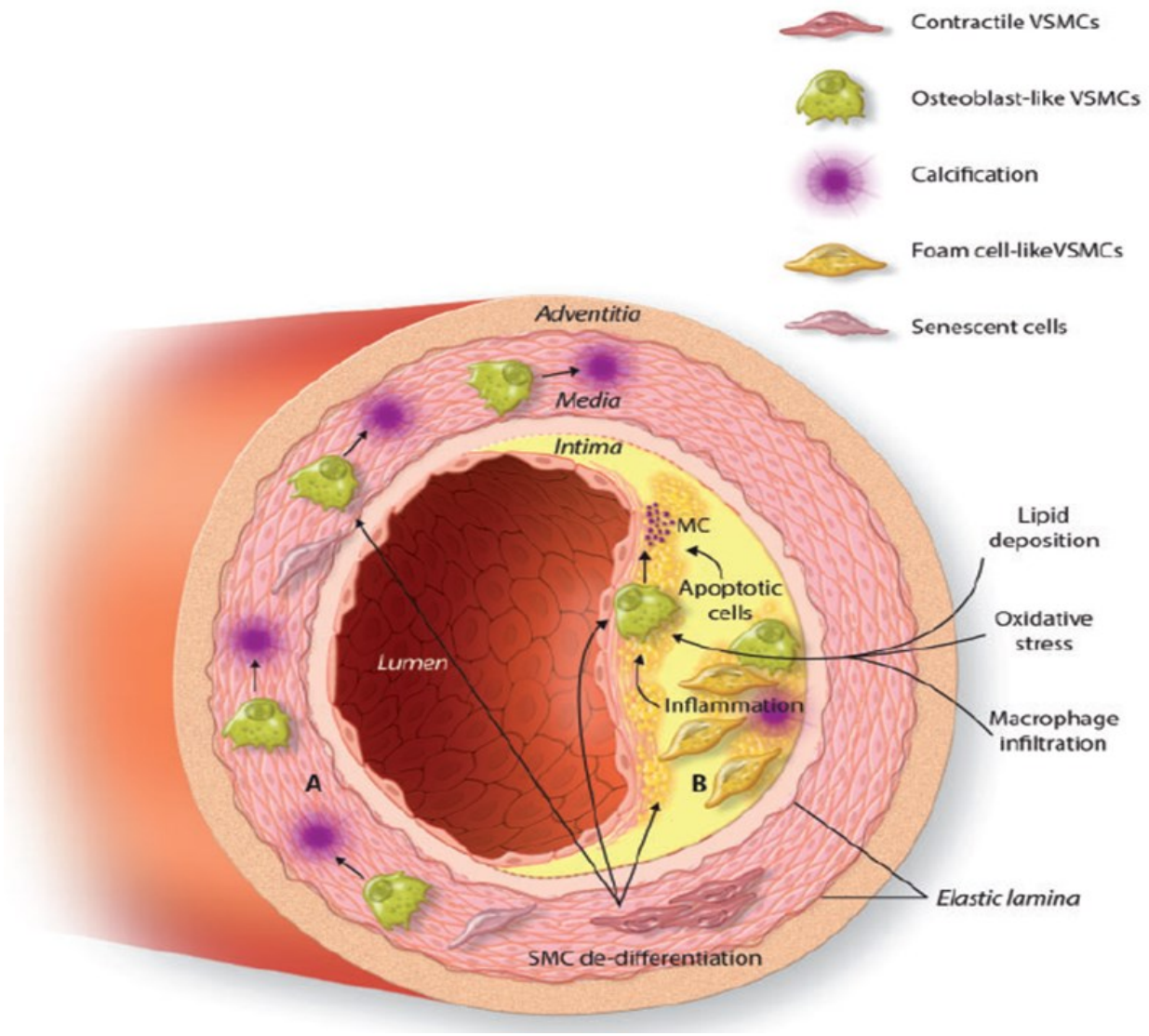


Fig. 4: Intimal and medial calcification as mediated by (vascular) smooth muscle cells.

Source: Durham et al, 2018

Growing evidence has implicated VSMCs as the main contributor of calcium deposits in the atherosclerotic plaque found in the arterial wall (Achim et al, 2022). As the atherosclerotic lesions develop and progress, VSMC phenotypic switch occurs leading to an increased calcification process via accelerated mineralization within the plaque (Cao et al, 2022; Tang et al, 2022). During the phenotypic switching, VSMC are converted to osteogenic or chondrogenic phenotype with development of calcifying vesicles also referred to as calcifying nodules (Shanahan et al, 2011). The atherosclerotic lesions are majorly composed of the fibrous materials (such as the fibrous cap, lipids etc.) and calcium during progression of atherosclerosis (Woo et al, 2023). This calcification tends to elevate the risk of

plaque rupture and cause plaque instability, which could result in severe cardiovascular consequences including heart attacks, myocardial infarction or strokes (Cao et al, 2022).

According to diverse studies vascular calcification has important consequences for the risk of developing CVDs (Karwowski et al, 2012; Bentzon et al, 2014; Chen et al, 2017; and Greenland et al, 2018). Calcification play a key role in stability and maintenance of atherosclerotic plaques, hence, in some cases leads to decreased plaque stability and susceptibility to rupture, thereby resulting in the formation of blood clots and abrupt blockages (Chen et al, 2017; Lee, Lee, and Jeon, 2020). The function of atherosclerotic calcification in plaque stability based on the location, shape and size of calcification gave rise to classes of calcification which are microcalcification and macrocalcification (Woo et al, 2023). In microcalcification, inflammatory activities negatively affect the plaque stability a risk for CVDs, on the other hand macrocalcification tends to enhance stability of the plaque (Harman and Jørgensen, 2019).

Furthermore, VC also reduces arterial flexibility, preventing the artery from adjust to changes in blood flow, which elevates blood pressure and increased risk of heart disease (Greenland et al, 2018; Mori et al, 2018). In addition, the existence and severity of calcification serve as indicators of total cardiovascular risk, aiding in risk assessment and directing preventative interventions (Bentzon et al, 2014); Roth et al, 2015). The likelihood of developing CVDs and the complications that accompany them can be significantly decreased by adopting a heart-protective habits, overseeing risk variables for calcification, such as high cholesterol and blood pressure, and seeking medical attention when essential (Coronado et al, 2022).

VC has so far only been controlled by risk factor management and attempts to control the dysfunctional calcium-phosphate metabolism (Herrmann et al, 2020). However, it is a complex pathophysiological process that requires further understanding to tackle the pathogenesis of VC. It has been widely studied via different research models which includes *in vitro*, *in vivo* and *ex vivo* (Borland et al, 2020; Herrmann et al, 2020; 2021; Poznyak et al, 2020; Radvar et al, 2021, Bogdanova et al, 2022).

In vitro models have emerged as indispensable tools in unravelling the intricate mechanisms underlying pathogenesis of several diseases including VC (Goto et al, 2019). These models offer controlled experimental environments that allow researchers to manipulate variables, dissect cellular interactions, and explore causative factors, thereby advancing our understanding of calcification and paving the way for novel therapeutic strategies. Using the *in vitro* model system, the most studied transition of SMCs

to osteogenic or chondrogenic phenotype have been explored (Durham et al, 2018). Although, it has been reported that in the *in vitro* model system such as the cell culture, there is no natural existence of calcification occurring except the cells are stimulated or induced to generate calcification (Herrmann et al, 2020).

In-vitro models offer a platform for studying the molecular players involved in calcification (Herrmann et al, 2020; Ceccherini *et al*., 2022). By genetically manipulating VSMCs or using inhibitors and activators, researchers can identify key molecules that regulate calcification processes, such as matrix vesicles, osteogenic markers, and calcium transporters (Holmar et al, 2020). These findings provide insights into potential therapeutic targets for preventing or mitigating vascular calcification.

Reviewing cell type models as suitable approaches for in-vitro studies of vascular calcification, Hermann *et al*. (2020) reported that *in vitro* cell culture models, particularly using VSMC enabling investigation of cellular responses to various stimuli. In similar vein, Radvar et al. (2021) notes that researchers can mimic the microenvironment of calcifying arteries by exposing VSMCs to factors such as elevated calcium and phosphate levels, inflammatory molecules, and oxidative stress. This approach facilitates the dissection of signalling pathways and molecular events that drive VSMC phenotypic switching from contractile to calcifying states. For instance, Alves et al. (2014) used *in vitro* method to study the genes and mechanisms associated with calcification in calcified smooth muscle cells and osteoblasts. Aghagolzadeh et al. (2016) studied interplay between inflammatory cytokines and calciprotein particles using cultured VSMCs *in vitro*. Kapustin et al. (2015) using *in vitro* model reported on the effect of matrix vesicles in VSMC calcification, they found that matrix vesicles (MVs) are exosomes, with their release by some factors they contribute to the process of calcification. Similarly, Aherrahrou et al. (2020) employed human patient samples and *in vitro* model and they reported that genetic variants play significant role in VSMCs function suggesting increased MIA3 expression to promote atheroprotective VSMC phenotypic transitions. The use of *in vitro* study only gives clear information about the calcification process hence, some studies have employed other forms of research models for calcification research.

It is evident from most studies that the use of *in vitro* method where cell culture technique is employed to investigate pathogenesis of VC involve the induction of calcification. This is basically because *in vitro* experiments, the induction of calcification is not spontaneous, rather a calcifying medium is required

for the experiment. Hermann et al, 2020 review highlighted the different types of supplement which have been widely used to induce calcification with their respective frequent concentration, which includes organic phosphate (beta-glycerophosphate), dexamethasone, inorganic phosphate (sodium hydrogen phosphate), ascorbic acid, sodium pyruvate etc.

Researches have shown that elevated concentration of serum phosphate causes calcification, hence, an individual's risk of developing CVDs especially seen in chronic kidney diseases (CKD) patients (Massy et al, 2015; Reiss et al, 2018). According to many VC researches it is evident that one of the factors that triggers calcification process is alteration of phosphate levels (either pyrophosphate (PPi) or extracellular phosphate (Pi)) in the cell (Pescatore et al, 2019). A common method for producing vascular calcification in experimental models, particularly in cell culture model, is the use of inorganic phosphate inducing method. This method entails increasing the amount of inorganic phosphate, a critical component of the hydroxyapatite crystals seen in calcified tissues, on vascular VSMCs or other pertinent cell types as calcifying nodules (Lu et al, 2022; Rui et al, 2022).

Cell culture media is supplemented with sodium phosphate salts or comparable substances to carry out the inorganic phosphate technique (Hermann et al, 2020). Efficiently simulating the mineral-rich environment that promotes the formation of hydroxyapatite crystals, increases the concentration of extracellular phosphate (Nagaishi et al, 2023). Because of this excess of phosphate, VSMCs, important contributors to vascular calcification, undergo a phenotypic change from their usual contractile state to a calcifying phenotype (Tsuda et al, 2020). This approach provides a controlled environment for researchers to examine the molecular processes and signalling networks that underlie vascular calcification. This technique enables the evaluation of prospective therapeutic interventions targeted at slowing down the process of calcification by adjusting phosphate concentrations and monitoring cellular responses. Serguienko et al, (2018) studied *in vitro* osteoblast differentiation by inducing calcification using dexamethasone, ascorbic acid and beta-glycerol, they were able to follow real time mineralization *in vitro*. Also, many retrospective researchers have discovered the significant role of phosphate in promoting atherosclerosis in arteries (Martin et al, 2015; Park et al, 2020).

It has been established that calcify VSMC tend to switch to the osteochondrogenic phenotype owing to induction of calcification, hence, transition into osteoblast-like cells (Woo et al, 2023). Inducing calcification in VSMCs culture using osteogenic medium (with and without inorganic phosphate) have

been explored as a way to initiate osteogenic differentiation in VSMC to osteocytes explaining its role in VC pathogenesis (Yao et al, 2021). Osteogenic medium contains organic phosphate which also plays a role in deposition and accumulation of calcium and phosphate in the extracellular matrix of VSMCs. Schack et al, (2013) reported the use of osteogenic medium with inorganic and organic phosphate, they reported the inorganic phosphate have better more effect on the osteoblast markers in comparison to the organic phosphate.

The well-established approach of staining methods (which includes Haematoxylin and Eosin, OsteoSense™680EX, von Kossa, Alizarin red stain etc.) are essential for observing and tracing changes in diverse biological processes thereby enabling the visualisation and analysis of cellular structures, molecules, and variations within tissues (Greco et al, 2022). As regards vascular calcification process, staining techniques have been explored and plays a significant role in the identification and quantification of mineralization (deposits and accumulation known as hydroxyapatite), calcified areas, and monitoring the progression of calcification in the artery (Liu et al, 2017). The detection of hydroxyapatite in cell culture and the vascular system cannot be overemphasized (Sato et al, 2020). For instance, both Alizarin Red (ARS) and von Kossa staining deposits of calcium and phosphate respectively. The use of these stains mostly calcein or ARS have advanced understanding in the field of VC process employing via *in vitro*, *in vivo* and *ex vivo* research model.

Calcein stain (also referred to as calcein green) is a non-permanent dye used in *in vitro* studies such as VMSCs, and primary mesenchymal stem cell culture for tracing and quantification on mineralization. When activated, the fluorescent dye calcein, which binds to calcium ions deposits which displays a potent green fluorescence. It has been used to monitor mineralization during the induction of calcification both *in vitro* and in vivo experiments. By adding calcein to cell cultures or animal models, researchers observed calcium ion deposition (Trillhaase et al, 2021; and Zhao et al, 2022). The brightness of the green luminescence, serves as a quantifiable measure of mineralization progress over time and reflects the degree of calcification. The staining enables the visualisation of calcified nodules and the assessment of the impact of experimental conditions or interventions on the amount of mineralization in cells.

These staining approaches are significant because they can give a clear visual picture of calcification, which is frequently difficult to see with more traditional techniques. For example, Serguienko et al,

(2018) studied the occurrence of mineralization in real time using cultured primary mesenchymal stem cells and found that the staining is very effective than the traditional staining (ARS) used in tracing VC.

Researchers can compare experimental settings, measure calcification progression statistically and qualitatively, and assess the effectiveness of proposed therapeutic interventions by using calcein and alizarin red staining. These staining methods advance our knowledge of the pathophysiological processes that support calcification-related disorders and aid in the creating specialized approaches for the management or prevention of calcification-related problems.

Reporter gene technique which involves the use of regulatory regions such as promoters and enhancers coupled with a reporter (usually a protein) that fluorescent upon activation of the regulatory region have extensively been used in different research to study gene expression and cell signalling (Serganova and Blasberg, 2019; Neefjes et al, 2021). Several approaches have been reported to be employed in packaging reporter such as biologic (e.g., bacterial or bacterial vectors), mechanical approach (such as microinjection or electroporation) and chemical method (including nanoparticle carriers or lipid) (Bartel et al, 2012; Yin et al, 2014; Lostale-Seijo et al, 2018). The reporter gene is usually delivered into a target (e.g. cell) by either transduction or transfection method.

The importance of the report system cannot be overemphasized, having the following advantages to further understand the molecular mechanism of VC. One of such advantages is to allow researchers in the field of vascular calcification to visually track cells which take part in VC at any specific time. Also, the system can help assess temporarily when calcification is triggered and its duration. Additionally, the strength or degree of the calcification response can be quantified using the fluorescence of the reporter. Likewise, rapid screening of large samples or experimental conditions can be explored in order to determine factors that may have an effect on SMCs role in calcification. Conclusively, the system can aid in validation of markers considered to be linked with calcification for their function and importance in actual cellular conditions.

Despite the significant increase in the knowledge on drivers of VC, additional study is still required to unravel the mechanism of phenotypic switching towards osteogenic phenotype in calcified VSMCs. To the best of our knowledge, no known study is reported to investigate tracing calcified VSMCs switch using promoter region of existing identified genetic markers with reporter genes in human coronary SMCs. Therefore, this study is aimed at dissociating and tracing transient SMCs dedifferentiating to

calcifying phenotype (osteoblast-like cells) *in vitro*. In future, the reporter system could be an effective fast assay that can be employed in either clustered regularly interspaced short palindromic repeats (CRISPR) or drug screening. This would allow for the identification of novel genes and drugs what can inhibit or enhance SMC calcification.

Materials and methods

Amplification and Extraction of Plasmids Construct

Five plasmid constructs for selected genes which include ALPL, RUNX2, ACAN, CHAD and COL2a1 associated with vascular calcification in SMCs were purchased from GeneCopoeia (Fig. 1). Each plasmid construct contains promoter of the above selected genes, reporter gene (mCherry which fluorescence in transduced cells) and antibiotics resistance gene (puromycyin) for selection of target cells expressing the gene of interest upon calcification. The plasmid for each gene was amplified using bacterial transformation.

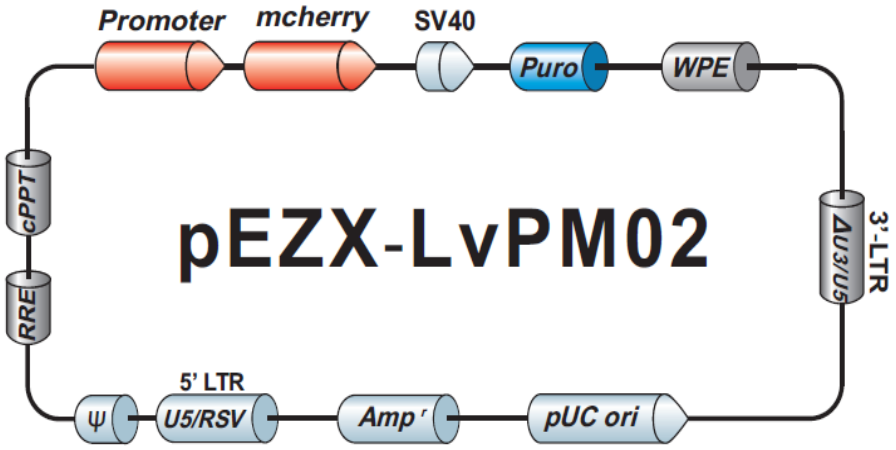


Fig. 4: Map and features of the designed plasmid construct for each selected gene

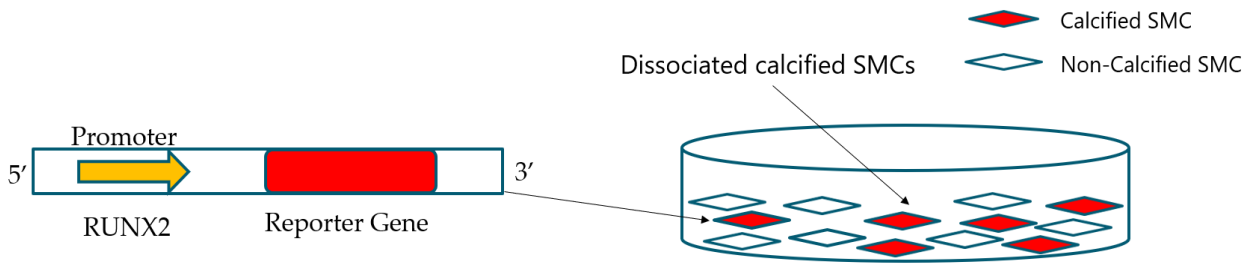


Fig.5: Example of the plasmid construct where promoter and gene replaced with mCherry is used for tracing calcification

The amplification of the plasmids was carried out by plasmids being cultured on bacterial medium. 2µl of plasmid constructs was mixed with 30µl of bacteria in separate tubes. The mixture was incubated on ice for 20mins, then subjected to a 40-second heat shock at 42^0C in water bath, followed by cooling on ice for 2 to 5 mins. 500µl of SOC medium was added to each tube and then rotated on a shaker for 45 mins at 37°C in the incubator. 200ul of the bacterial cells were plated on LB (Ampicillin) plate (20ml LB-Agar and 40ul 50mg/ml ampicillin 100µg/ml). Plates were allowed to dry, placed upside down and kept at 37°C in incubator overnight (approximately 16-18 hours). One colony of bacteria from each plate was collected into a beaker containing 20ml of 5x LB, 80ml of sterile milliQ water and 200ul of ampicillin. The plasmid extraction was performed following the protocol from NucleoBond Xtra plasmid Midi EF kit. The concentration and purity of the extracted plasmid were measured using DeNovix spectrophotometer (DS-11 FX+). The extracted plasmids were used to prepare and generate lentiviral vector at the national virus vector laboratory at AIVI.

Restriction Enzyme Digestion

In order to validate the quality of the amplified plasmid, they were subjected to restriction enzyme digestion. The fragments should correspond to the expected size and number of sizes when digested with required restriction enzymes which are EcoRI (ER0271) and BamH1 (ER0051) (ThermoScientific). Amplified plasmids (template DNA) were digested following the manufacturer's protocol (ThermoScientific Fast Digest restriction enzymes). 2 µl template DNA (up to 1µg), 2 µl 10× buffer, 1 µl restriction enzyme, and 15 µl nuclease-free water (Thermo Fisher Scientific) were mixed to a final volume of 20 µl. The mixture was incubated at 37^0C on a heat block, 2 µl of FastDigest Green Buffer (Thermo Scientific) was added for visualization on the gel.

