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Patient-Specific Induced Pluripotent stem cell models of variant angina derived from peripheral blood

말초혈액유래 역분화 줄기세포를 이용한 이형 협심증 기전 연구

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ABSTRACT

Cardiovascular disease is the one of the most common causes of death in the world. Specially, variant angina is more prevalent in Korean and Japanese than in Caucasian population. Variant angina is caused by a spasm in the coronary arteries and is considered a fatal disease, since it leads to myocardial infarction, arrhythmia, and sudden death. Until now, however, the mechanisms of this illness have not been elucidated.

I isolated circulating multipotent stem (CiMS) cells from normal and variant angina patient peripheral blood to generate human induced pluripotent stem cells (hiPSC). Human vascular smooth muscle cells (VSMCs) were differentiated from hiPSC and their calcium efflux was analyzed using with the fluorescent dye FLUO4. Moreover, endothelial cells (EC) were differentiated from hiPSC and characterized with immunocytochemistry and observed immunofluorescent using confocal microscopy.

Using the calcium efflux model, I observed a high intensity of intracellular calcium and specific calcium efflux in the variant angina group, but not in the normal group. I identified differences between normal and variant angina vascular smooth muscle cells

contractility using an *in vitro* calcium fluorescence model. Increased expression of *Serca2* was also observed in variant angina VSMCs. I also confirmed increased *Serca2* protein expression and its SUMOylation, which is the key factor to regulate VSMC hyperactivity.

I isolated CiMS from normal and variant angina patients and observed hyperactivity in samples from variant angina patients and overepxression of *Serca2* as well as an increase in sumoylated *Serca2*, which might be a mechanism underlying the cause of artery spasms. These newly identified mechanisms of variant angina showed clinical relevance, suggesting that these mechanisms could be promising for the development of new drugs and effective therapy.

Keywords: hiPSC, Variant angina, Vascular smooth muscle, Endothelial cell, Differentiation, calcium efflux, FLUO4, SERCA2, Sumoylation

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LIST OF ABBREVIATIONS

VA, Variant angina

PBMC, Peripheral blood mononuclear cells

iPSC, Induced pluripotent stem cell

CiMS, Circulating multipotent stem cells

ECs, Endothelial cells

SMCs, Smooth muscle cells

AP, Alkaline phosphatase

hEGF, Human epidermal growth factor

VEGF, Vascular endothelial growth factor

bFGF, Basic fibroblast growth factor

NCM, non-colony type monolayer

CNN1, Calponin 1

SMA, Smooth muscle actin

SERCA2, Sarco/Endoplasmic Reticulum Ca^{2+}ATPase 2

SUMO1, Small ubiquitin-like modifier 1

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INTRODUCTION

Coronary artery spasms play a significant role in many types of ischemic heart disease, not only in angina pectoris, but also in myocardial infarction with or without ST-segment elevation, and ventricular arrhythmia, which cause sudden death[1-3]. Coronary artery spasm represents an important type of variant angina and is more prevalent in Asian populations than Caucasian populations.

Printzmetal and his colleagues first reported variant angina in 1959[4]. This syndrome of ischemic pain is accompanied by ST elevation and occurs at rest, most often between midnight and morning. Variant angina is a more generalized form of coronary artery disease with higher occurrence in Japanese and Korean than Caucasians populations[5]. The incidence of variant angina in patients can occur in a much younger age than stable or unstable angina.

Over sixty years after the first description of variant angina, the understanding of the causes of and mechanisms underlying the syndrome, as well as the methods of direct diagnosis are still lacking.

According to recent studies, the mechanisms of this syndrome have been suggested to include: 1) endothelial cell dysfunction, 2) hyperactivity of vascular smooth muscle cells (VSMCs), 3) heavy smokers, excessive alcohol, inflammation, 4) genetic factors.

The endothelium plays a significant role in coronary vascular tone, releasing several vasodilators including nitric oxide (NO), which is considered to be the most important vasodilated factor. Therefore, critical injury of the endothelium causes vasoconstriction[6]. It is important to understand that diverse vasoactive stimuli, such as acetylcholine, histamine, and serotonin, cause NO release by the endothelium, leading to vasoconstriction through direct stimulation of vascular smooth muscles[7].