Cell culture of Human Coronary Smooth Muscle Cells (HCASMCs)

Materials: Human Vascular Smooth Muscle Cell Basal medium (Gibco M-231-500), smooth muscle growth supplement SMGS (Gibco S-007-25), 1% penicillin-streptomycin, 10cm dish (Greiner bio-one), Serological pipette (5, 10, 25 and 50 ml Sarstedt), Eppendorf CellXpert incubator.

To study the role of Smooth muscle cells (SMCs) reported as the primary source of vascular calcification, Human coronary smooth muscle cells (HCASMCs) donated from the CADGEN research group was cultured with Human Vascular Smooth Muscle Cell Basal medium (Gibco M-231-500) with smooth muscle growth supplement (Gibco S-007-25) and 1% penicillin-streptomycin as previously described. Cells were cultured in 10cm dish (Greiner bio-one) at 37°C in Eppendorf CellXpert incubator. Confluence cells grown on 10 cm dish were split (1:2) into 15cm dish in order to produce high number of cells for the experiment.

Transduction and Selection of Stable Clones

To generate stable lines to study the activity of the of the selected reporter genes on calcification, cultured HCASMCs were transduced with lentiviral vectors (HIV-based) to deliver the plasmids into VSMCs.

Materials: Lenti-Viral stock (20µl aliquots), 10cm dish (Greiner bio-one), polybrene (Gibco TR-1003-G), puromycin 2µg/ml (Gibco A11138-03), Human Vascular Smooth Muscle Cell Basal medium (Gibco M-231-500), 10cm dish (Greiner bio-one), Eppendorf CellXpert incubator.

1.5 x 10^6 cells were seeded in 10cm dish for each gene, after 24hrs the cells were at 80% confluence. The medium was changed and replaced with medium containing polybrene (Gibco TR-1003-G), aliquots of thawed Lentiviral stock containing plasmids were added to the cells. The plates were kept in the fridge for 1-2 hours at 4°C and moved to the incubator at 37°C. 24 hours after transduction, medium containing viruses was changed and replaced with normal medium. After 48 hours, selection of stable clone was performed by adding directly into the medium puromycin 2µg/ml (Gibco A11138-03). Antibiotics selection was completed in 12-14 days.

Induction of calcification

Once the stable lines were generated, they were treated with calcifying medium (inorganic phosphate and osteogenic medium). Since then, it has been established that a high concentration of phosphate in cells induces deposit and accumulation of phosphate and calcium forming hydroxyapatite. Hence, calcifying medium containing high inorganic phosphate was used on SMCs.

Calcifying medium (CM)

Materials: StemXVivo Osteogenic/Adipogenic Base media (CCM007) and Human Osteogenic supplement 20X (CCM008), 1% penicillin-streptomycin, 15ml and 25ml falcon tubes (Greiner bio-one), disodium hydrogen phosphate ($Na_2HPO_4.7H_20$); Merck (Darmstadt, Germany) and sodium dihydrogen phosphate ($NaH_2PO_4.H_20$ Merck (Darmstadt, Germany) and 0.2µl filter (Cat No.15206869).

The protocol for the inorganic phosphate was adopted from Holmar et al, 2020. Two different calcifying media was used for the experiment. Osteogenic medium was prepared from StemXVivo Osteogenic/Adipogenic Base media (CCM007) and Human Osteogenic supplement 20X (CCM008) and 1% penicillin-streptomycin. The medium was aliquoted in 25ml falcon tubes and stored in -20^{0}C for further use. Inorganic phosphate at concentration of 3.7mM was prepared by adding in 1:1 ratio disodium hydrogen phosphate ($Na_2HPO_4.7H_20$); Merck (Darmstadt, Germany) and sodium dihydrogen phosphate ($NaH_2PO_4.H_20$ Merck (Darmstadt, Germany) to normal medium. 100mM stock solution of the mix was prepared and filter sterilized with 0.2µl filter (Cat No.15206869). The stock was aliquoted in 15ml falcon tube and kept in fridge at +4°C until use.

Induction of calcification

Materials: 12-well plate (Greiner bio-one), Calcifying Media (CM).

1.5 x 10^6 cells were seeded in 12-well plate in normal medium, at 80% confluence the normal medium was changed to calcifying medium (CM) which causes the deposit of mineral in the cells. The CM was changed every 72 hours for 7 days. The first day of adding CM was defined as day zero (0).

Staining of calcification

Upon induction of calcification the calcified areas referred to as calcifying nodules were stained using calcein and Alizarin Red Stain (ARS) for visualization and quantification of calcification for each treatment condition.

Calcein Staining

Materials: Calcein stain (C0875, Sigma-Aldrich Merck), 0.1 M of NaOH, Molecular grade water, 0.2µl filter (Cat No.15206869), Incucyte S3.

The staining of calcification was adopted and modified from Serguienko *et al*., 2018. 10mM of calcein stain (C0875, Sigma-Aldrich Merck) solution was prepared with 0.1 M of NaOH. 1mM of the mixture was prepared by diluting the stock in 1:10 ratio as the working stock solution. The working solution was filter sterilized with 0.2µl filter (Cat No.15206869), stored at room temperature in dark till further use. The calcein solution was added directly to the medium in each well 24 hours prior to imaging. To enhance quality imaging by reducing fluorescent background, medium containing calcein was replaced with normal medium prior to images acquisition. The green fluorescence was detected with an incucyte.

Alizarin Red Stain (ARS)

Materials: Phosphate Buffered Saline (PBS) Gibco, 4% Paraformaldehyde (PFA), MilliQ water, Alizarin Red Stain (ARS), Plate Shaker

Cell medium was aspirated from the wells, washed with 500ul PBS and fixed with 500ul 4% PFA for 20 min at room temperature. The fixative was removed and cells washed with PBS. 250 ul of ARS was added to the well and incubated for 20 mins on a shaker at room temperature. The ARS was removed and cells were incubated with 1 ml milliQ water for 5 mins on shaker (this was repeated 4 times). Images were taken with inverted microscope.

Collagenase Assay

When calcification occurs, it is difficult to dissociate the calcified SMCs into single cells, hence, collagenase assay containing EDTA—collagenase was used to separate the cells.

Materials: Phosphate Buffered Saline (PBS) Gibco, EDTA, Hanks' Balanced Salts Solution (Cat No: ECM0507L), Gibco Collagenase lyophilized non-sterile prepared from Cl. histolyticum (Cat No: 17018-029), Plate shaker, 1M Calcium Chloride (VWR Sterile E506), Trypan Blue Stain 0.4% (Cat no- 2365784 Invitrogen by ThermoFisher Scientific), Eppendorf tubes, Countess Cell Counter, Beckman Coulter CytoFlex S.

CM was aspirated and cell washed twice with PBS. 500µl of 30mM EDTA was added to each well and incubated for 15 mins at RT. The EDTA was aspirated and added once again for another 15 min. Then, the EDTA was aspirated and 500µl of sterile 8mg/ml collagenase in Hanks' Balanced Salts Solution HBSS/4mM calcium chloride $CaCl_2$ was added to the well incubating for 10 mins at 37°C on a plate

shaker. Afterwards, pipette the lysate up and down then continue incubation for 20 mins at 37°C on a plate shaker. Transfer the lysate to 1.5ml Eppendorf tube, and spin at 500g for 5 mins at RT. The supernatant was aspirated and pellet resuspended in 500µl trypsin, then mix gently (approximately 20x). The tubes are then incubated for 15 mins in water bath at 37°C. Tubes were removed from water bath (Thermo Scientific) and lysate pipette up and down with further 15 min time incubation. 500µl media was added to the lysate and spin at 500g for 5 mins at RT. The supernatant was aspirated and pellet resuspended in 500µl media. Cells were counted with trypan blue stain 0.4% (Cat no- 2365784 Invitrogen by ThermoFisher Scientific) using the countess cell counter. The single cells from the assay were subject to FACS sorting to determine the percentage positive of mCherry fluorescence using Beckman Coulter CytoFlex S.

Data analysis

Using the incucyte software the small green areas synonymous to calcium deposits were selected by adjusting the threshold GCU: Maximum mean intensity: Edge Sensitivity: and Area. Quantification of calcified area (count) was done using Fiji/ImageJ. Statistical analysis of data generated was done using GraphPad prism (version 10.0.2 (232)). Data are presented as mean ± SEM.

Results

PLASMID CONCENTRATION

The plasmids were amplified and then extracted to achieve the required concentration for lentiviral production (Table 1). Furthermore, restriction enzyme digest indicated that each gene had been successfully amplified through bacterial transformation (Table 2).

Table 1: Obtained concentration (ng/µl) and purity of plasmids using spectrophotometer.

GENE MARKERS	CONCENTRATION (ng/µl)	Purity (260/280)
ALPL	846.34	1.91
RUNX2	792.77	1.92
ACAN	1033.65	1.92
CHAD	824.18	1.91
COL2A1	922.99	1.92

ALPL- Alkaline phosphatase; RUNX2 - Runt-related transcription factor 2; ACAN- Aggrecan; CHAD- Chondroadherin; COL2a1 - Collagen type II Alpha 1

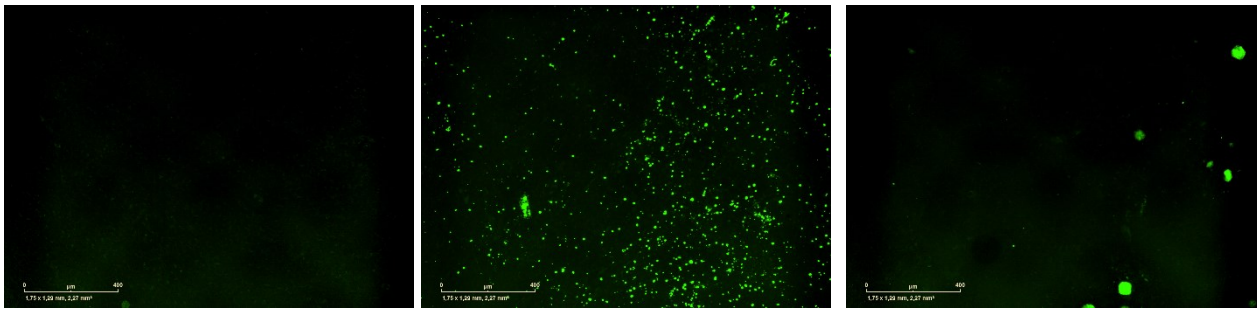
Table 2: The number of expected fragments upon restriction digest of amplified plasmid for each selected gene marker.

GENE MARKERS	EcoR1	BamH1	NUMBER OF EXPECTED BAND
ALPL	7970, 8273	9532	3
RUNX2	7970	9622	2
ACAN	7970, 8558	9512	3
CHAD	7970	9237	2
COL2A1	7970	9553	2

Calcification in HCASMCs

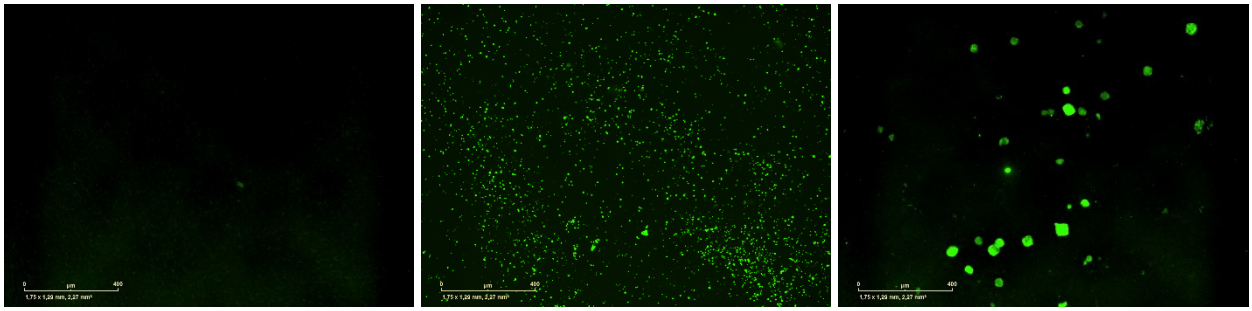
Calcein stain nodules of calcification

In order to study the reporter gene expression of cell lines stably expressing the reporter gene, we induced calcification via IP and OM in stable HCASMC lines expressing specific selected genes of interest. Then, calcification activity was monitored with calcein staining for 7 days with one-time image scan by the incucyte zoom. Fig. 6A-E show the fluorescent images and calcification areas with nodules (green dots). In all samples induced with IP, calcification was more evident by the green dots. However, in the OM, crystals were observed in the osteogenic medium which are not recognized as calcified nodules due to the very large size of the green dots. RUNX2 and Col2a1 showed the highest calcification (with more calcium deposits) in IP treated cells when compared to the other reporter systems (Fig. 11 Appendix 1-3)



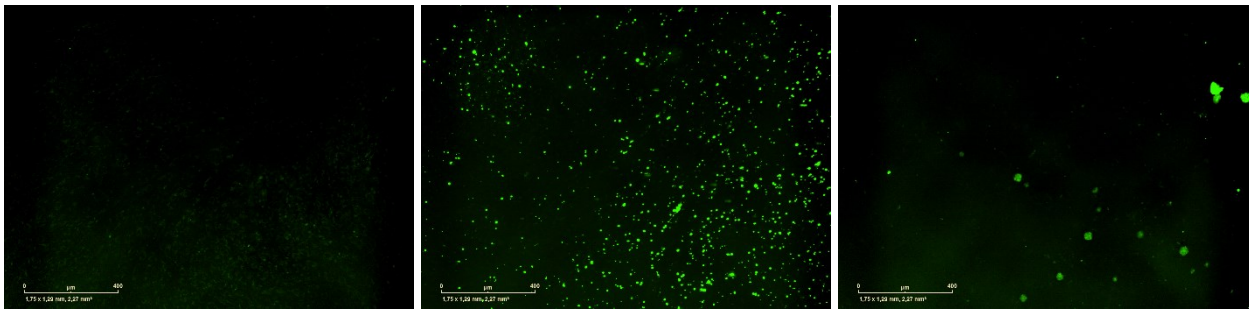
Control Inorganic phosphate Osteogenic medium

Fig. 6A: Cells stably expressing ALPL reporter gene, stained with calcein to identify calcified areas with nodules (deposited calcium-phosphate) using the incucyte S3 machine at day 7.



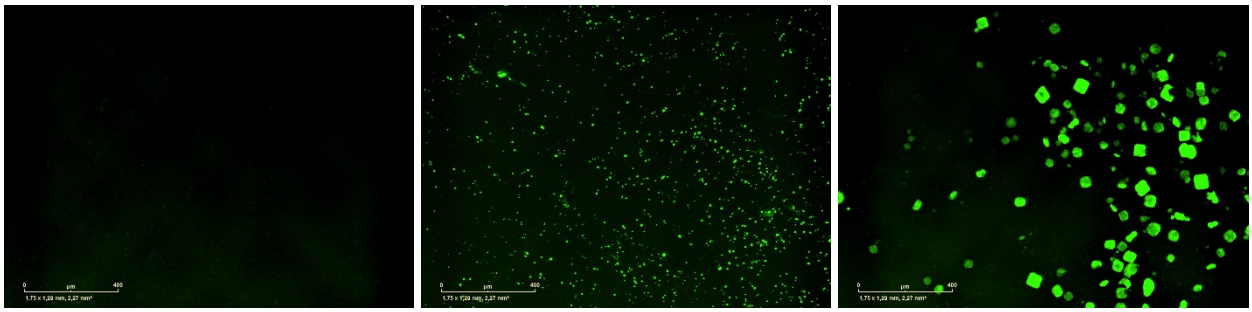
Control Inorganic phosphate Osteogenic medium

Fig.6B: Cells stably expressing RUNX2 reporter gene, stained with calcein to identify calcified areas with nodules (deposited calcium-phosphate) using the incucyte S3 machine at day 7.



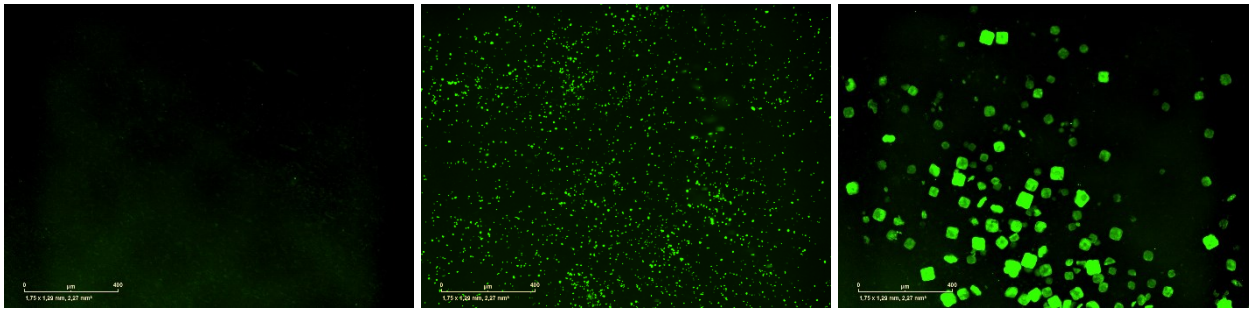
Control Inorganic phosphate Osteogenic medium

Fig. 6C: Fig: Cells stably expressing ACAN reporter gene, stained with calcein to identify calcified areas with nodules (deposited calcium-phosphate) using the incucyte S3 machine at day 7.



Control Inorganic phosphate Osteogenic medium

Fig. 6D: Cells stably expressing CHAD reporter gene, stained with calcein to identify calcified areas with nodules (deposited calcium-phosphate) using the incucyte S3 machine at day 7.

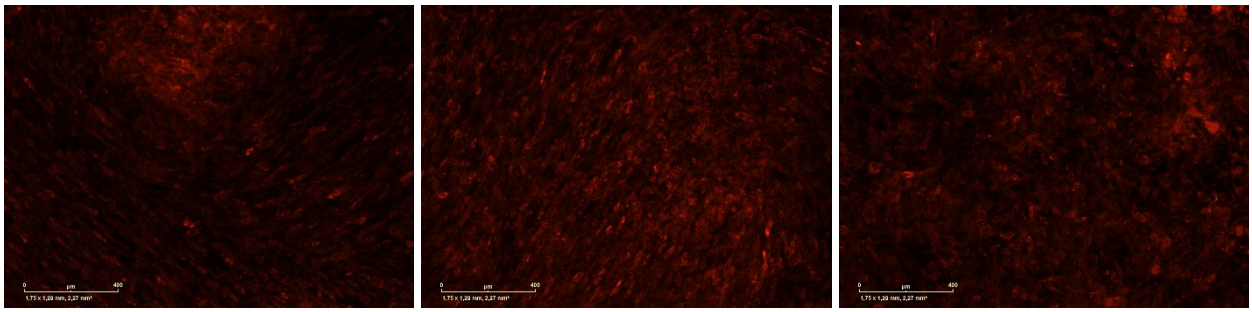


Control Inorganic phosphate Osteogenic medium

Fig. 6E: Cells stably expressing COL2a1 reporter gene, stained with calcein to identify calcified areas with nodules (deposited calcium-phosphate) using the incucyte S3 machine at day 7.

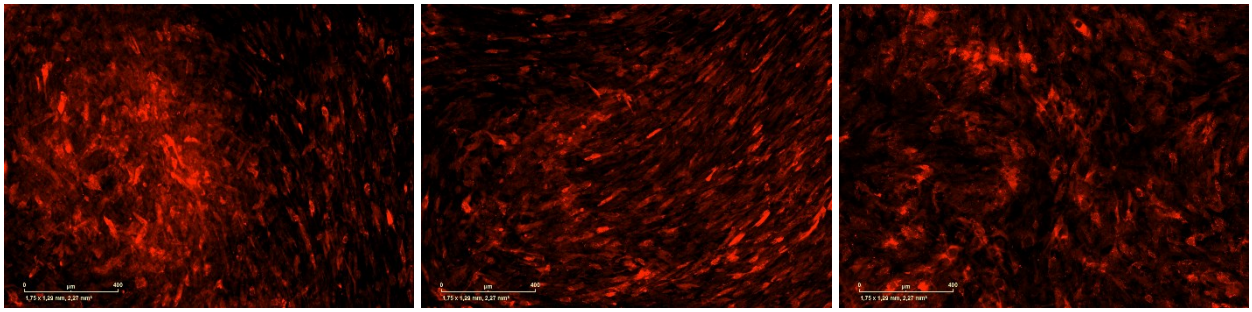
To further elucidate on the activity of the marker genes upon induction of calcification for seven (7) days, we also captured images from the red channel of the incucyte S3 for both basal (control) and treated conditions for mCherry fluorescence of stable lines.

Fig.7A-E shows the fluorescence (mcherry) of the cells upon induction of calcification. It was observed that there was increase in the fluorescence in the treated conditions as compared to the control. In addition, in the cells treated with osteogenic medium, it was observed that the cells morphology changed when compared to the control and inorganic phosphate cells.



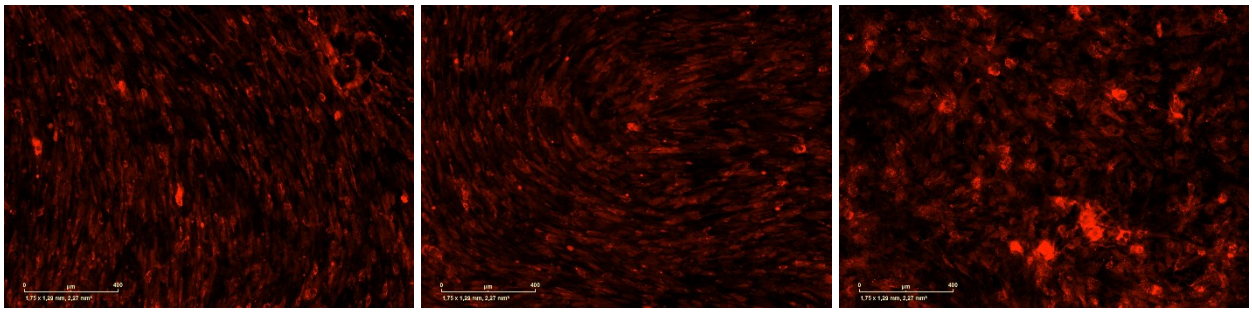
Control Inorganic phosphate Osteogenic medium

Fig.7A: The ALPL-reporter construct showed mCherry fluorescence in calcified areas, as detected by the incucyte S3.



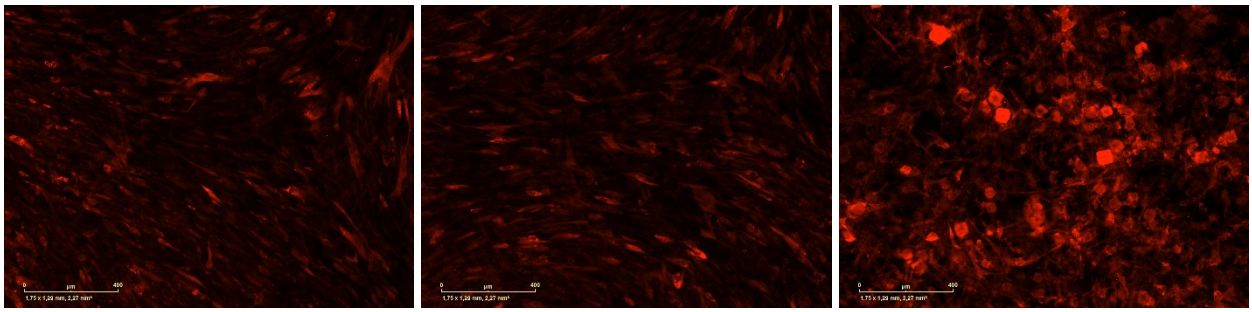
Control Inorganic phosphate Osteogenic medium

Fig. 7B: The RUNX2-reporter construct showed mCherry fluorescence in calcified areas, as detected by the incucyte S3.



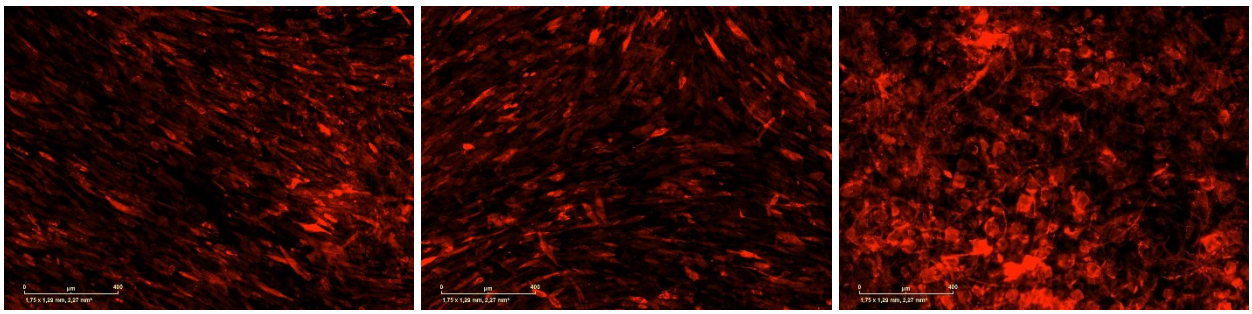
Control Inorganic phosphate Osteogenic medium

Fig. 7C: The ACAN-reporter construct showed mCherry fluorescence in calcified areas, as detected by the incucyte S3.



Control Inorganic phosphate Osteogenic medium

Fig. 7D: The CHAD-reporter construct showed mCherry fluorescence in calcified areas, as detected by the incucyte S3.



Control Inorganic phosphate Osteogenic medium

Fig.7E: The COL2a1-reporter construct showed mCherry fluorescence in calcified areas, as detected by the incucyte S3.

Alizarin Red Stain (ARS)

To further validate calcification in transduced cells, the calcified areas were stained with ARS. It was clearly seen that the calcification was high in osteogenic medium than in inorganic phosphate (Fig. 8).

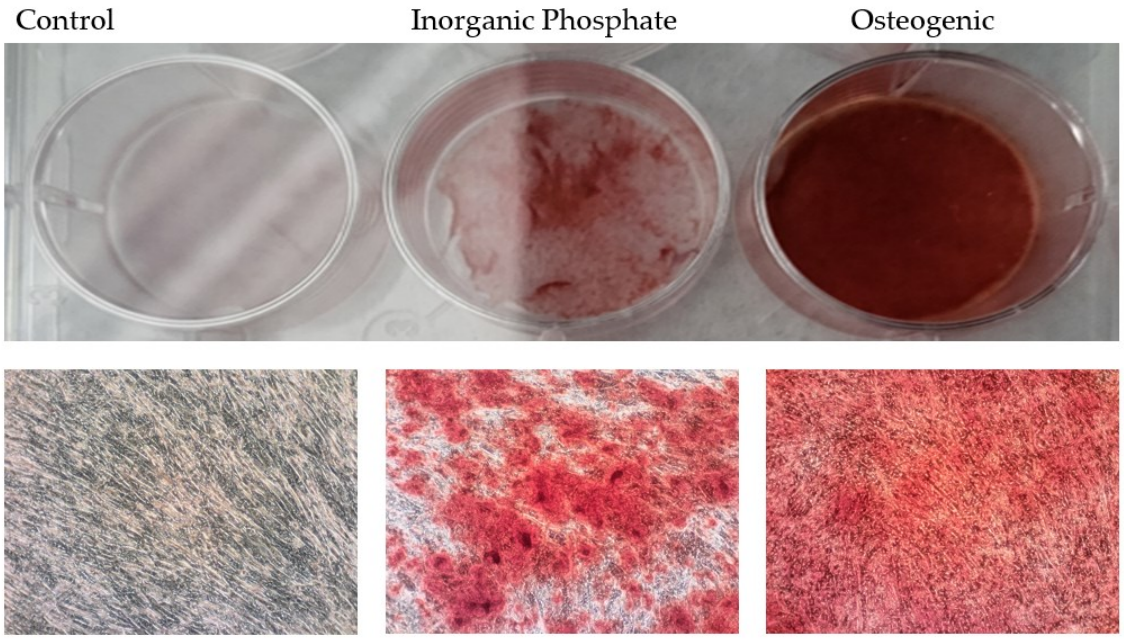


Fig.8: Cells showing calcified areas stained with ARS after treatment with IP and osteogenic medium

Fluorescent-activated cell sorting of calcified smooth muscle cell

We employed the EDTA-collagenase dissociation assay to separate the calcified VSMCs into single cells. The single cells were analyzed with fluorescence activates cell sorting to determine the percentage of mCherry fluorescence and the best calcification marker to trace calcification. Fig. 9 shows the percentage mCherry fluorescence of control cells (without transduction) where no mCherry fluorescence was observed as compared to the transduced control cells with mCherry fluorescence ranging between 46.28% and 98.53%. RUNX2 showed the highest mCherry fluorescence and CHAD the lowest fluorescence in both IP and OM treated cells (Fig.11B-F). In IP condition, the reporter construct transduced cells demonstrated mCherry fluorescence ranging between 49.29% and 98.69%, while osteogenic treated cells mCherry fluorescence of stable cells were observed to range between 85.31% and 99.49% (Fig.12-13 Appendix 2-1).

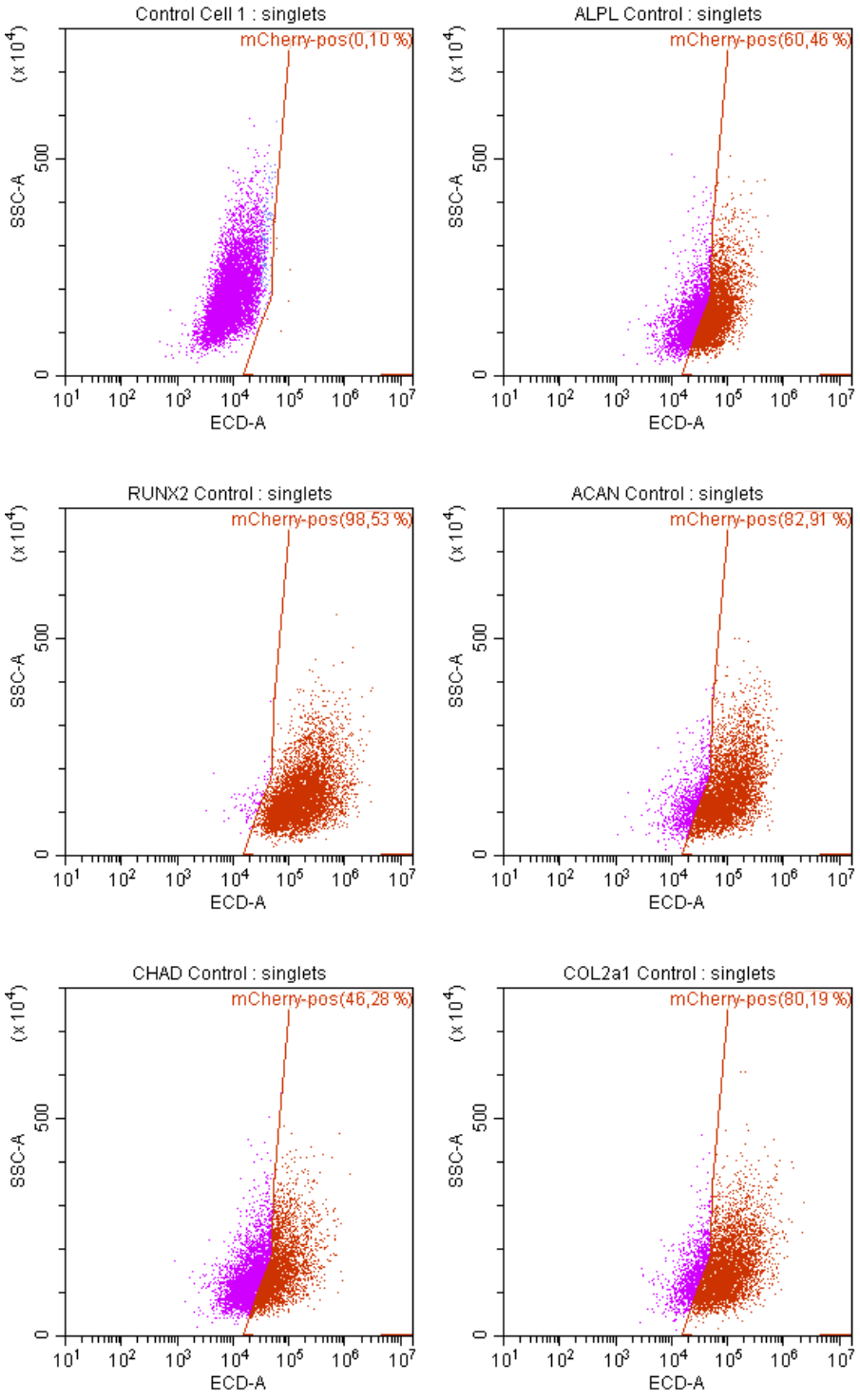


Fig. 9: Representative images from the showing difference in mCherry fluorescence between control (non transduced cell and control transduced cells)

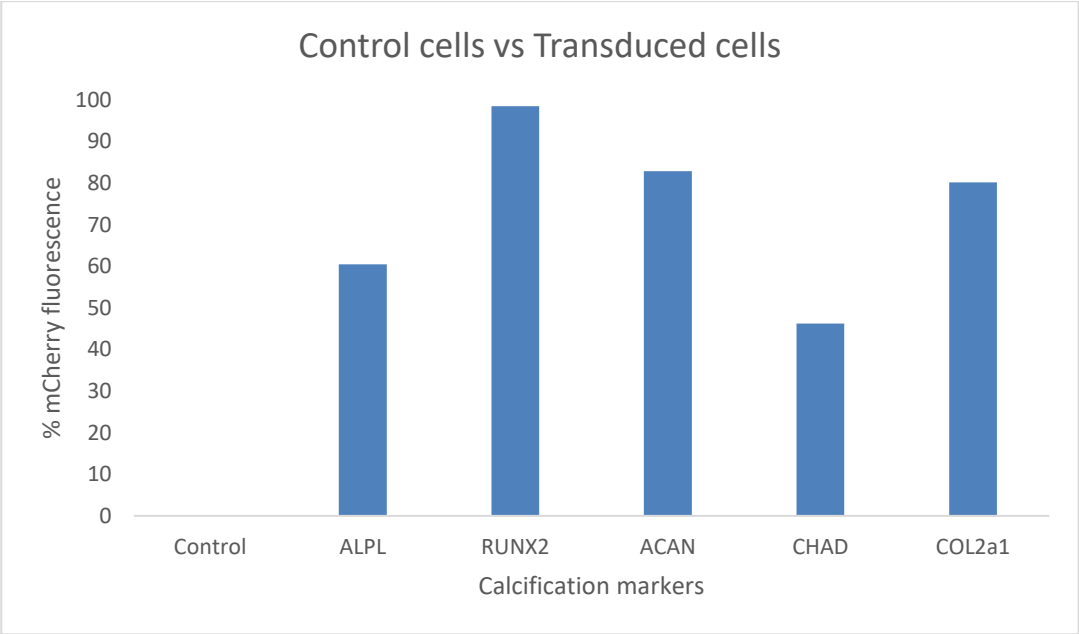


Fig.10A: Comparison of the mCherry fluorescence (%) of single cells between control cells (non-transduced cell) and transduced cells for selected calcification marker

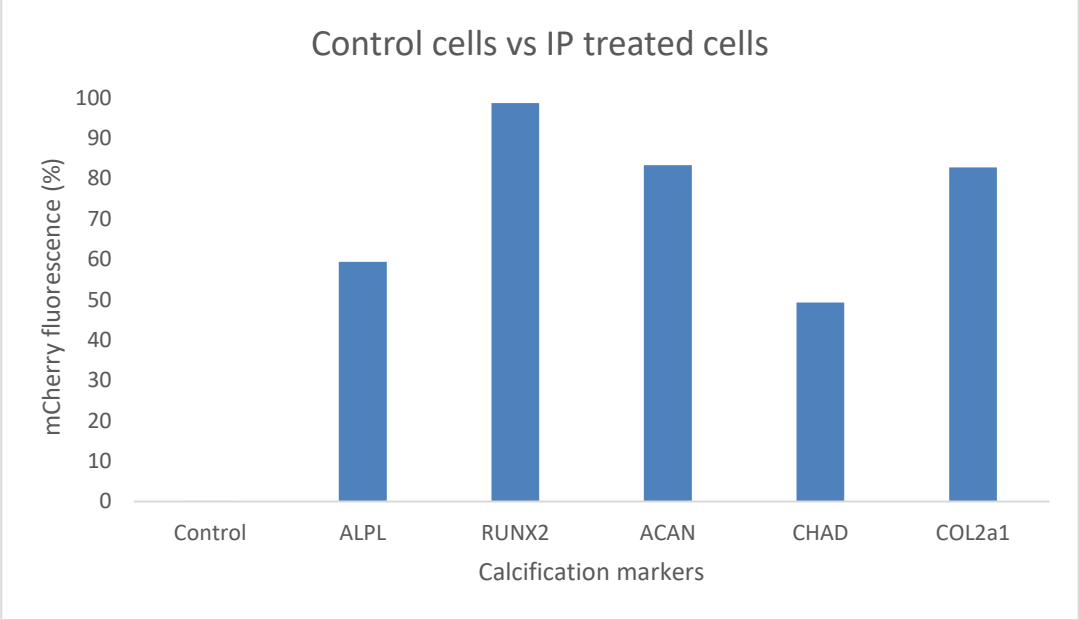


Fig.10B: Comparison of the mCherry fluorescence (%) of single cells between control cells (transduced cell) and IP treated transduced cells for selected calcification marker

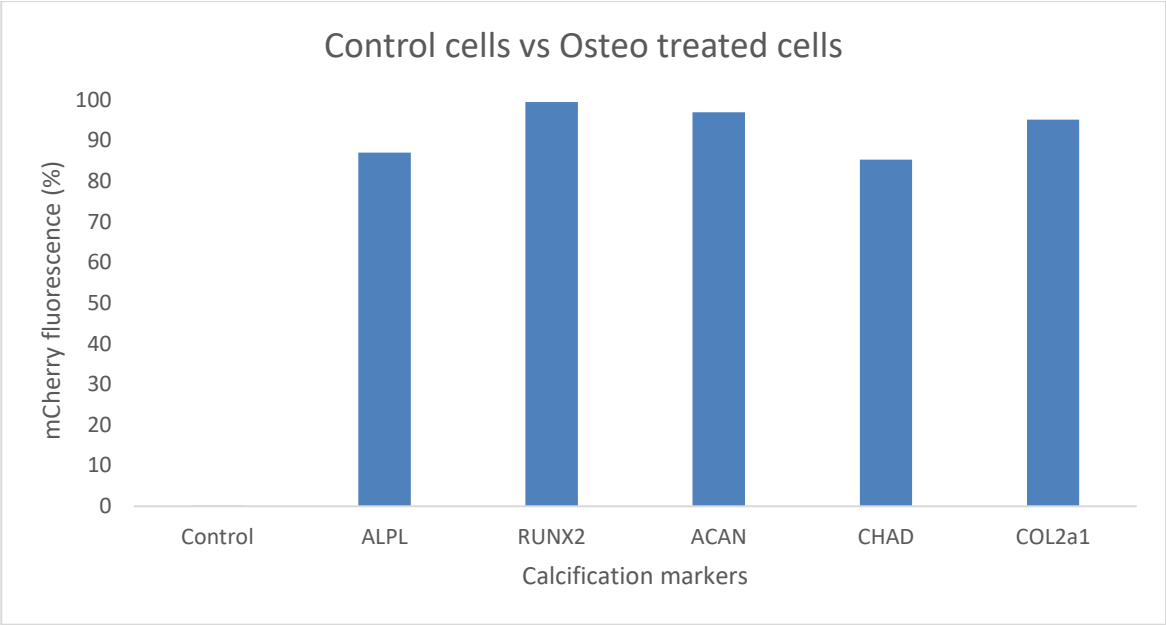
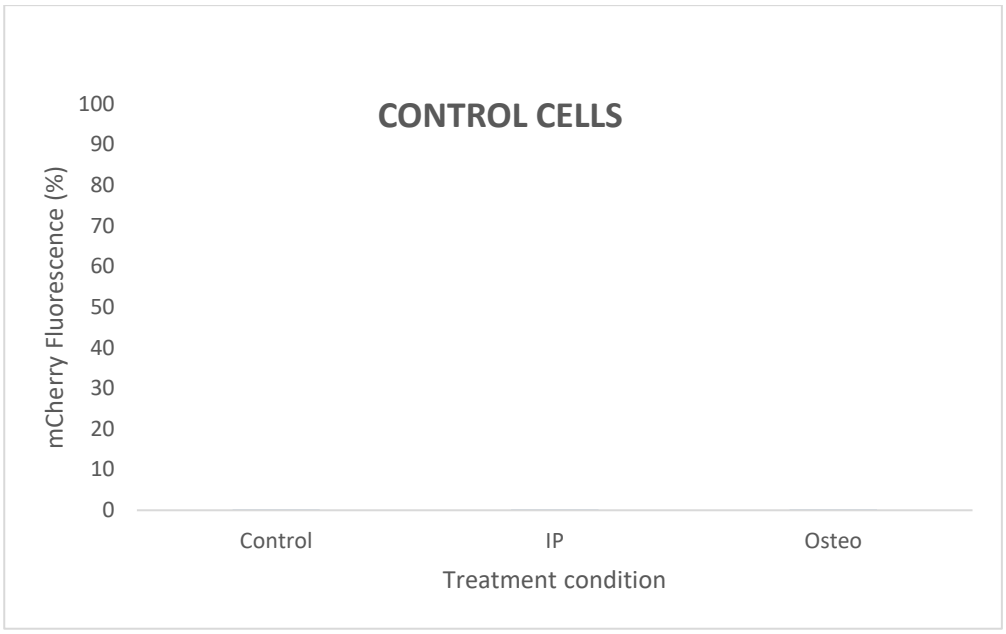
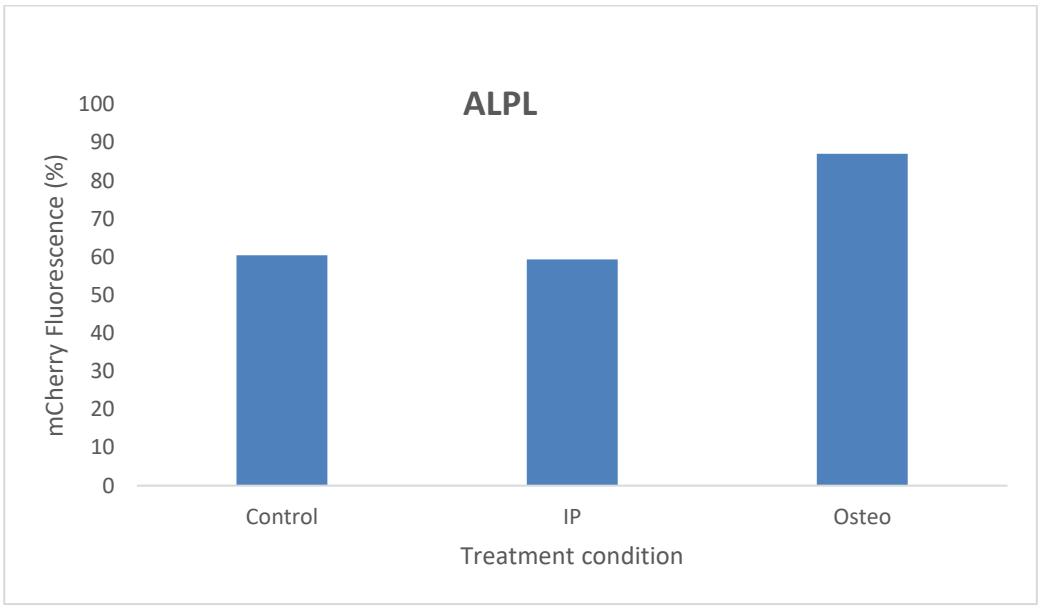