In patients with variant angina, vascular smooth muscle cell (VSMC) hyperactivity is persistent. VSMC contraction, however, is regulated by a complex mechanism, which not yet elucidated but known to involve diverse G-proteins, enzymes, and regulatory pathways. The contraction of the vascular smooth muscle is regulated by intracellular calcium signals[8]. The contractile stimulations are provoked by an elevation of cytosolic

calcium concentration[9]. The elevation of intracellular calcium increases the level of calcium-bound calmodulin, which activates myosin light chain kinase. Abnormality in any one of numerous pathways might be responsible for coronary vascular smooth muscle cell hyperactivity[10]. There are many other potential hypotheses for variant angina. A majority (75%) of heavy smokers show a significant association with coronary artery spasm. How smoking is associated with coronary artery spasm, however, remains unknown[11]. Excessive alcohol consumption has also been associated with variant angina, but the evidence is not robust[12]. Inflammation might be a potential marker by which coronary artery spasm could be detected. In previous studies of inflammatory cells, master cells in particular were discussed[13]. In recent studies, adventitial coronary tissues released inflammatory cytokines that were found to be the cause of spasms for vascular smooth muscle cells in pigs *in vivo*[14].

Several genetic mutations have been discussed as being potentially involved in variant angina. Much of the focus on the genetic aspects of variant angina has focused on the gene encoding NO synthase[7], but polymorphisms have been also described in other genes, such as serotoninergic receptors and

adrenergic receptors[15,16], antioxidant enzymes, and inflammatory cytokines[17,18]. However, there is no consensus in the field. Furthermore, family history has been found to be not a risk factor for variant angina, suggesting that genetic factors may not be a major component in the pathogenesis of variant angina.

Due to the rarity of the illness, and the tissue that is affected is the coronary artery, biopsy analysis has been limited. Much of the information describing the pathophysiology of variant angina has come from studies on patient-derived circulating multipotent stem (CiMS) cells. CiMS cells are a recently characterized population, which can be derived from human peripheral blood. These cells are mobilized from the endocardium.

Here, I describe an induced pluripotent stem cell (iPSC) model of variant angina. iPSC lines were made from patient-derived CiMS cells and differentiated into vascular smooth muscle cells and endothelial cells to analyze the mechanisms of intracellular calcium release on the functional properties of the cells and investigate the differences between normal and variant angina patients. I found that induced intracellular calcium levels were increased in variant angina VSMCs after using a variety of stimulatory vasoactive factors such as carbachol, acetylcholine

and ergometrine maleate, but no such increase was seen in normal VSMCs. These results support the hypothesis that the stimulation-induced intracellular calcium increases calcium uptake by sumoylated-SERCA2 in endoplasmic reticulum (ER).

In vascular smooth muscle cells, two *SERCA* genes are expressed: *ATP2A2* and *ATP2A3*. The major VSMC *Serca2* isoform can be either *SERCA2a* or *SERCA2b*, depending on the cell phenotype[19,20]. After expressing the *SERCA2a* isoform in VSMCs, an agonist-induced elevation of cytosolic calcium triggers VSMC contraction[20,21]. *SERCA2a* is a critical ATPase controlling calcium re-uptake during excitation-contraction coupling[22]. SUMOylation at lysine 480 and 585 is essential for maintaining *SERCA2a* ATPase activity and stability in mouse and human cells[22].

In this study, I identified a novel variant angina mechanism using cells differentiated from human induced pluripotent stem cells. Moreover, I also examined their potential use for diagnosis *in vitro* and overall clinical relevance.

MATERIALS AND METHODS

1. Peripheral blood mononuclear cell (PBMNC) isolation and circulating multipotent stem (CiMS) cell culture

Human peripheral blood samples (10 mL) were obtained from blood donors with informed consent. PBMNCs were isolated from the blood samples by density gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) according to the manufacturer's recommendations. Freshly isolated mononuclear cells were suspended with the EGM-2MV BulletKit system (Lonza, Basel, Switzerland), which contains endothelial basal medium-2, 5% fetal bovine serum, human epidermal growth factor (hEGF), vascular endothelial growth factor (VEGF), human fibroblast growth factor-basic (hFGF-B), insulin growth factor-1 (R3-IGF-1), and ascorbic acid. Mononuclear cells were seeded on non-coated six-well plates (Sigma, St. Louis, MO) at 6×10^6 cells per well and incubated in a 5% CO_2 incubator at 37°C.

The medium was changed daily for up to two weeks after plating. Adherent CiMS cells were observed as early as three days after the start of culture and gradually formed colonies. CiMS cell

colonies grown in culture were maintained with EGM-2MV media and sub-cultured using 0.05% Trypsin-EDTA solution. CiMS cells were passaged every three to four days.