Fig. 10C: Comparison of the mCherry fluorescence (%) of single cells between control cells (transduced cell) and osteogenic treated transduced cells for selected calcification marker

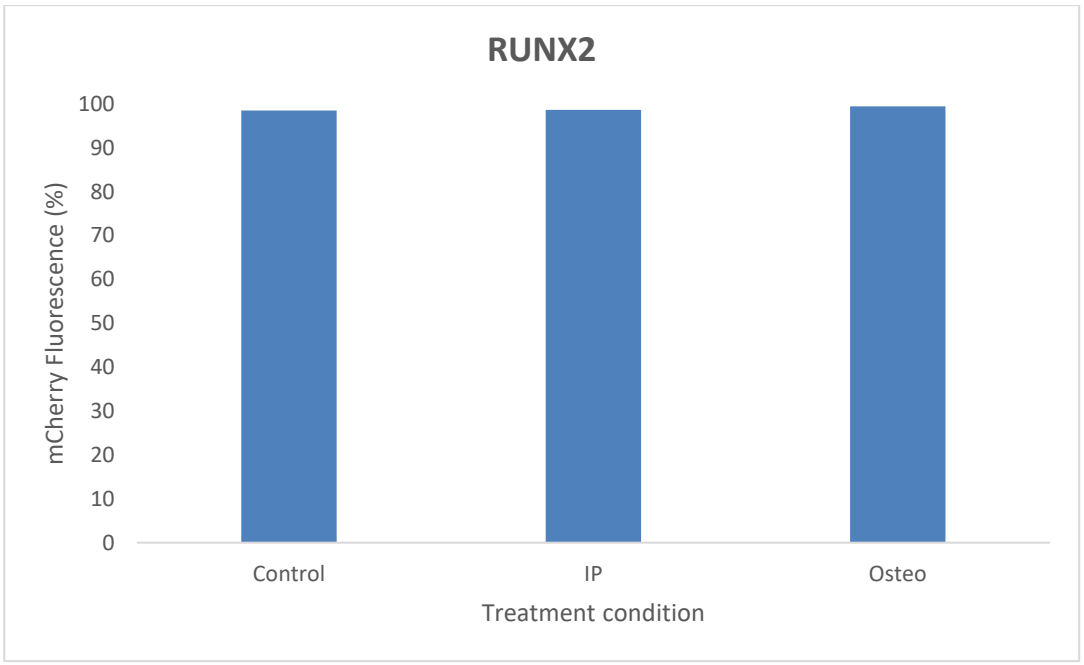
Fig.11A shows comparison of the mCherry fluorescence within the same group for control non-transduced cells and stable cells for the selected marker genes. It was observed that the control group without transduction showed no mCherry fluorescence as compared to the stable cells. RUNX2 showed the highest mCherry fluorescence within the group when compared to ALPL, ACAN, CHAD and Col2a1 (Fig. 11C). There was no clear difference between the control and treatment condition (Fig.11B-F). It was observed that the difference between the control and IP treated stable cells was almost similar when compared to the osteogenic treated cells (Fig.11B-F). ALPL and CHAD showed significant differences comparing the difference between control and osteogenic treated cells (Fig. 11B and 11E). In addition, the replicate images of the mCherry fluorescence for ALPL and CHAD generated from the incucyte correlates with the observed differences in the mCherry fluorescence for FACS sorted cell in ALPL and CHAD (Fig.12-13 Appendix 2-1).


CONTROL CELLS
mCherry Fluorescence (%)
100
90
80
70
60
50
40
30
20
10
0
Control
IP
Osteo
Treatment condition

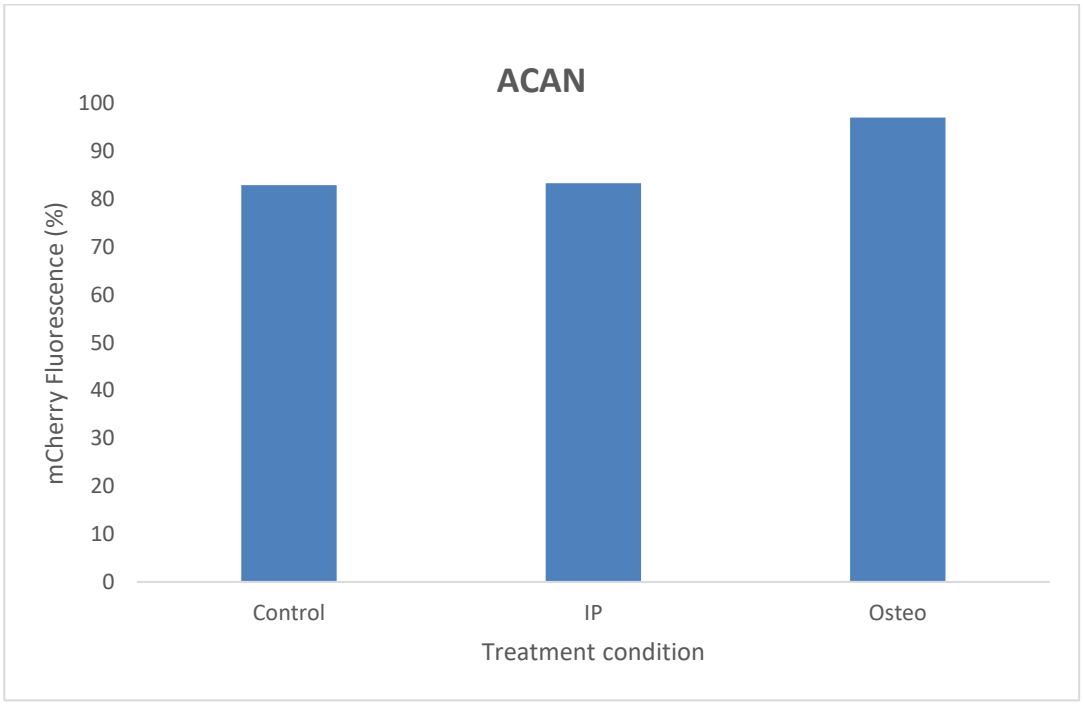
A


ALPL
mCherry Fluorescence (%)
100
90
80
70
60
50
40
30
20
10
0
Control
IP
Osteo
Treatment condition

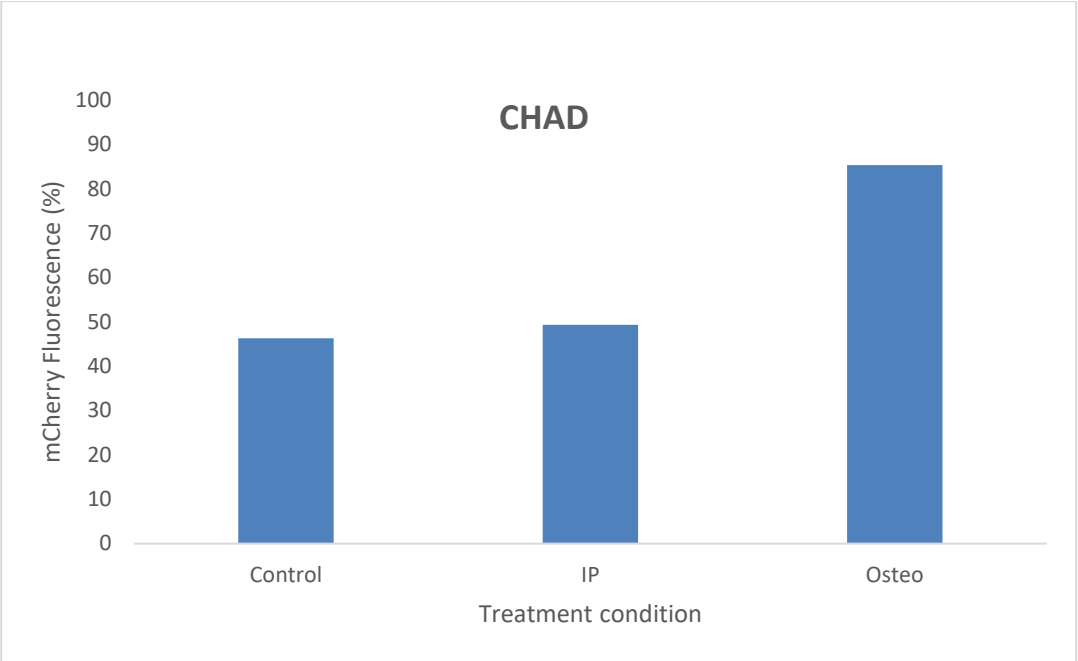
B


RUNX2
100
90
80
70
60
50
40
30
20
10
0
mCherry Fluorescence (%)
Control
IP
Osteo
Treatment condition

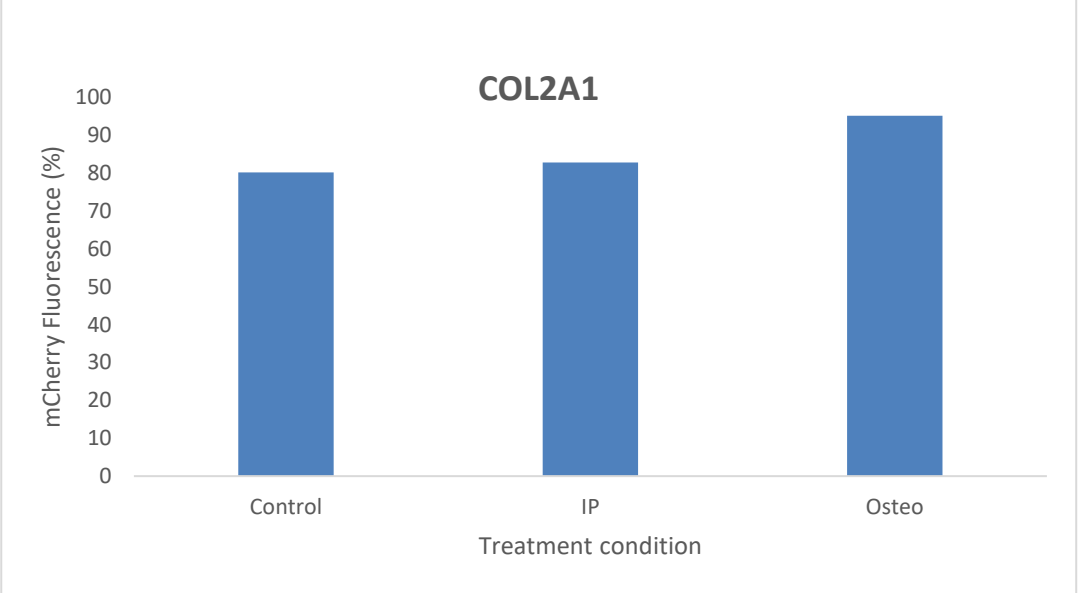
C


ACAN
100
90
80
70
60
50
40
30
20
10
0
mCherry Fluorescence (%)
Control
IP
Osteo
Treatment condition

D



E



F

Fig.11: Comparison of mCherry fluorescence within the same group for control and treated cells. A. Non-transduced cells (B-F) stable clones.

Discussion

Cardiovascular disease remains the leading cause of death globally affecting both males and females. In Europe, CVDs have been reported to cause 45% of death, hence, remains one of the major public health challenges (Townsend et al, 2022). Therefore, it is a paramount area of research to be explored. VC is one of the major hallmarks and strong predictor of CVDs such as atherosclerosis, myocardial infarction, stroke, CAD etc. (Woo et al, 2023). VSMCs have been discovered to be the primary source of VC implicated in CVDs development and progression such as found in atherosclerotic plaques (Durham et al, 2018; Sorokin et al, 2022).

There are diverse significant studies revealing the process involved in initiation and progression of VC in pathological conditions such as atherosclerosis, diabetes mellitus and chronic kidney disease (CKD) (Durham et al, 2018). It is believed that VC is an actively regulated process involved in deposit and accumulation of calcium and phosphate. Reports on several studies on the molecular mechanisms of VC such as phenotypic switch of VSMCs, changes in calcium and phosphate level, identification and validation of genetic markers have been investigated using *in vitro*, *in vivo* and *ex vivo* research model. However, further research is required to unravel the molecular mechanisms of VC. We hypothesize that the regulatory regions (promoter) of genes expressed during phenotypic switch towards osteocyte-like cells in SMCs play significant function in understanding pathogenesis of calcification. The functions include real time tracking of calcification in cells, response time of calcification, quantification and validation of markers and high throughput screening of large samples with different experimental condition can be achieved.

Reiss et al, 2018 reported that the high concentration of phosphate in the serum is linked to the risk of developing CVDs. The increase in the amount of phosphate and calcium in the cell have been found to lead to the process of calcification (Zhou et al, 2021). In addition, mineralization process occurring in the extracellular matrix involves phosphate binding with calcium released upon calcification to form hydroxyapatite (Rui et al, 2022). In this study, VC was successfully induced via inorganic phosphate (at a concentration of 3.7mM) and osteogenic medium in VSMCs (Fig. 6A-E and 7A-E).

Calcification was observed using calcein staining which binds to the deposited calcium and phosphate in the cells. We found that the calcein staining had no effect on the viability and morphology of VSMCs which agrees with the findings by Serguienko et al, (2018). However, we discovered that there was

calcification in cell treated with inorganic phosphate (day 7) compared to the osteogenic medium treated cells. Studies have proven that cells induced with inorganic phosphate show calcification at early stage (evident with the calcifying nodule) than those treated with osteogenic medium supplemented with organic phosphate (Schäck et al, 2013; Lu et al, 2020; Rui et al, 2022). Robert et al, (2020) reported that the time point for analysis for induced calcification plays a significant role in appearance of markers and calcified nodules. This suggest why there was no calcification nodules yet in the cells treated with osteogenic medium.

However, we found that cells treated with osteogenic medium showed change in their morphology without calcifying nodules rather we observed crystals (Fig. 1-5) (Appendix 1-1). We therefore suggest that the osteogenic medium might have an effect on the formation of the crystals rather than calcifying nodules. According to Lu et al., (2022) the visibility of calcification in cells treated with osteogenic medium requires long time (between 14-21 days), hence, this correlates with what we observed in the osteogenic treated cells with no visible calcified nodules at the seven days of treatment. Twine et al, 2014 also reported that enriched gene expression is more observed during the late stage of osteogenesis.

In vitro studies on both normal physiology and pathological mineralization via genome-wide gene expression analysis have discovered and shown the role of different genes in VC during phenotypic switching process (Alves et al, 2014). In addition, reporter gene assay has proven to be a good system for tracing the activity of regulatory regions (such as promoters and enhancers) coupled to the reporter (fluorescent or bioluminescent) when activated (Serganova and Blasberg, 2019). This study employed the reporter assay system where identified calcification genetic markers (which includes ALPL, RUNX2, ACAN, CHAD and Col2a1) which play significant role in the transition of VSMCs to the osteochondrogenic phenotype are coupled with reporter gene (mCherry).

This study revealed that the promoter activity of selected genes was increased due to the induction of calcification through the calcifying medium (Fig. 7A-E). Although, we did not observe calcified nodules in the osteogenic treated cells, we found that there was a significant mCherry fluorescence difference between the basal condition and the treated conditions (Fig. 6-10) (Appendix 1-2). Furthermore, in the osteogenic treated cells we discovered that there was an increase in the fluorescence of the mCherry between basal condition and osteogenic treated cells. In contrast, the inorganic phosphate treated cells

showed no major difference between the basal and treated conditions (Fig.11B-F). RUNX2 showed almost similar mCherry fluorescence when comparing the basal (control) condition and treated conditions, hence, we suggest to optimize the reporter system, we might need to select clones with lowered basal activity due to integration site differences of the reporter system.

One of the major challenges associated with calcified SMCs are dissociation and identification of calcified and non-calcified cells. To the best of our knowledge, for the very first time in this study we report the successful dissociation of calcified SMCs into single cells. To validate the findings from the images generated from the incucyte upon induction of calcification, single calcified cells were subjected to fluorescence-activated cell sorting (FACS). The sorted cells were used to identify the best genetic calcification marker for tracing calcification in VSMCs based on the promoter activity of the genes. We found from the study that ALPL and CHAD outperformed the other reporters for tracing calcification since there was significant difference between the basal condition and osteogenic treated cells (Fig.11B and E). This agrees with previous studies which have reported the high expression of these genes when VSMCs transition into the osteochondrogenic phenotype due to VC (Alves et al, 2014; Robert et al, 2020).

Conclusion

It is therefore concluded in this study that the use of our reporter system has proven to be a positive and useful technique to monitor calcification real time. Secondly, for the very first time we report the successful dissociation of calcified SMCs into single cells which will enable further studies such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) screening and ATAC-seq to validate our findings. Finally, CHAD and ALPL are promising reliable markers for tracing vascular calcification. In future, for researchers the reporter system could serve as a quick assay for effectiveness, where the system can be used for either (CRISPR) or drug screening to identify novel genes or drugs that can inhibit or enhance SMCs contribution to vascular calcification.

Future Perspective

This experiment will be carried out with treatment conditions extended to 14 and 21 days so that calcification in osteogenic medium treated cells can be established by clearly observing the calcifying nodules. Also, the single cells generated from the study can be used for CRISPR and ATAC-Seq.

Acknowledgements

It has been a great opportunity to have my master's thesis completed at the Cardiovascular genomics research group in A.I.Virtanen Institute, University of Eastern Finland, Finland. The few times spent in the lab was an awesome and memorable moment to once again use my research skills and learn new skill in the field of cardiovascular research. It was an exciting moment completing my first optimizations in the laboratory. It has been a period of mixed feelings from the understanding that everything might not go as planned in your research to curiosities that actually motivate me as a scientist to explore more. I would like to use this medium to appreciate everyone who has contributed to the success of completing this thesis.