2. Retro virus infection and iPSC generation

We co-transfected human embryonic kidney (HEK) 293T cells, which were plated at 8.3×10^3 cells per 100 mm dish and incubated overnight. The next day, the cells were transfected with pMXs vectors with polyethylenimine (Polyscience, Warrington, PA, USA) transfection reagent (Open Biosystems). Forty-eight hours after transfection, the medium was collected as the first virus-containing supernatant and replaced with fresh medium, which was collected after twenty-four hours later as the second virus-containing supernatant. Normal CiMS and variant angina CiMS were seeded at 5×10^5 cells per 35 mm dish one day before transduction. The virus-containing supernatants were filtered through a 0.22 mm pore-size filter and concentrated using ultrahigh centrifugation at 25000 rpm for 90 min. Viral supernatant was supplemented with 10 μg/mL polybrene. Equal amounts of supernatants containing each of the four retroviruses were mixed, transferred to the CiMS cell dish,

and incubated overnight. Twenty-four hours after transduction, the virus-containing medium was replaced with EGM-2MV medium. Six days after transduction, CiMS cells were harvested by trypsinization and re-plated at 2.5×10^5 cells per 35 mm dish on an STO feeder cell layer. Two days later, the medium was replaced with Primate ES Cell Medium supplemented with 10 ng/mL bFGF. The medium was changed every other day. Fourteen days after transduction, colonies were picked and transferred into 2 mL of Primate ES Cell Medium.

3. Immunofluorescent staining and confocal microscopy analysis

Immunostaining was performed as described previously[21] with slight modifications. Briefly, cells were fixed with 4% paraformaldehyde for 30 minutes, permeabilized with 100% MeOH for five minutes, and blocked with 1% bovine serum albumin in PBS for one hour. After blocking, cells were incubated overnight at 4℃ with specific primary antibodies: anti-NANOG (Cell Signaling Technology, Danvers, MA, USA), anti-Oct3/4 (Cell Signaling Technology, Danvers, MA, USA), and anti-calponin (Sigma-aldrich, MO, USA), anti-smooth muscle actin (SMA) (Abcam, CA, USA), and followed by incubation with

fluorescent tagged secondary antibodies (Invitrogen, Carlsbad, CA, USA). The images were acquired using a confocal laser scanning microscope system (LSM 710, Carl Zeiss AG, Oberkochen, Germany) and processed with Zen 2008 software (Carl Zeiss AG, Oberkochen, Germany). A water- or oil-immersion objective (40×, or 63×, 1.4 numerical aperture, NA) with the pinhole set for a section thickness of 0.8μm (pinhole set to 1 airy unit in each channel) was used.

4. Alkaline phosphatase (AP) staining and karyotyping

For detecting AP activity, the BCIP/NBT Substrate System was obtained from DAKO (DAKO, California, USA) and performed following the manufacturer's instruction. Karyotype analysis was conducted by using standard protocols for the chromosomal Giemsa (G)-banding at the Samkwang Health Medical Center.

5. Adaptation to single-cell based non-colony type monolayer culture of iPSC

Human iPSC cells, initially grown as colonies on STO feeder cells, were cultured on hESC-quantified Matrigel (BD) for five days. The cells were dissociated by incubation with Accutase

(Invitrogen) for 15 min. Dissociated single cells were plated on hESC-qualified Matrigel (BD) coated 6-well dishes, following the manufacturer's instruction. Approximately 2.5×10^5 cells/well were seeded in one well of a 6-well plate (Eppendorf) in mTESR (Stemcell Technologies). Twenty-four hours later, media was replaced with RPMI (Gibco) containing B27 (Gibco) and 10 μM CHIR99021 (Cayman).

6. Three germ layer differentiation assay

Human induced pluripotent stem cells were differentiated into each of the three germ layers using the base media and differentiation supplements provided in the human pluripotent stem cell functional identification kit (R&D Biosystems). After differentiation, all cells were fixed with MeOH for 10 minutes at -20°C. Cells were washed and blocked with 5% BSA and incubated overnight with anti-Otx (1:100), anti-Brachyury T (1:100), and anti-Sox19 (1:100) at 4°C. The next day, cells were washed and incubated with Alexa-anti-mouse 488 (Invitrogen), Alexa-anti-mouse 555 (Invitrogen), Alexa-anti-mouse 647 (Invitrogen) for two hours. The nuclei were counterstained with DAPI (blue).

The three-germ layer differentiated cells were viewed under confocal laser scanning microscope system (LSM 710, Carl Zeiss AG, Oberkochen, Germany) and processed with Zen 2008 software (Carl Zeiss AG, Oberkochen, Germany).

7. *in vitro* Differentiation

Vascular smooth muscle differentiation: To induce VSMCs, hiPSC were treated with RPMI (Gibco) containing B27 and 10 μM CHIR99021 (Cayman) for two days to differentiate the mesoderm lineage. Single mesoderm lineage cells were plated on a Collagen Type IV (Sigma Aldrich) coated dish. For the vascular smooth muscle cell differentiation, 20 μg/mL PDGF-BB (R&D systems), 2 μg/mL TGF-β1 (Peprotech) containing RPMI-B27 medium was changed every day. On day five, the cells were collected through digestion with Trypsin 0.25% (Invitrogen) and seeded at a concentration of 5×10^5 cells/well on Collagen Type IV coated 6-well plates. Differentiation media was changed after 20 days and mature VSMCs were used.

Endothelial cell differentiation: To induce EC differentiation, hiPSC were treated with RPMI (Gibco) containing 2% B27 (Invitrogen) and CHIR99021 (Cayman) for two days to

differentiate the mesoderm lineage. Single mesoderm lineage cells were stimulated with endothelial differentiation media (RPMI, 2% B27) containing 100 ng/mL VEGF and 50 ng/mL bFGF for 10 days. Single cells were dissociated using Accutase (Gibco) and CD34 positive cells were isolated by MACS and plated on fibronectin (Sigma Aldrich) coated dishes and the medium was changed every day.

8. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen). One microgram of total RNA was utilized for reverse transcription reaction with Power cDNA synthesis kit (Applied Biosystems) and Oligo dT primers, according to the manufacturer's instructions. PCR was performed with AmpliTaqGold (Applied Biosystems). PCR reactions were performed as follows: 94° C for 12 min, followed by 30 cycles 94° C-45 sec, 58° C-45 sec, 72° C-50 sec. Amplified PCR products were analyzed by electrophoresis on a 2% agarose gel. *Nanog*, *Oct3/4*, *Brachyury T*, *CNN1*, *SMA*, *SMA22a*, *Serca2*, *PECAM*, *VE-CAD* gene expression were analyzed using the Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA,

USA). The reaction was carried out according to the manufacturer's protocol using SYBR Green master mix (ROCHE, Mannheim, Germany). Gene expression levels were normalized to the level of GAPDH or 18S RNA. Gene expression was quantified using the double delta Ct method.

9. Western blot analysis

We evaluated protein amounts from whole-cell lysates, quantified using the BCA assay (Thermo), and loaded 50 μg of protein from each of the indicated samples per well into a 10% SDS-PAGE gel (BioRAD). Proteins were transferred to a nitrocellulose membrane (BioRAD), blocked for one hour using 5% skim milk. Membranes were washed and incubated overnight with anti-CNN1 (1:1000, Sigma-Aldrich), anti-SMA (1:1000, ABCAM), anti-tubulin (1:1000, Santa Cruz), anti-SERCA2 (1:1000, ABCAM), anti-SUMO1 (1:1000, Cell Signaling), anti-UBA2(1:1000, Cell Signaling), anti-Ubc9 (1:1000, Cell Signaling), anti-SENP1 (1:1000, Cell Signaling), anti-actin (1:1000, Santa Cruz). The next day, membranes were washed and incubated for two hours with either anti-rabbit HRP (1:2500. Abcam), anti-mouse HRP (1:2500, Abcam), or anti-goat HRP

(1:2500, Abcam), washed, exposed to film, and developed using an auto developer. Images were quantified using Image J software.

10. Functional contraction assays

Briefly, hiPSC derived vascular smooth muscle cells were cultured and dissociated using Accutase. Single cells were re-plated on 35 mm-confocal dishes (iBIDi) and incubated overnight at 37° C. Vascular smooth muscle cells were washed and induced for contraction by incubation with 1 mM carbachol (Sigma Aldrich) in DMEM medium for 60 minutes. Images were quantified using Image J software.

11. Lentiviral transduction

We used lentiviral vectors PLK0.1 shVector from Open Biosystems and PLVX overexpression vector from Clontech. To further investigate the effect of gene overexpression, we subcloned a construct encoding *SERCA2* cDNA (Open Biosystems, Huntsville, AL, USA) into the lentiviral vector.