First, I would like to thank my supervisors, Redouane Aherrahrou and Prof. Minna Kaikkonen-Määttä who played a significant role in the success of this research, taking time out of their very busy schedule to discuss my thesis work with me and contributing their wealth of knowledge and ideas. I say a big thank you to Prof. Minna Kaikkonen-Määttä for the great opportunity given to me to do my thesis in her research group. I want to thank her for making me feel as a team member in the group. I want to sincerely appreciate Dr. Redouane Aherrahrou for his support and believe in me throughout the project.

This project would not have had any headway without the assistance of the team members in CADGEN research group. I sincerely want to thank Merja Lukkari for her help from the early stage of the research where we had to complete the amplification and concentration of plasmids used for the study. She made available everything I needed to do my lab work. Prosanta and Mari gave me the best support during the optimization phase of the thesis where I had to work with the smooth muscle cells. They were both always ready to listen to my queries. Thank you, Tiit Ord for making available your inorganic phosphate to optimize the induction of calcification. I enjoy working with everyone in the lab.

I also want to appreciate people outside the lab who helped me. Janne Capra thank you for the assistance with the use of the incucyte for imaging and analysis. Thank you to Larry Mesner and Buo, Atum Michael Lionel from the University of Virginia, USA, for the advice on the staining protocols, induction of calcification and collagenase assay.

I want to thank my flatmates and friends (Bella, Dipo, Fasasi, Amina, Isa, Yemi) for their prayers and moral support throughout the project time. Fasasi A. Salahudeen will always offer to drive me to the lab in the cold though not convenient for him.

To my friends and mentors back home In Nigeria (Prof. Joy Okpuzor, Dr. Fagbayi, Richard Adewole) who supported me when I had to discuss protocols and thoughts with you, you all gave listening ears. I say thank you

Lastly, I want to appreciate my family (Agbabiaje and Bamidele) for the prayer, moral support they gave me during my down times completing the project. I want to especially thank my wonderful and loving wife (Mrs Ronke Agbabiaje) who stood by me in every step of the project when I wept, was scared and worried, she was there for me. I remember you came for vacation but you did not have so much time for fun because of my lab work but you never complained. Thank you for everything my Jewel.

References

Achim A., Kákonyi K, Nagy F, Jambrik Z, Varga A., Nemes A, Chan, JSK, Toth GG, Ruzsa Z (2022) Radial Artery Calcification in Predicting Coronary Calcification and Atherosclerosis Burden. *Cardiol Res Pract* 2022: 5108389.

Aghagolzadeh P, Bachtler M, Bijarnia R, Jackson C, Smith ER, Odermatt A, Radpour R, Pasch A (2016) Calcification of vascular smooth muscle cells is induced by secondary calciprotein particles and enhanced by tumor necrosis factor-α. *Atherosclerosis*. 251: 404-414.

Aherrahrou R, Guo L, Nagraj VP, Aguhob A, Hinkle J, Chen L, Yuhl Soh J, Lue D, Alencar GF, Boltjes A, van der Laan SW, Farber E, Fuller D, Anane-Wae R, Akingbesote N, Manichaikul AW, Ma L, Kaikkonen MU, Björkegren JLM, Önengüt-Gümüşcü S, Pasterkamp G, Miller CL, Owens GK, Finn A, Navab M, Fogelman AM, Berliner JA, Civelek M (2020) Genetic Regulation of Atherosclerosis-Relevant Phenotypes in Human Vascular Smooth Muscle Cells. *Circ Res* 127(12): 1552-1565.

Ahmad FB, Cisewski JA, Anderson RN (2022) Provisional Mortality Data — United States, 2021. *MMWR. Morbidity and Mortality Weekly Report* 71, pp. 597–600.

Alencar GF, Owsiany KM, Karnewar S, Sukhavasi K, Mocci G, Nguyen AT, Williams CM, Shamsuzzaman S, Mokry M, Henderson CA, Haskins R, Baylis RA, Finn AV, McNamara CA, Zunder ER, Venkata V, Pasterkamp G, Björkegren J, Bekiranov S, Owens GK (2020) Stem Cell Pluripotency Genes Klf4 and Oct4 Regulate Complex SMC Phenotypic Changes Critical in Late-Stage Atherosclerotic Lesion Pathogenesis. *Circulation* 142(21): 2045-2059.

Alves RD, Eijken M, van de Peppel J, van Leeuwen JP (2014) Calcifying vascular smooth muscle cells and osteoblasts: independent cell types exhibiting extracellular matrix and biomineralization-related mimicries. *BMC Genomics* 15, 965.

Anwar MA, Shalhoub J, Lim CS, Gohel MS, Davies AH (2012) The Effect of Pressure-Induced Mechanical Stretch on Vascular Wall Differential Gene Expression. *J Vasc Res* 49(6): 463–478.

Augstein A, Mierke J, Poitz DM, Strasser RH (2018) Sox9 is increased in arterial plaque and associated with synthetic phenotype of vascular smooth muscle cells and causes alterations in extracellular matrix and calcification. *Biochim Biophys Acta Mol Basis Dis*. 1864(8): 2526-2537.

Bartel MA, Weinstein JR, Schaffer DV (2012) Directed evolution of novel adeno-associated viruses for therapeutic gene delivery. *Gene Ther*. 19: 694–700.

Bentzon JF, Otsuka F, Virmani R, Falk E (2014) Mechanisms of Plaque Formation and Rupture. *Circ Res*, 114(12): 1852–1866.

Björkegren JLM, Lusis A.J (2022) Atherosclerosis: Recent Developments. *Cell* 185(10): 1630–1645.

Bogdanova MV, Zabirnyk A, Malashicheva A, Semenova D, Kvitting JPE, Kaljusto ML, Pérez MM, Kostareva A, Stensløkken KO, Sullivan GJ, Rutkovskiy A and Vaage J (2022) Models and Techniques to Study Aortic Valve Calcification *in vitro*, ex Vivo and in Vivo. An Overview. *Frontiers in Pharmacology*, 13.

Borland SJ, Behnsen J, Ashton N, Francis, SE, Brennan K, Sherratt MJ, Withers PJ and Canfield AE (2020) X-ray Micro-Computed Tomography: An Emerging Technology to Analyse Vascular Calcification in Animal Models. *Int J Mol Sci* 21(12): 4538.

Cao G, Xuan, X, Hu J, Zhang R, Jin H and Dong H (2022) How Vascular Smooth Muscle Cell Phenotype Switching Contributes to Vascular Disease. *Cell Communication and Signaling* 20(1).

Ceccherini E, Cecchettini A, Gisone I, Persiani E, Morales MA and Vozzi F (2022) Vascular Calcification: *in vitro* Models under the Magnifying Glass. *Biomedicines*, 10(10), 2491.

Centres for Disease Control and Prevention (CDC) (2019). *Heart disease risk factors*. [online] Centres for Disease Control and Prevention. Available at: https://www.cdc.gov/heartdisease/risk_factors.htm.

Chen J, Budoff MJ, Reilly MP, Yang W, Rosas SE, Rahman M, Zhang X, Roy JA, Lustigova E, Nessel L, Ford V, Raj D, Porter AC, Soliman EZ, Wright JT, Wolf M and He J (2017) Coronary Artery Calcification and Risk of Cardiovascular Disease and Death Among Patients with Chronic Kidney Disease. *JAMA Cardiol* 2(6), 635.

Cheng P, Wirka RC, Kim JB, Kim HJ, Nguyen T, Kundu R, Zhao Q, Sharma D, Pedroza A, Nagao M, Iyer D, Fischbein MP, Quertermous T (2022) Smad3 regulates smooth muscle cell fate and mediates adverse remodeling and calcification of the atherosclerotic plaque. *Nat Cardiovasc Res* 1(4): 322-333.

Chignon A, Bon-Baret V, Boulanger MC, Bossé Y, Mathieu P (2021) Oxyphospholipids in Cardiovascular Calcification. *Arterioscler Thromb Vasc Biol* (1): 11-19.

Chow B and Rabkin SW (2015) The Relationship between Arterial Stiffness and Heart Failure with Preserved Ejection fraction: A Systemic meta-analysis. *Heart Fail Rev* 20(3), 291–303.

Coronado F, Melvin SC, Bell RA and Zhao G (2022) Global Responses to Prevent, Manage, and Control Cardiovascular Diseases. *Prev Chronic Dis*, 19: E84.

Côté N, El Husseini D, Pépin A, Guauque-Olarte S, Ducharme V, Bouchard-Cannon P, Audet A, Fournier D, Gaudreault N, Derbali H, McKee MD, Simard C, Després JP, Pibarot P, Bossé Y, Mathieu P (2012) ATP acts as a survival signal and prevents the mineralization of aortic valve. *J Mol Cell Cardiol* 52(5): 1191-202.

Dal Canto E, Ceriello A, Rydén L, Ferrini M, Hansen TB, Schnell O, Standl E and Beulens JW (2019) Diabetes as a cardiovascular risk factor: An overview of global trends of macro and micro vascular complications. *Eur J Prev Cardiol* 26(2), 25–32.

Dube P, DeRiso A, Patel M, Battepati D, Khatib-Shahidi B, Sharma H, Gupta R, Malhotra D, Dworkin L, Haller S, Kennedy D (2021) Vascular Calcification in Chronic Kidney Disease: Diversity in the Vessel Wall. *Biomedicines* 9(4): 404.

Durham AL, Speer MY, Scatena M, Giachelli CM and Shanahan CM (2018) Role of Smooth Muscle Cells in Vascular calcification: Implications in Atherosclerosis and Arterial Stiffness. *Cardiovasc Res* 114(4), 590–600.

Espinosa-Diez C, Mandi V, Du M, Liu M and Gomez D (2021) Smooth muscle cells in atherosclerosis: Clones but not carbon copies. *JVS Vasc Sci*. 2: P136-148.

Frostegård J (2013) Immunity, Atherosclerosis and Cardiovascular Disease. *BMC Medicine*, 11(1).

Fuchs FD and Whelton PK (2019) High Blood Pressure and Cardiovascular Disease. *Hypertension*, 75(2), 285–292.

Goto S, Rogers MA, Blaser MC, Higashi H, Lee LH, Schlotter F, Body SC, Aikawa M, Singh SA, Aikawa E (2019) Standardization of Human Calcific Aortic Valve Disease *In vitro* Modeling Reveals Passage-dependent Calcification. *Front. Cardiovasc Med* 6, 49.

Greco A, Herrman J, Babic M, Gummi MR, van der Giet M, Tölle M and Schuchardt, M (2022) Molecular Imaging and Quantification of Smooth Muscle Cell and Aortic Tissue Calcification *In vitro* and Ex Vivo with a Fluorescent Hydroxyapatite-Specific Probe. *Biomedicines*, 10, 2271.

Greenland P, Blaha MJ, Budoff MJ, Erbel R and Watson KE (2018) Coronary Calcium Score and Cardiovascular Risk. *Journal of the American College of Cardiology*, 72(4): 434–447.

Guembe MJ, Fernandez-Lazaro CI, Sayon-Orea C, Toledo E and Moreno-Iribas C (2020) Risk for Cardiovascular Disease Associated with Metabolic Syndrome and Its components: a 13-year Prospective Study in the RIVANA Cohort. *Cardiovas Diabetol* 19(1): 195.

Harman JL, Jørgensen HF (2019) The role of smooth muscle cells in plaque stability: Therapeutic targeting potential. *Br J Pharmacol* 176(19):3741-3753.

Herrmann J, Babic M, Tölle M, Markus and Schuchardt M (2020) Research Models for Studying Vascular Calcification. *Int J Mol Sci* 21(6): 2204–2204.

Himmelsbach A, Ciliox C, Goettsch C (2020) Cardiovascular calcification in chronic kidney disease—therapeutic opportunities. *Toxins (Basel)* 12(3): 181.

Holmar J, Noels H, Böhm M, Bhargava S, Jankowski J and Orth-Alampour S (2020) Development, Establishment and Validation of *in vitro* and Ex Vivo Assays of Vascular Calcification. *Biochem Biophys Res Commun* 530(2): 462–470.

Jankowski J, Floege J, Fliser D, Böhm M and Marx N (2021) Cardiovascular Disease in Chronic Kidney Disease. *Circulation*, 143(11): 1157–1172.

Jiang W, Zhang Z, Li Y, Chen C, Yang H, Lin Q, Hu M, Qin X. (2021) The cell origin and role of osteoclastogenesis and osteoblastogenesis in vascular calcification. *Front in Cardiovasc Med* 8: 639740.

Jiang, Y, Qian, HY (2023) Transcription factors: key regulatory targets of vascular smooth muscle cell in atherosclerosis. *Mol Med* 29(1):2.

Kapustin AN, Chatrou ML, Drozdov I, Zheng Y, Davidson SM, Soong D, Furmanik M, Sanchis P, De Rosales RT, Alvarez-Hernandez D, Shroff R, Yin X, Muller K, Skepper JN, Mayr M, Reutelingsperger CP, Chester A, Bertazzo S, Schurgers LJ, Shanahan CM (2015) Vascular smooth muscle cell calcification is mediated by regulated exosome secretion. *Circ Res*. 116(8):1312–1323.

Karwowski W, Naumnik B, Szczepański M and Myśliwiec M (2012) The Mechanism of Vascular Calcification – a Systematic Review. *Med Sci Monit* 18(1), pp.RA1–RA11.

Kawtharany L, Bessueille L, Issa H, Hamade E, Zibara K, Magne D (2022) Inflammation and Microcalcification: A Never-Ending Vicious Cycle in Atherosclerosis? *J Vasc Res* 59(3): 137-150.

Kuhn, O and Lynch S (2016) Heart Attacks. *The Recorder*, 41.

Lai CF, Shao JS, Behrmann A, Krchma K, Cheng SL, Towler DA (2012) TNFR1-activated reactive oxidative species signals up-regulate osteogenic Msx2 programs in aortic myofibroblasts. *Endocrinology* 153: 3897–3910.

Lanzer P, Hannan FM, Lanzer JD, Janzen J, Raggi P, Furniss D, Schuchardt M, Thakker R, Fok PW, Saez-Rodriguez J, Millan A, Sato Y, Ferraresi R, Virmani R, St Hilaire C (2021) Medial Arterial Calcification: JACC State-of-the-Art Review. *J Am Coll Cardiol* 78: 1145–1165.

Lee HY, Lim S, Park S (2021) Role of Inflammation in Arterial Calcification. *Korean Circ J* 51(2): 114-125

Lee JS, Morrisett JD, Tung CH (2012) Detection of hydroxyapatite in calcified cardiovascular tissues. *Atherosclerosis* 224(2): 340–347.

Lee SJ, Lee IK and Jeon JH (2020). Vascular Calcification—New Insights into Its Mechanism. *Int J Mol Sci* 21(8): 2685.

Leon BM and Maddox TM (2015) Diabetes and Cardiovascular disease: Epidemiology, Biological mechanisms, Treatment Recommendations and Future Research. *World Journal of Diabetes*, 6(13): 1246.

Lim J, Ehsanipour A, Hsu JJ, Lu J, Pedego T, Wu A, Walthers CM, Demer LL, Seidlits SK, Tintut Y (2016) Inflammation drives retraction, stiffening, and nodule formation via cytoskeletal machinery in a three-dimensional culture model of aortic stenosis. *Am J Pathol* 186: 2378–2389.

Lin ME, Chen TM, Wallingford MC, Nguyen NB, Yamada S, Sawangmake C, Zhang J, Speer MY, Giachelli CM (2016) Runx2 deletion in smooth muscle cells inhibits vascular osteochondrogenesis and calcification but not atherosclerotic lesion formation. *Cardiovasc Res* 112(2):606–616.

Liu J Xiao X, Shen Y, Chen L, Xu C, Zhao H, Wu Y, Zhang Q, Zhong J, Tang Z, Liu C, Zhao Q, Zheng Y, Cao R, Zu X (2017) MicroRNA-32 promotes calcification in vascular smooth muscle cells: Implications as a novel marker for coronary artery calcification. *PLoS One* 12(3): e0174138

Liu L, Zeng P, Yang X, Duan Y, Zhang W, Ma C, Zhang X, Yang S, Li X, Yang J, Liang Y, Han H, Zhu Y, Han J, Chen Y (2018) Inhibition of Vascular Calcification. *Arterioscler Thromb Vasc Biol* 38(10): 2382-2395.

Long F (2012) Building strong bones: molecular regulation of the osteoblast lineage. *Nat Rev Mol Cell Biol* 13: 27-38.

Lostale-Seijo I, Montenegro J. 2018 Synthetic materials at the forefront of gene delivery. *Nat Rev Chem* 2: 258–277

Lu C, Dong X, Yu WP, Ding JL, Yang W, Gong Y, Liu JC, Tang YH, Xu JJ, Zhou JL (2020) Inorganic phosphate-osteogenic induction medium promotes osteogenic differentiation of valvular interstitial cells via the BMP-2/Smad1/5/9 and RhoA/ROCK-1 signaling pathways. *Am J Transl Res* 12(7): 3329-334

Lusis AJ (2000) Atherosclerosis. *Nature*, 407(6801): 233–241

Mahmood SS, Levy D, Vasan RS, Wang TJ (2014) The Framingham Heart Study and the epidemiology of cardiovascular disease: a historical perspective. *Lancet* 383(9921): 999-1008.

Major RW, Cheng MRI, Grant RA, Shantikumar S, Xu G, Oozeerally I, Brunskill NJ and Gray LJ (2018) Cardiovascular disease risk factors in chronic kidney disease: A systematic review and meta-analysis. *PLOS One* 13(3): 0192895.

Martín M, Valls J, Betriu A, Fernández E, Valdivielso JM (2015) Association of serum phosphorus with subclinical atherosclerosis in chronic kidney disease. sex makes a difference. *Atherosclerosis* 241: 264–270.

Massy ZA, Drüeke TB (2015) Magnesium and cardiovascular complications of chronic kidney disease. *Nat Rev Nephrol* 11: 432–442.

McAloon CJ, Osman F, Glennon P, Lim PB and Hayat SA (2016) Global Epidemiology and Incidence of Cardiovascular Disease. In: *Cardiovascular Diseases: Genetic Susceptibility, Environmental Factors and Their Interaction*. pp.57–96. Academic Press.

Mori H, Torii S, Kutyna M, Sakamoto A, Finn AV and Virmani R (2018) Coronary Artery Calcification and Its Progression: What Does It Really Mean? *JACC: Cardiovascular Imaging* 11(1): 127–142.

Murray CJL, Aravkin AY, Zheng P, Abbafati C, Abbas KM, Abbasi-Kangevari M, Abd-Allah F, Abdelalim A, Abdollahi M, Abdollahpour I, Abegaz KH, Abolhassani H, Aboyans V, Abreu LG, Abrigo MRM., Abualhasan A, Abu-Raddad LJ, Abushouk AI, Adabi M and Adekanmbi V (2020). Global burden of 87 risk factors in 204 countries and territories, 1990–2019: a systematic analysis for the Global Burden of Disease Study (2019) *The Lancet*, 396(10258): 1223–1249.

Nagaishi Y, Murata D, Yoshizato H, Nonaka T, Itoh M, Hara H and Nakayama K (2023) Scaffold-free Human Vascular Calcification Model Using a bio-three-dimensional Printer. *Biofabrication* 15: 1–11.

Neefjes M, Housmans BAC, van den Akker GGH, van Rhijn LW, Welting TJM, van der Kraan P.M (2021) Reporter gene comparison demonstrates interference of complex body fluids with secreted luciferase activity. *Sci Rep* 11, 1359.

Obisesan OH, Kou M, Wang F, Boakye E, Honda Y, Uddin S, Dzaye O, Osei AD, Orimoloye OA, Howard-Claudio, CM, Coresh J, Blumenthal RS, Hoogeveen RC, Budoff MJ, Matsushita K, Ballantyne CM, Blaha, MJ (2022) Lipoprotein(a) and Subclinical Vascular and Valvular Calcification on Cardiac Computed Tomography: the Atherosclerosis Risk in Communities Study. *J Am Heart Assoc* 11(11).

Pan H, Xue C, Auerbach BJ, Fan J, Bashore AC, Cui J, Yang DY, Trignano SB, Liu W, Shi J, Ihuegbu CO, Bush EC, Worley J, Vlahos L, Laise P, Solomon RA, Connolly ES, Califano A, Sims PA, Zhang H, Li M, Reilly MP (2020) Single-Cell Genomics Reveals a Novel Cell State During Smooth Muscle Cell Phenotypic

Switching and Potential Therapeutic Targets for Atherosclerosis in Mouse and Human. *Circulation* 142(21):2060-2075.

Park KS, Lee Y, Park GM, Park JH, Kim YG, Yang DH, Kang JW, Lim TH, Kim HK, Choe J, Lee SW, Kim YH (2020) Association between serum phosphorus and subclinical coronary atherosclerosis in asymptomatic Korean individuals without kidney dysfunction. *Am J Clin Nutr* 112: 66–73.