We co-transfected lentiviral constructs and packaging vectors into 293T cells with polyethylenimine (Polyscience, Warrington, PA, USA) and collected the supernatants containing lentiviral particles after 48 hours. These lentiviral particles were subsequently used to transfect the target cells.

12. Isolation of CD34+ cells by magnetic sorting

To dissociate differentiated hiPSC into single cells, differentiated hiPSC were treated with Accutase (Invitrogen) for 10 minutes at 37°C and, after gentle pipetting, passed through 40 μm cell strainers (BD Biosciences). CD34+ cells were then isolated by MACS MagneticBead columns (Miltenyi Biotec) using an antibody against CD34, according to the manufacturer's instructions.

13. Quantification and statistical analysis

We obtained images for comparative analysis under identical conditions of light, contrast, and magnification. The contracting area of the iPSC derived vascular smooth muscle was automatically measured by Image J software (NIH, MD, USA). To calculate the area, we drew lines on the image along the area

and then measured the length of the lines using the software. The differences between experimental groups were analyzed by Student' s t-test or one-way ANOVA using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). To analyze the effect of time and treatments, we performed a two-way ANOVA.

RESULTS

Generation and characterization of human induced pluripotent stem cells (iPSC)

CiMS were isolated from 10 normal volunteer (Normal) and 10 variant angina (VA) patient peripheral blood mononuclear cells (PBMCNs). After a few days of culture, these cells showed a diverse morphology (Fig1.A). I reprogrammed the twenty CiMS lines by retrovirus infection of cells at passage 2-4, using the "Yamanaka" four factors: Oct4, Sox2, Klf4 and c-Myc. Infected CiMS cells were cultured on murine feeder cells in human ESC medium. iPSC colonies were observed two to three weeks later and picked. All of the colonies were expanded in human ES cell medium with bFGF (10 ng/mL). I observed flat and compact cell morphology with clear-cut round edges with no distinguishable difference between normal volunteer and variant angina patient derived iPSC (Fig1.B). Clones from both groups stained positively for alkaline phosphatase activity and expressed the pluripotent markers NANOG and Oct3/4, as determined by immunocytochemistry (Fig1.C). I verified germ layer markers in differentiated iPSC. Both lines were

differentiated into each of the three germ layers. Ectoderm differentiated cells were stained for Otx2 and mesoderm differentiated cells stained for Brachyury (T). Endoderm differentiated cells were stained for Sox19 and nuclei were counterstained with DAPI (Fig1.D) We analyzed Normal and VA iPSC cell lines karyotypes and found normal karyotypes in all hiPSC tested (Fig1.E). Using Reverse transcription-polymerase chain reaction (RT-PCR) analysis, I found that the expression of the pluripotency markers *Oct3/4*, *Sox2*, *c-myc*, and *Klf4* (Fig1.F). Using quantitative reverse-transcriptase-polymerase chain reaction (qRT-PCR) analysis, I found that the expression of the pluripotency markers *Nanog* and *Oct3/4* showed no difference between iPSC and human embryonic stem cells. Consistent with the activation of endogenous pluripotency gene expression, reprogramming of normal- and VA-derived lines were accompanied by karyotyping and teratoma formation. All sections of teratoma exhibited three germ layers and showed no differences between normal and VA iPSC (Fig1. G,H). Overall, our data indicated that CiMS cells from variant angina patients can be reprogrammed into iPSC with characteristics that are similar to human embryonic stem cells.

Differentiation of iPSC into smooth muscle cells and their characterization

Next, I evaluated the smooth muscle cell (SMC) differentiation from non-colony type monolayer (NCM) culture methods [23]. I proceeded both iPSC cell lines under NCM culture conditions to differentiate these stem cells into cell types representative of mesoderm cell lineage. The efficient derivation of mesoderm subtypes from hiPSC was induced by treatment with a GSK inhibitor (GSKi) [24]. A more detailed differentiation method is depicted in Fig2.A. Comparing several concentrations of the GSKi (CHIR99021), significantly elevated levels of Brachyury T positive cells were seen after 48 hours (data not shown). Based on FACS and quantitative PCR analysis, the upregulation of Brachyury T at day two is only possible with a concentration of 10 μM CHIR99021 and no difference was seen between iPSC cell lines and hESCs (Fig2. B,C). Brachyury T positive cells were cultured in EGM-2 medium supplemented with PDGF-BB and TGF-β for 16 days. We observed SMC morphology and stained for calponin (CNN1) and smooth muscle actin (SMA) (FIG2. D). RT-PCR analysis showed high expression of SMA markers, such as CNN, SMA22a, SMA and Western blot analysis also showed

expression of SMC markers (FIG2. E,F). We quantified SMC markers using qRT-PCR and found no difference between hiPSC derived SMCs and human vascular smooth muscle cells(FIG2.G). Based on the phenotypes and expression data, there was no difference between hiPSC differentiated into the SMC lineage and human vascular smooth muscle cells.