Pescatore LA, Gamarra LF and Liberman M (2019) Multifaceted Mechanisms of Vascular Calcification in Aging. *Arterioscler Thromb. Vasc. Biol*. 39: 1307–1316.

Pustlauk W, Westhoff TH, Claeys L, Roch T, Geißler S, Babel N (2020) Induced osteogenic differentiation of human smooth muscle cells as a model of vascular calcification. *Sci Rep* 10, 5951.

Radvar E, Griffanti G, Tsolaki E, Bertazzo S, Nazhat SN, Addison O, Mata A, Shanahan CM, Elsharkawy S (2021) Engineered *In vitro* Models for Pathological Calcification: Routes Toward Mechanistic Understanding. *Advanced NanoBiomed Research* 1(8): 2100042.

Reiss AB, Miyawaki N, Moon J, Kasselman LJ, Voloshyna I, D'Avino R Jr, De Leon J (2018) CKD, arterial calcification, atherosclerosis and bone health: inter-relationships and controversies. *Atherosclerosis* 278: 49–59.

Robert AW, Marcon BH, Dallagiovanna B, Shigunov P (2020) Adipogenesis, Osteogenesis and Chondrogenesis of Human Mesenchymal Stem/Stromal Cells: A Comparative Transcriptome Approach. *Front Cell Dev Biol* 8: 561.

Roth GA, Forouzanfar MH, Moran AE, Barber R, Nguyen G, Feigin VL, Naghavi M, Mensah GA and Murray CJL (2015) Demographic and Epidemiologic Drivers of Global Cardiovascular Mortality. *N Engl J Med* 372(14): 1333–1341.

Rui S, Kubota T, Ohata Y, Yamamoto K, Fujiwara M, Takeyari S and Ozono K (2022) Phosphate promotes osteogenic differentiation through non-canonical Wnt signaling pathway in human mesenchymal stem cells. *Bone* 164, 116525.

Sato Y, Hiroyuki Jinnouchi H, Sakamoto A, Cornelissen A, Mori M, Kawakami R, Kawai K, Virmani R and Finn AV. (2020) Calcification in human vessels and valves: from pathological point of view. *AIMS Mol Sci* 7(3): 183–210.

Savinov AY, Salehi M, Yadav MC, Radichev I, Millán JL, Savinova OV (2015) Transgenic Overexpression of Tissue-Nonspecific Alkaline Phosphatase (TNAP) in Vascular Endothelium Results in Generalized Arterial Calcification. *J Am Heart Assoc* 4(12): e002499.

Schäck LM, Noack S, Winkler R, Wißmann G, Behrens P, Wellmann M, Jagodzinski M, Krettek C, Hoffmann A (2013) The Phosphate Source Influences Gene Expression and Quality of Mineralization during *In vitro* Osteogenic Differentiation of Human Mesenchymal Stem Cells. *PLoS ONE* 8(6): e65943.

Schurgers LJ, Akbulut AC, Kaczor DM, Halder M, Koenen RR and Kramann R (2018). Initiation and Propagation of Vascular Calcification Is Regulated by a Concert of Platelet- and Smooth Muscle Cell-Derived Extracellular Vesicles. *Front. Cardiovasc. Med* 5: 36.

Serganova I and Blasberg RG (2019) Molecular Imaging with Reporter Genes: Has Its Promise Been Delivered? *J Nucl Med* 60: 1665–1681.

Serguienko A, Wang MY and Myklebost O (2018) Real-Time Vital Mineralization Detection and Quantification during *In vitro* Osteoblast Differentiation. *Biological Procedures Online*, 20: 14.

Shaban SM, Jo SB, Hafez E, Cho JH, Kim D-H (2022) A comprehensive overview on alkaline phosphatase targeting and reporting assays. *Coord Chem Rev* 465, 214567.

Shanahan CM, Crouthamel MH, Kapustin A, Giachelli CM 2011. Arterial calcification in chronic kidney disease: key roles for calcium and phosphate. *Circ Res* 109(6): 697–711.

Shao C, Wang J, Tian J, Tang YD 2020. Coronary Artery Disease: From Mechanism to Clinical Practice. *Adv Exp Med Biol* 1177:1-36.

Shao S, Liu Y, Hong W, Mo Y, Shu F, Jiang L, Tan N (2023) Influence of FOSL1 Inhibition on Vascular Calcification and ROS Generation through Ferroptosis via P53-SLC7A11 Axis. *Biomedicines*, 11(2), 635–635.

Sheen CR, Kuss P, Narisawa S, Yadav MC, Nigro J, Wang W, Chhea TN, Sergienko EA, Kapoor K, Jackson MR, Hoylaerts MF, Pinkerton AB, O'Neill WC, Millán JL. (2015) Pathophysiological role of vascular smooth muscle alkaline phosphatase in medial artery calcification. *J Bone Miner Res* 30(5): 824-36.

Sorokin V, Vickneson K, Kofidis T, Chin Cheng Woo CC, Lin XY, Foo R, Catherine M. Shanahan CM (2020) Role of Vascular Smooth Muscle Cell Plasticity and Interactions in Vessel Wall Inflammation. *Front. Immunol* 11: 599415.

St. Hilaire C (2022) Medial Arterial Calcification: A Significant and Independent Contributor of Peripheral Artery Disease. *Arterioscler Thromb and Vasc Biol* 42(3): 253–260.

Stabley JN, Towler DA, (2017) "Arterial calcification in diabetes mellitus," *Arterioscler Thromb and Vasc Biol* 37(2): 205–217.

Sun Y, Byon CH, Yuan K, Chen J, Mao X, Heath JM, Javed A, Zhang K, Anderson PG, Chen Y (2012) Smooth muscle cell-specific runx2 deficiency inhibits vascular calcification. *Circ Res* 111(5): 543-52.

Tang HY, Chen AQ, Zhang H, Gao XF, Kong XQ and Zhang JJ (2022) Vascular Smooth Muscle Cells Phenotypic Switching in Cardiovascular Diseases *Cells* 11(24): 4060.

Townsend N, Kazakiewicz D, Lucy Wright F, Timmis A, Huculeci R, Torbica A, Gale CP, Achenbach S, Weidinger F and Vardas P (2022) Epidemiology of cardiovascular disease in Europe. *Nat Rev Cardiol* 19: 133–143.

Trillhaase A, Schmidt B, Märtens M, Haferkamp U, Erdmann J and Aherrahrou Z (2021) The CAD Risk Locus 9p21 Increases the Risk of Vascular Calcification in an iPSC-derived VSMC Model. *Stem Cell Res Ther* 12(1): 166.

Trion A, van der Laarse A (2004) Vascular smooth muscle cells and calcification in atherosclerosis. *Am Heart J* 147(5): 808–814.

Tsuda T, Imanishi M, Oogoshi M, Goda M, Kihira Y, Horinouchi Y, Zamami Y, Ishizawa K, Ikeda Y, Hashimoto I, Takahashi T and Izawa-Ishizawa Y (2020) Rho-associated Protein Kinase and Cyclophilin a Are Involved in Inorganic phosphate-induced Calcification Signaling in Vascular Smooth Muscle Cells. *J Pharmacol Sci* 142(3): 109–115.

Twine NA, Chen L, Pang CN, Wilkins MR, Kassem M (2014) Identification of differentiation-stage specific markers that define the ex vivo osteoblastic phenotype. *Bone* 67: 23–32.

Vaduganathan M, Mensah GA, Turco JV, Fuster V and Roth GA (2022) The Global Burden of Cardiovascular Diseases and Risk: A Compass for Future Health. *J Am Coll of Cardiol* 80(25): 2361-2371.

Valdivielso JM, Rodríguez-Puyol D, Pascual J, Barrios C, Bermúdez-López M, Sánchez-Niño MD, Pérez-Fernández M, Ortiz A. (2019) "Atherosclerosis in chronic kidney disease,". *Arterioscler Thromb Vasc Biol* 39(10): 1938–1966.

Vieceli Dalla Sega F, Fortini F, Severi P, Rizzo P, Gardi I, Cimaglia P, Rapezzi C, Tavazzi L and Ferrari R (2022) Cardiac Calcifications: Phenotypes, Mechanisms, Clinical and Prognostic Implications. *Biology*, 11(3): 414.

Woo SH, Kim DY, Choi JH (2018) Roles of Vascular Smooth Muscle Cells in Atherosclerotic Calcification. *J Lipid Atheroscler* 12(2): 106-118.

Woo SH, Kyung D, Lee SH, Park KS, Kim M, Kim K, Kwon HJ, Won YS, Choi I, Park Y-J, Go D-M, Oh J-S, Yoon WK, Paik SS, Kim JH, Kim YH, Choi JH, and Kim DY 2023. TXNIP Suppresses the Osteochondrogenic Switch of VSMCs in Atherosclerosis. *Circu Res* 132:00–00.

Wu H, Whitfield TW, Gordon JA, Dobson JR, Tai PW, van Wijnen AJ, Stein JL, Stein GS, Lian JB (2014) Genomic occupancy of Runx2 with global expression profiling identifies a novel dimension to control of osteoblastogenesis. *Genome Biol* 15(3): R52.

Wu M, Rementer C and Giachelli CM (2013) Vascular Calcification: An Update on Mechanisms and Challenges in Treatment. *Calcif Tissue Int* 93(4): 365–373.

Yao J, Wu X, Qiao X, Zhang D, Zhang L, Ma J, Cai X, Boström KI and Yao Y (2021) Shifting osteogenesis in vascular calcification. *JCI Insight* 6(10): e143023.

Yin H, Kanasty RL, Eltoukhy AA, Vegas AJ, Dorkin JR, Anderson DG (2014) Nonviral vectors for gene-based therapy. *Nat Rev Genet* 15: 541–555.

Zhao M, Lin XH, Zeng YH, Su HZ, Wang, C, Yang K, Chen YK, Lin BW, Yao XP and Chen WJ (2022) Knockdown of myorg leads to brain calcification in zebrafish. *Mol Brain*, 15(1): 65.

Zhu D, Mackenzie NCW, Millán JL, Farquharson C, MacRae VE (2011) The Appearance and Modulation of Osteocyte Marker Expression during Calcification of Vascular Smooth Muscle Cells. *PLoS One* 6(5): e19595.

APPENDIX 1-1

Calcification images of calcified areas stained with calcein generated from the incucyte in triplicates (Fig. 1-5). The green fluorescent dots are the deposits of calcium and phosphate in the cell

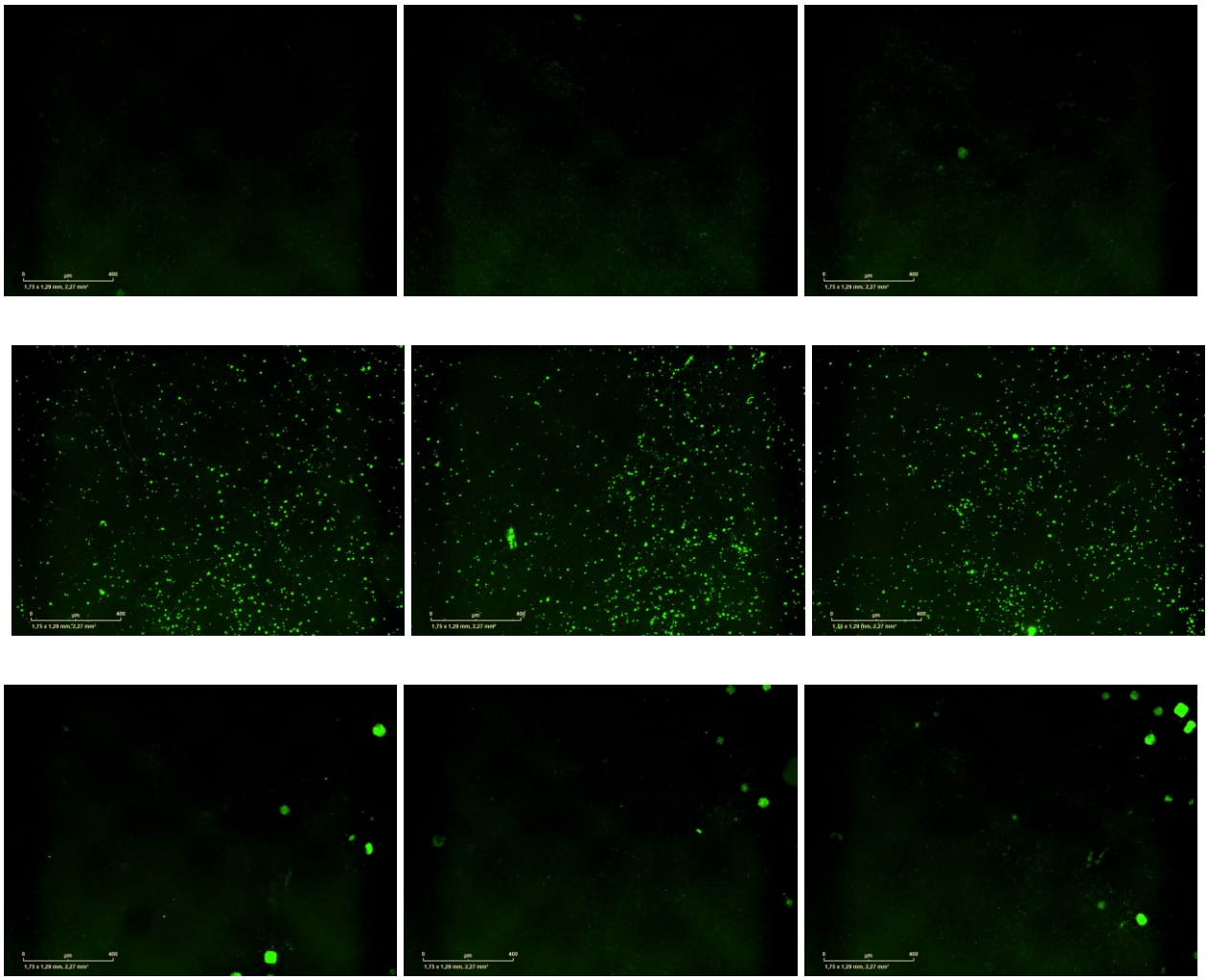


Fig.1: Representative images of calcified area as observed in calcein stained stable cells expressing ALPL reporter gene. Treatment was done in triplicates as seen above.

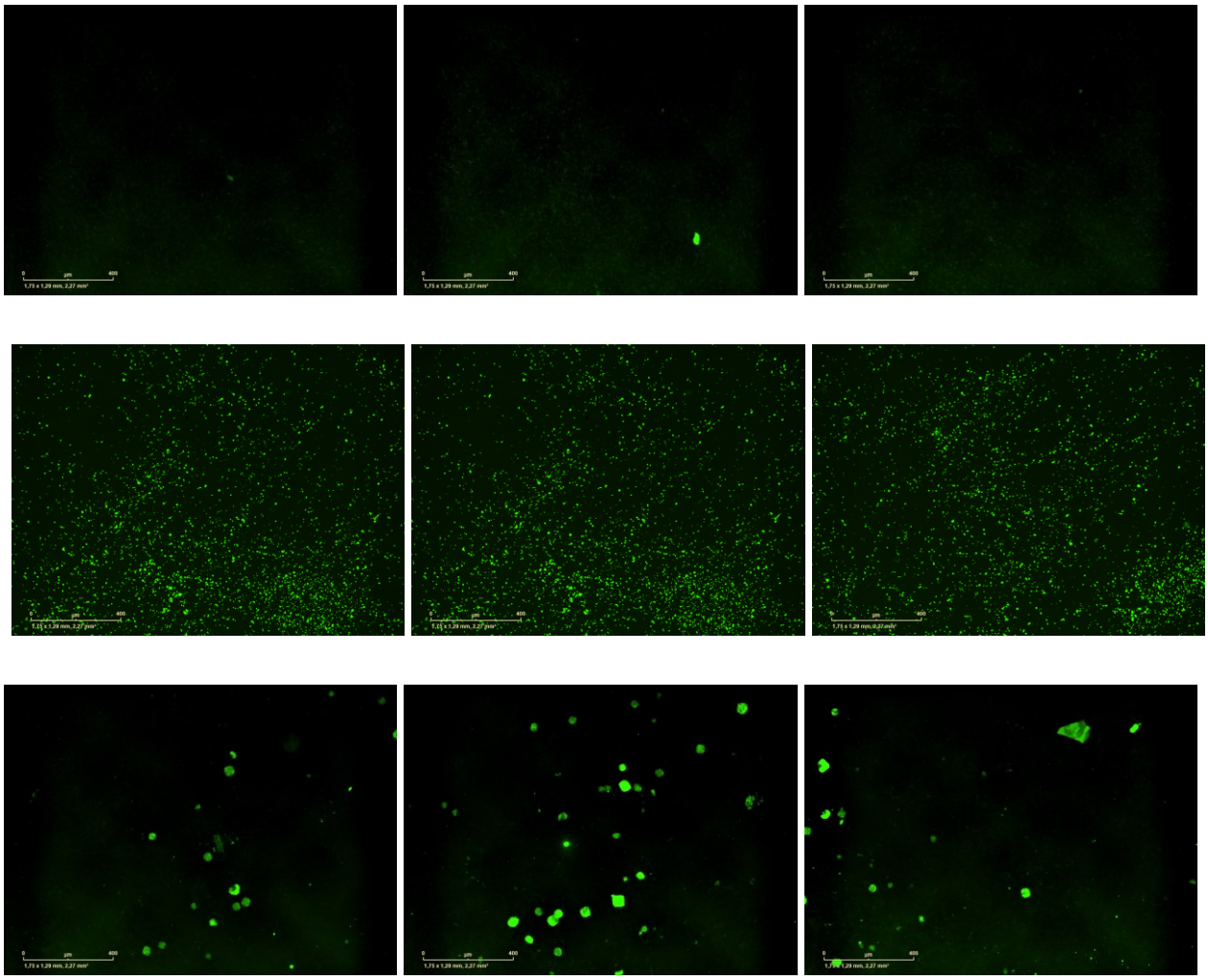


Fig.2: Representative images of calcified area as observed in calcein stained stable cells expressing RUNX2 reporter gene. Treatment was done in triplicates as seen above.

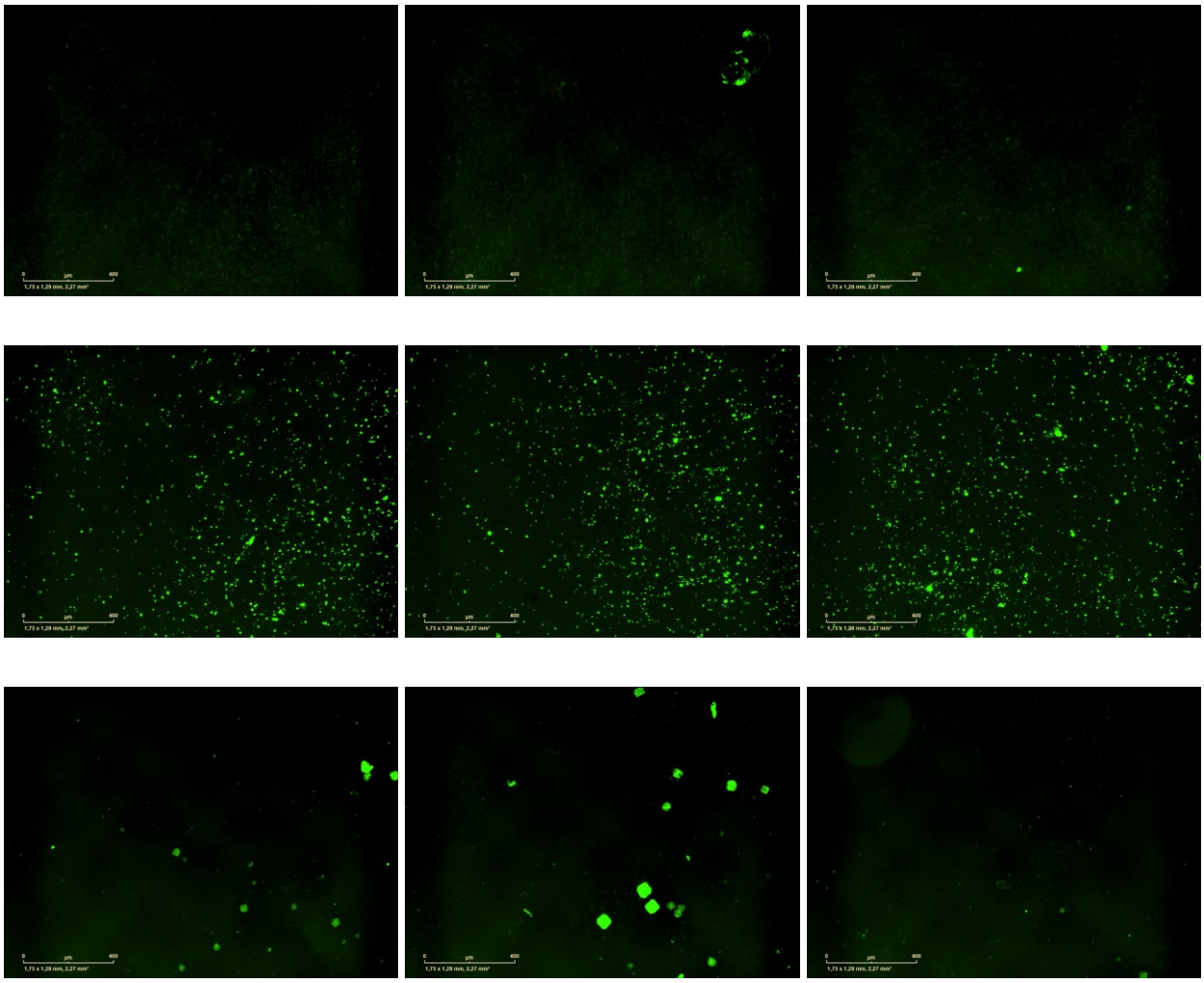


Fig.3: Representative images of calcified area as observed in calcein stained stable cells expressing ACAN reporter gene. Treatment was done in triplicates as seen above.

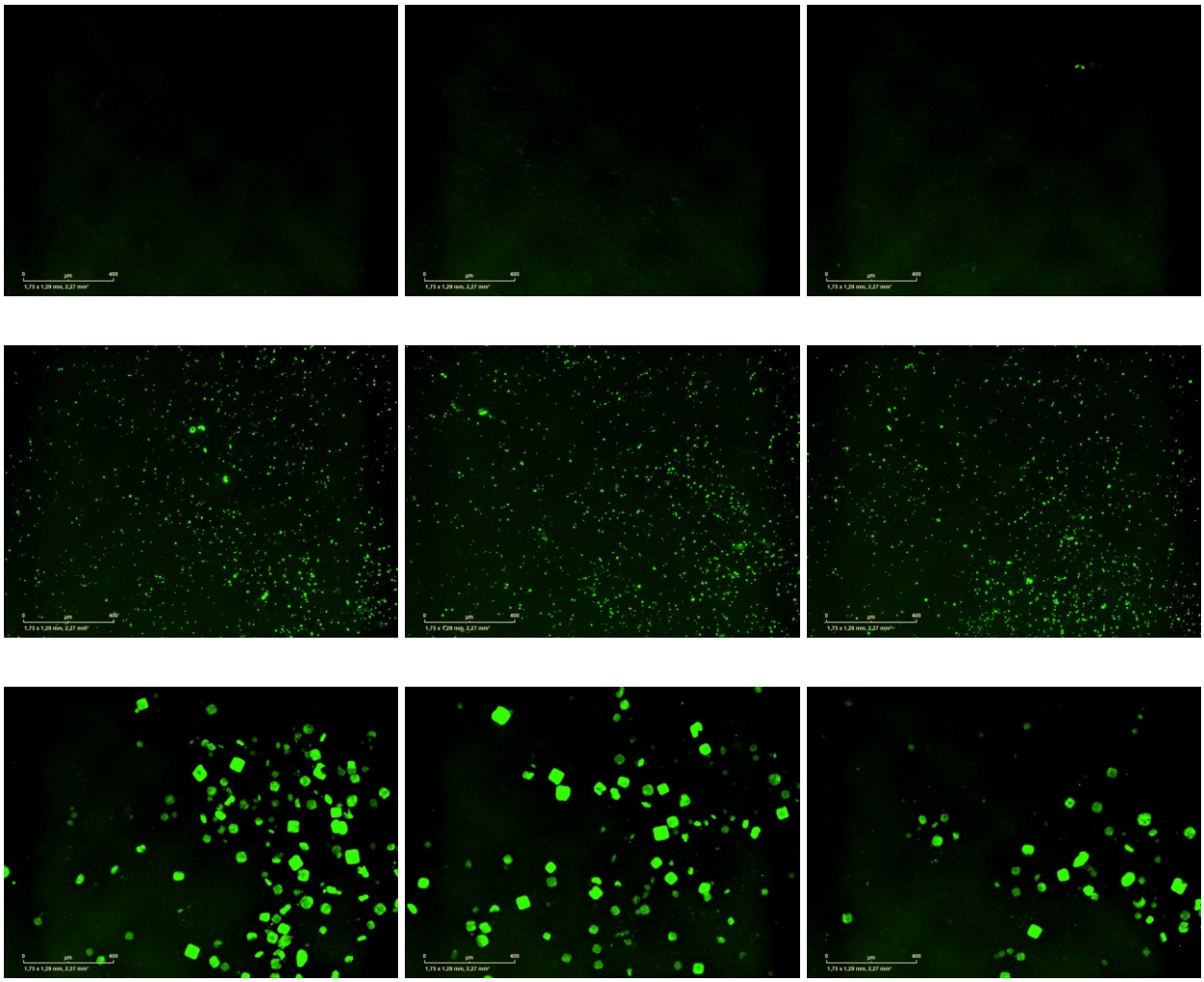


Fig.4: Representative images of calcified area as observed in calcein stained stable cells expressing CHAD reporter gene. Treatment was done in triplicates as seen above.

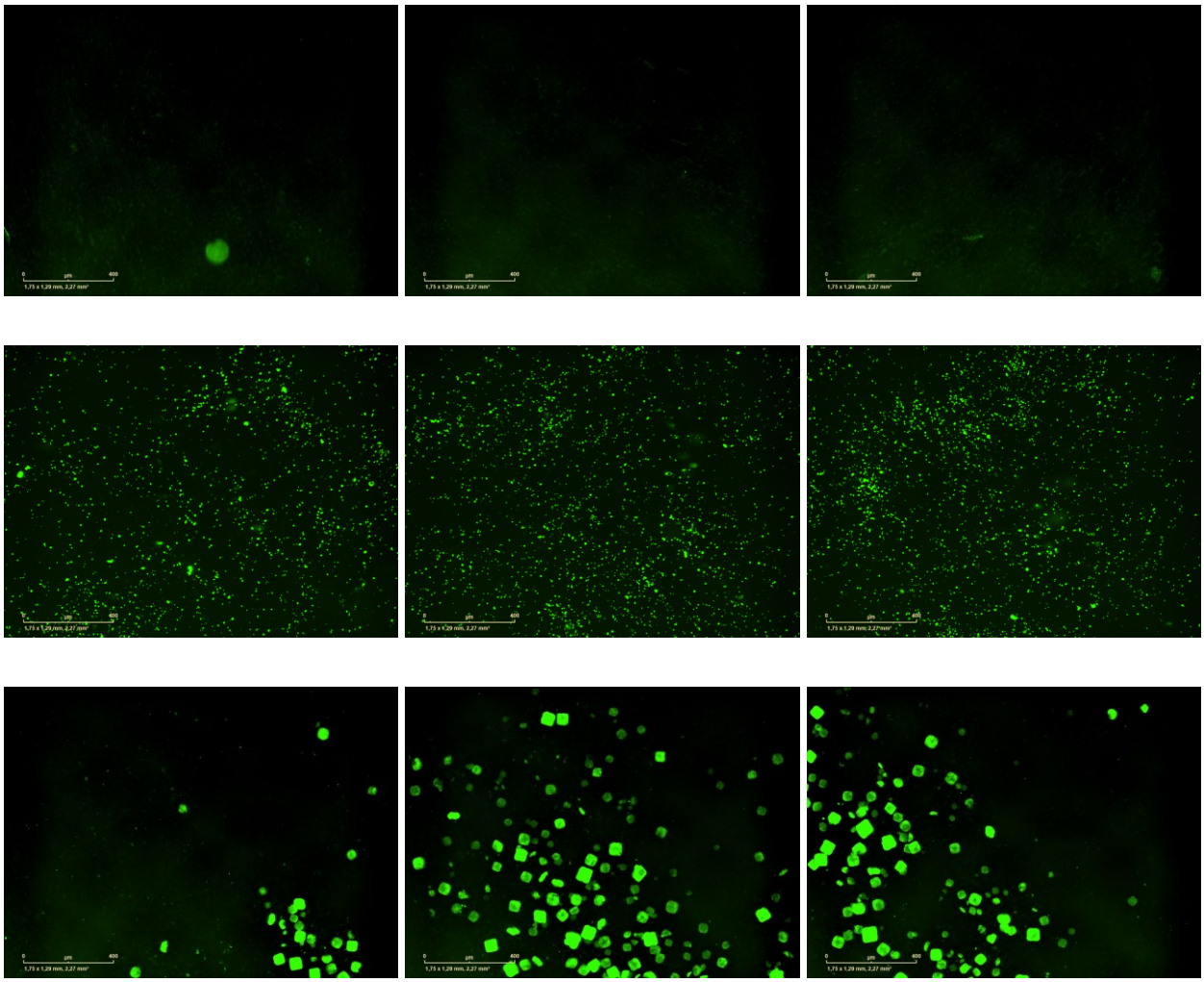


Fig.5: Representative images of calcified area as observed in calcein stained stable cells expressing COL2a1 reporter gene. Treatment was done in triplicates as seen above.

First row: Control; Second row: Inorganic phosphate treatment; Third row: Osteogenic medium treatment

APENDIX 1-2

Images of the cells showing promoter activity for reporter gene upon induction of calcification in the stable lines. Fig. 6-10.

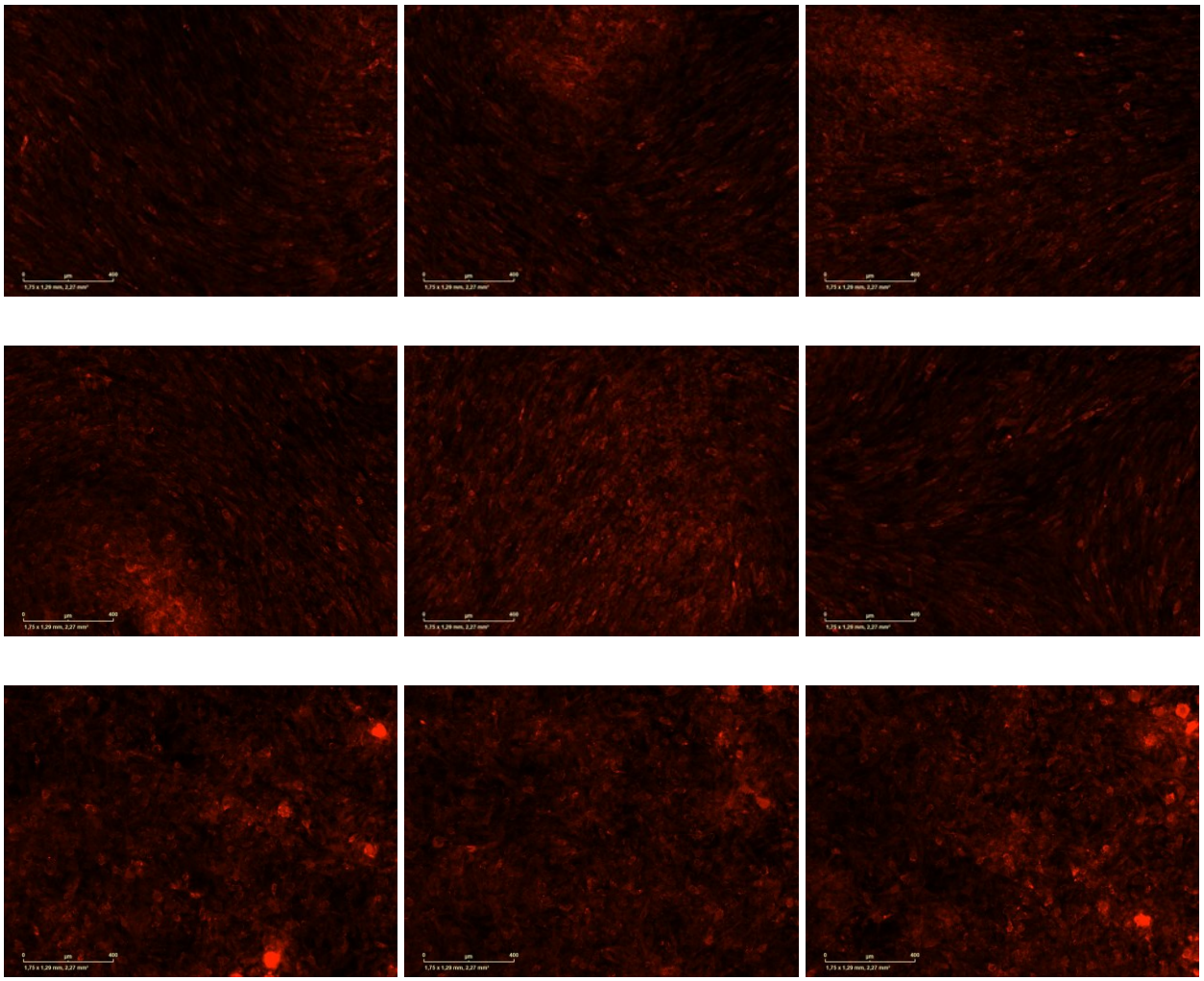


Fig.6: Representative images of mCherry fluorescence in stable cells expressing ALPL reporter gene when calcification was induced us. Treatment was done in triplicates as seen above.

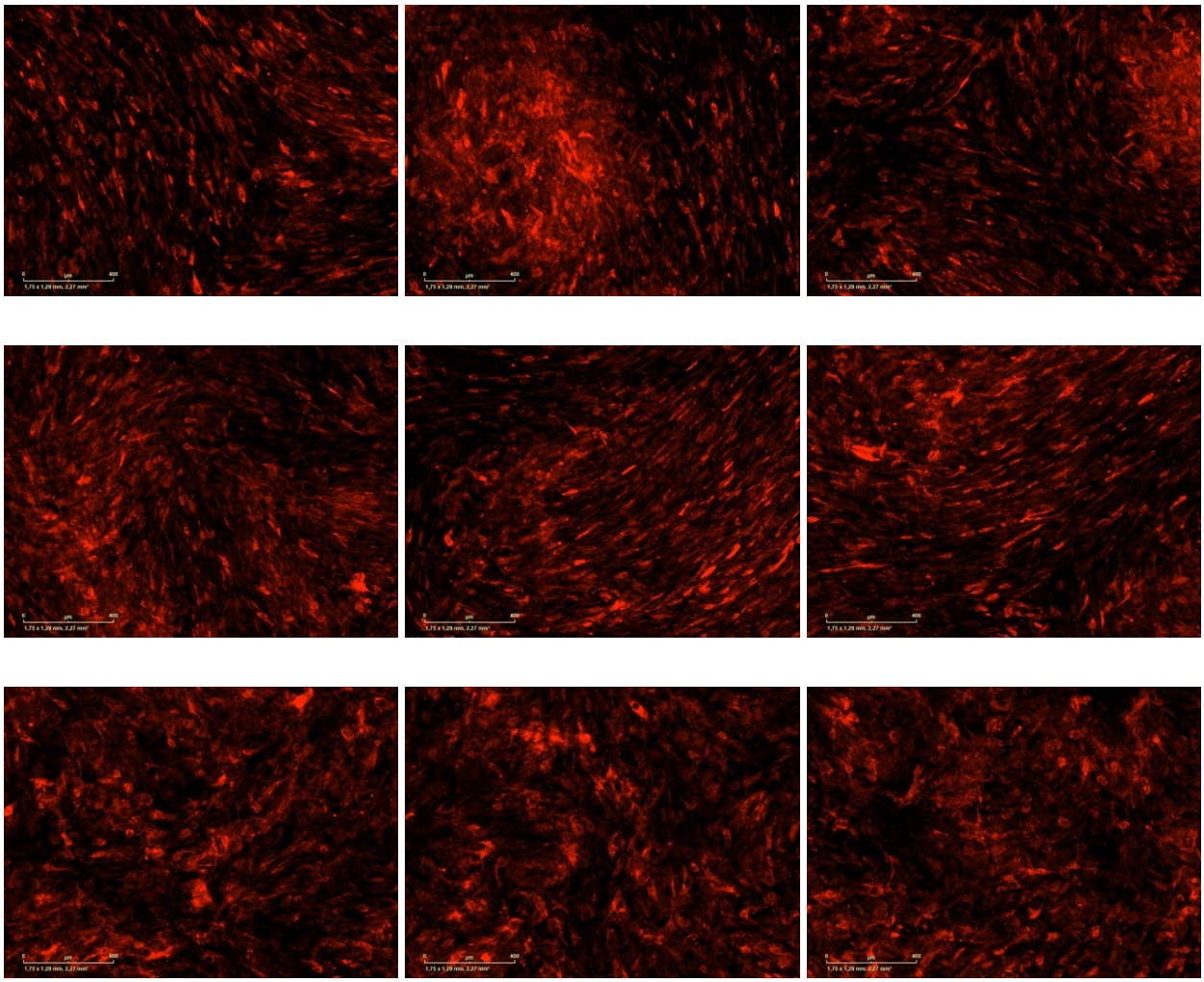


Fig.7: Representative images of mCherry fluorescence in stable cells expressing RUNX2 reporter gene when calcification was induced us. Treatment was done in triplicates as seen above.

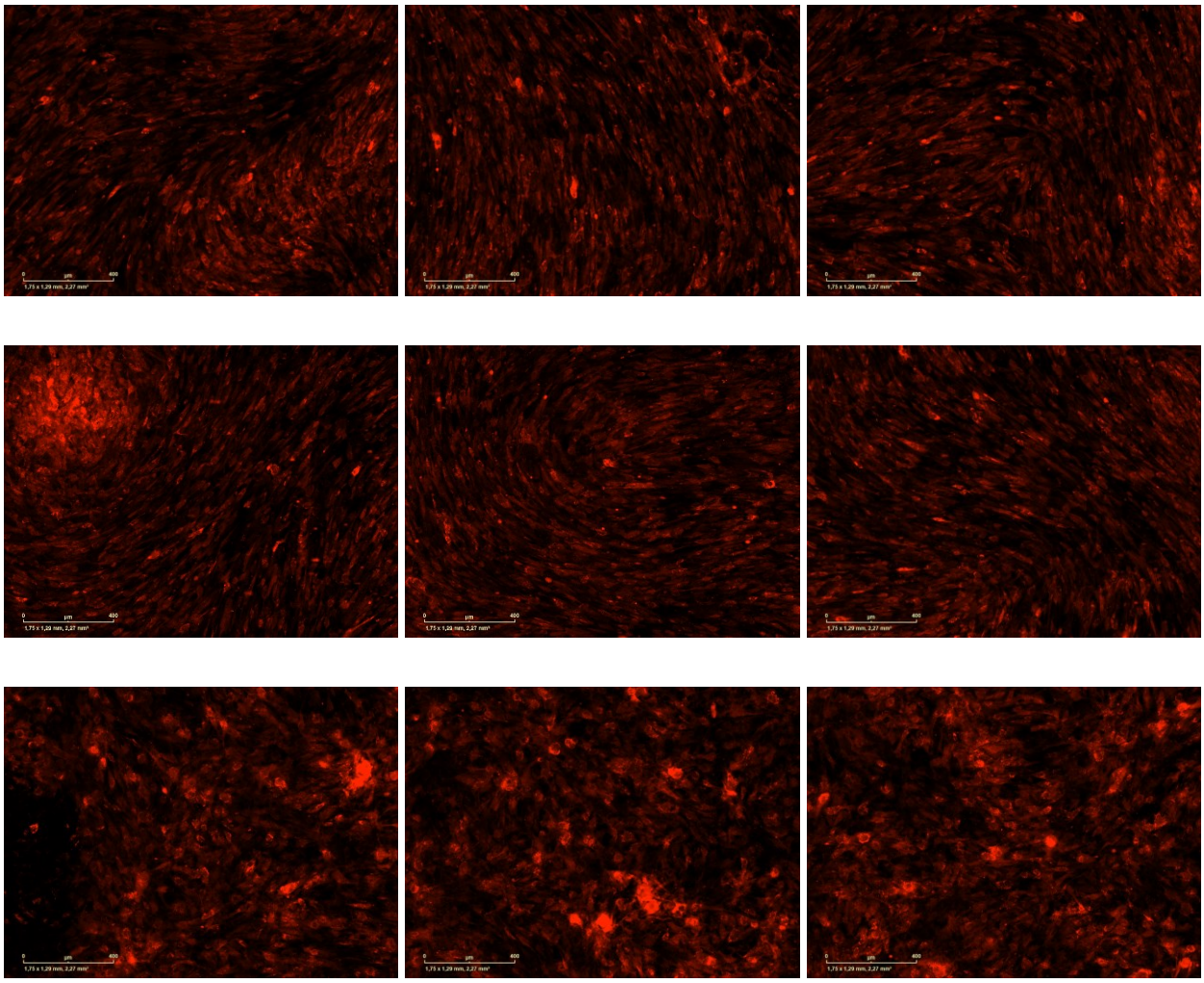


Fig.8: Representative images of mCherry fluorescence in stable cells expressing ACAN reporter gene when calcification was induced us. Treatment was done in triplicates as seen above.

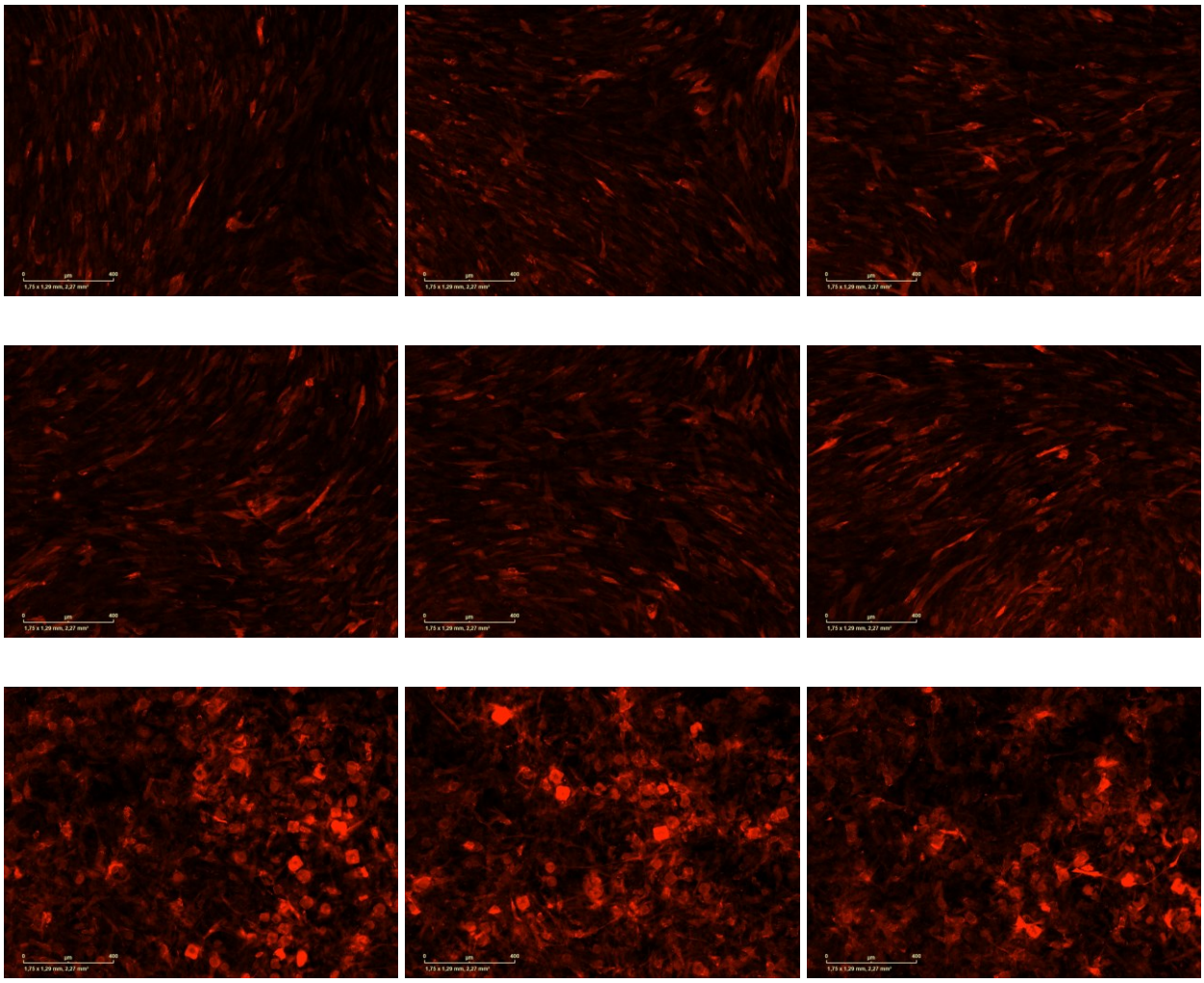


Fig.9: Representative images of mCherry fluorescence in stable cells expressing CHAD reporter gene when calcification was induced us. Treatment was done in triplicates as seen above.

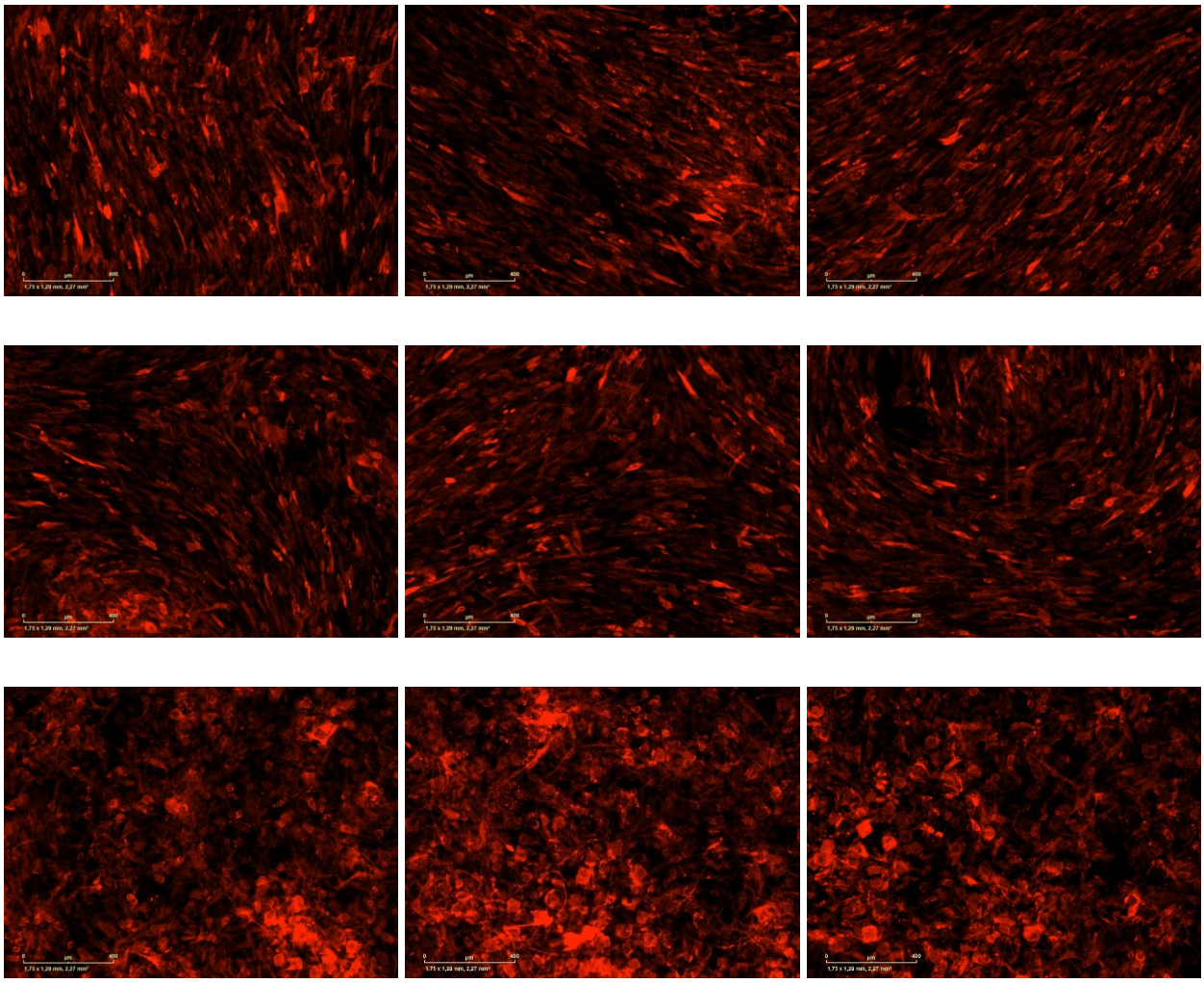


Fig.10: Representative images of mCherry fluorescence in stable cells expressing Col2a1 reporter gene when calcification was induced us. Treatment was done in triplicates as seen above.

First row: Control; Second row: Inorganic phosphate treatment; Third row: Osteogenic medium treatment

*Applicable to all images both green and red channel

APPENDIX 1-3

The table below shows the quantification data for the calcified areas in images from the stable lines stained with calcein. The green dots in the images were counted and analysed using the Fiji/imageJ.

Table 1: Green dot (calcified area) count from the images generated for inorganic phosphate treated stable cells. The treatment was done in triplicates n=3

GENETIC MARKER	REPLICATE 1	REPLICATE 2	REPLICATE 3	Mean
ALPL	650	695	804	716
RUNX2	1206	1523	1322	1350
ACAN	684	752	793	743
CHAD	717	764	747	743
Col2a1	1018	1343	1386	1249

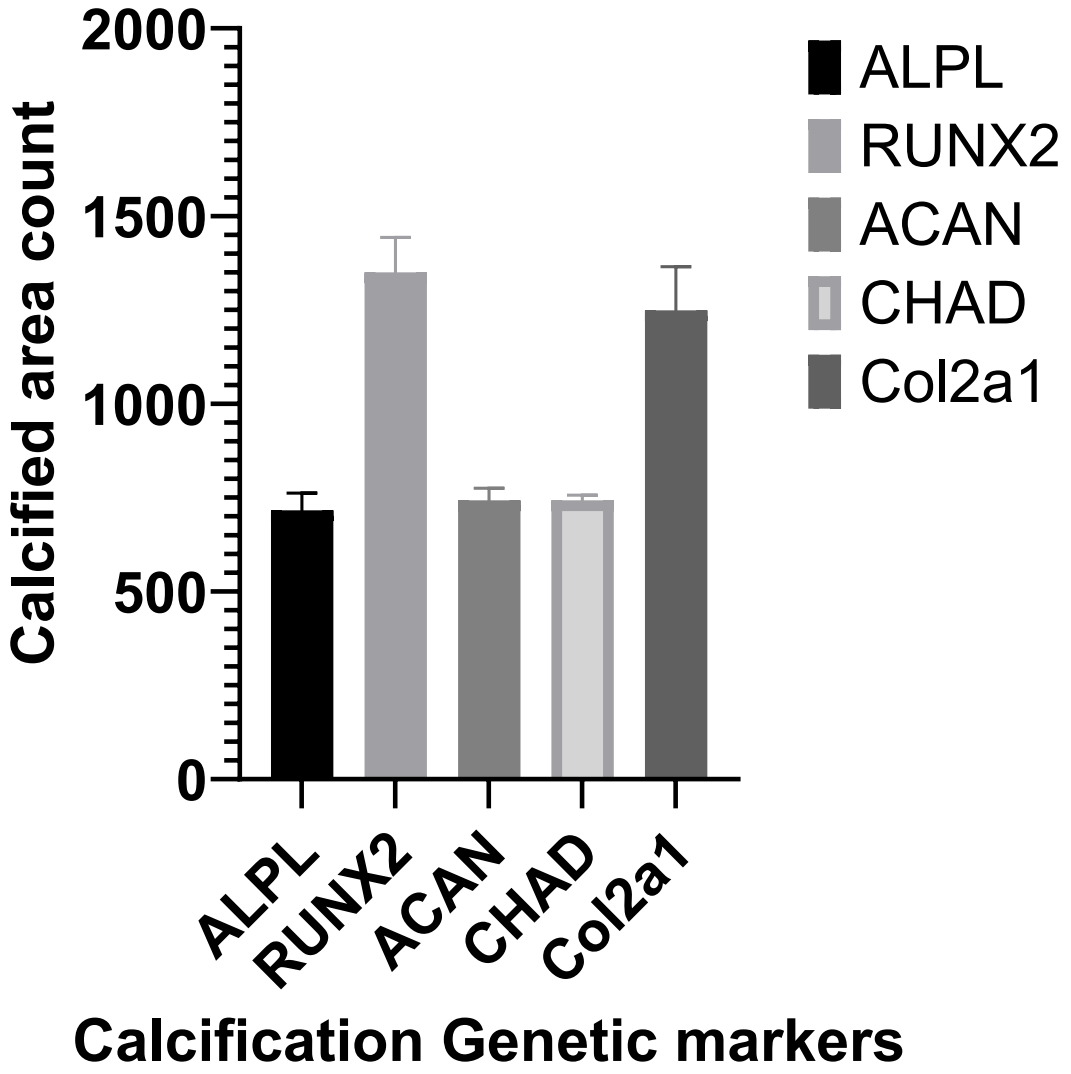


Fig. 11.: The count for green dot in images showing calcified areas in reporter systems upon inorganic phosphate treatment in stable cells. n=3

APPENDIX 2-1

Calcified SMCs were dissociated using collagenase assay, the singles cells were sorted by FACS to compared the mCherry fluorescence to identify which of the reporter gene is best used for tracing calcification (Fig. 12 and 13).

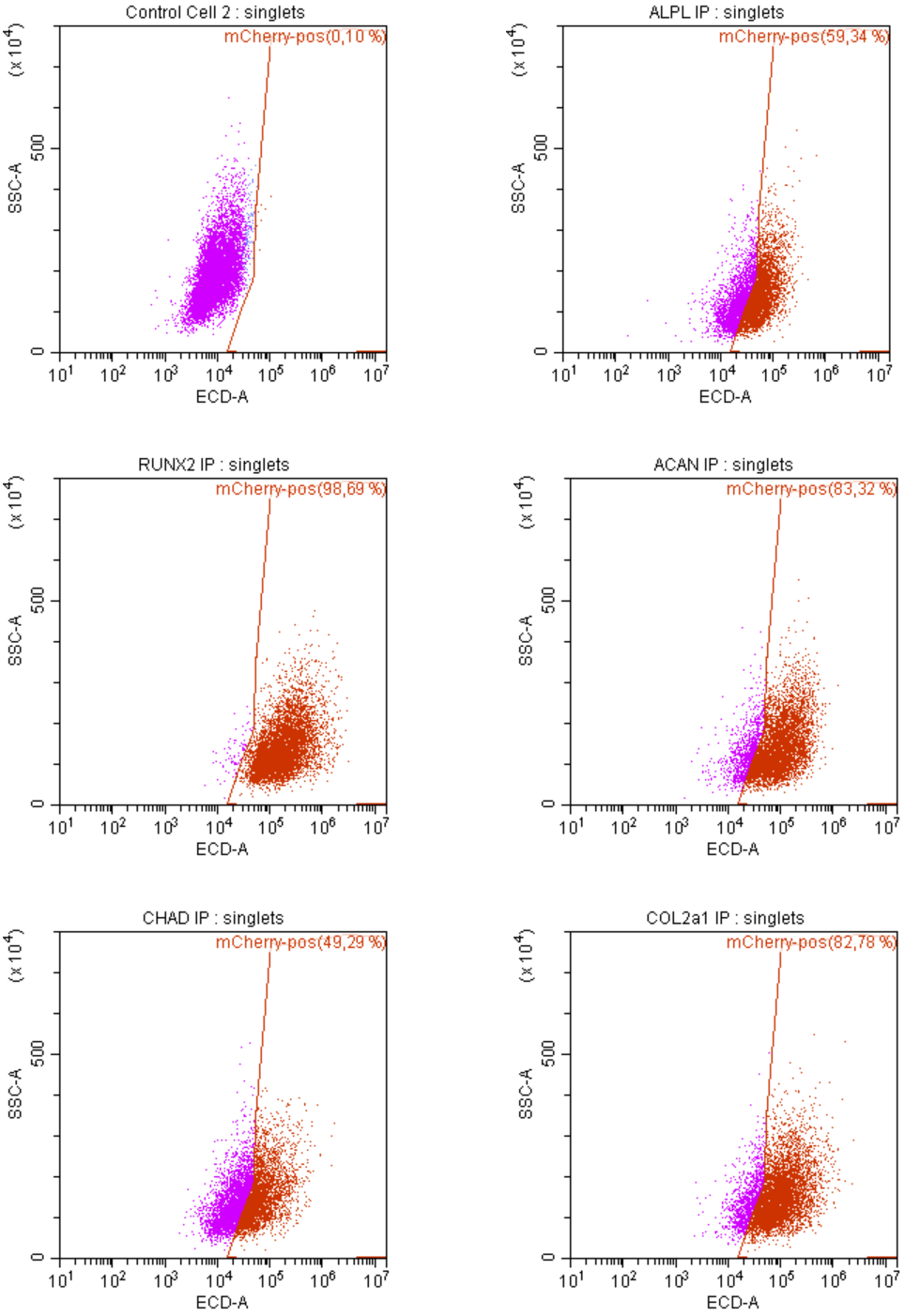


Fig. 12: Comparison of FACS sorted single calcified cells between control and IP treated cells

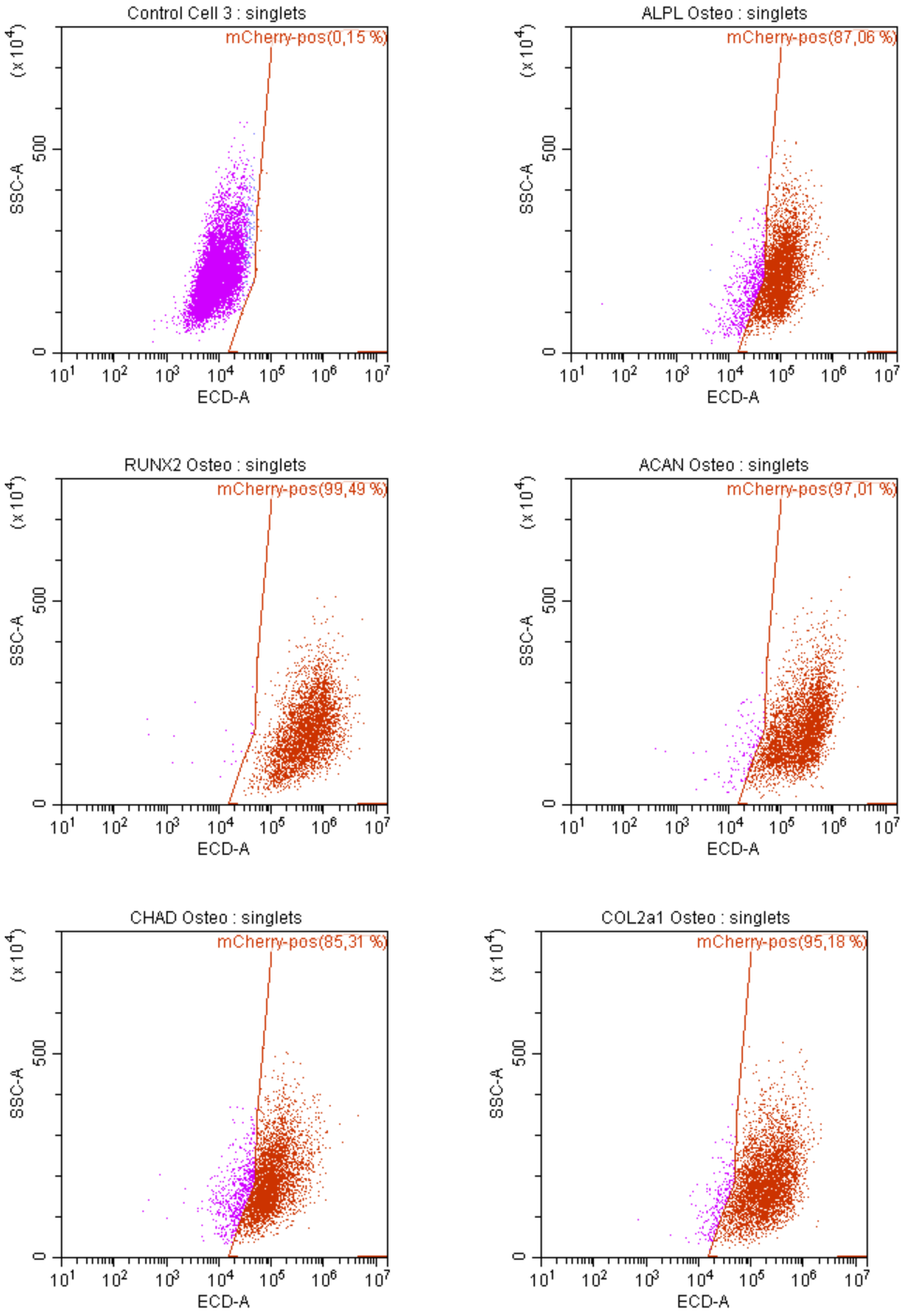


Fig. 13: Comparison of FACS sorted single calcified cells between control and osteogenic medium treated cells