There is consistent evidence suggesting that in patients with variant angina, there is primary VSMC nonspecific hyperactivity[3]. VSMCs are the key cells where the abnormality responsible for variant angina occurs. To test the primary function of VSMCs cells, we sought to analyze their capacity to contract in response to charbachol, which is a vasoactive agonist. We quantified contraction area, comparing normal patient-iPSC derived VSMCs and variant angina patient-iPSC derived VSMCs. Surprisingly, variant angina patient-iPSC derived VSMCs showed very strong contraction when induced with charbachol (Fig2.H), suggesting that hyperactivity in VSMCs is the most important factor to cause variant angina.

Differentiation of iPSC into endothelial cells and characterization

Next, I evaluated the endothelial differentiation using NCM

methods[23]. I proceeded with iPSC cell lines from both CTL and VA patients under NCM conditions to differentiate the cells into cell types representative of mesoderm cell lineage. The efficient derivation of mesoderm subtypes from hiPSC is achieved by treatment with GSK inhibitors[24]. Such differentiation method is shown in Fig3. A. As described previously in Figure 2, the only successful condition was using a concentration of GSKi of 10μM treating cells for two days. After the GSKi stage, Brachyury T positive cells were cultured in EGM-2MV medium supplemented with VEGF-bb and bFGF for 10 days. A CD34 marker was used to isolate vascular progenitor cells by magnetic selection from NCM grown in differentiation media. The CD34 positive cells were grown and expanded in endothelial differentiation medium containing VEGF-bb and bFGF supplements. I observed endothelial cell morphology and stained for PECAM (CD31) and Ve-CAD (Fig3.B). Based on these phenotypes and expression data, there were no differences between normal and variant angina derived ECs in terms of characteristics.

Variant angina iPSC-derived VSMCs demonstrate vasoactive agonist-induced intracellular calcium levels and calcium sparks

Calcium sparks have been observed in cardiac[25], skeletal[26,27], and smooth muscle cells[28]. These measurements have been made using laser scanning confocal microscopy and the fluorescent calcium indicator FLUO4[29].

To assess VSMC contractile potential, the cells were preloaded with the calcium sensitive dye FLUO4. Carbachol (250 μM), which is a vasoactive agonist, stimulated an increased in FLUO4 fluorescence intensity in the derived VSMCs within 1 min of treatment, indicating increased intracellular calcium efflux. FLUO4 intensity decreased, within 5 min. The same trend was observed in the hVSMCs and normal iPSC-derived VSMCs, but not in variant angina iPSC-derived VSMCs. Next, I tested ten variant angina iPSC-derived VSMC cell lines and found that all showed over two times higher level of intracellular calcium efflux than normal VSMC cell lines. Also, I observed specific calcium efflux only in variant angina iPSC-derived VSMC (Fig4.A). The same trend was observed when I used other vasoactive agonists, such as Ergometrine maleate (Fig4.B), Acetylcholine (Fig4.C). Based on this data, the variant angina group displayed more than two times higher intracellular calcium levels than the normal group. Moreover, I observed patient specific calcium efflux, after

treatment with a variety of vasoactive agonist in the variant angina derived VSMCs. I measured ten normal and variant angina lines and found that intracellular calcium efflux ratios were three to five times higher in the variant angina lines. Overall, this finding strongly suggests that the abnormality of calcium efflux in variant angina could be due to induced VSMC hyperactivity.

SERCA2 induced VSMCs hyperactivity in variant angina derived VSMCs

Sarco/Endoplasmic Reticulum Ca^{2+}ATPase (SERCA) is the only active Ca^{2+} transporter in the sarcoplasmic reticulum (SR). In muscle cells, SERCA controls the SR calcium store that is mobilized during muscle contraction and it decreases cytosolic calcium concentration to allow muscle relaxation[30]. Because of the hyperactivity in variant angina VSMCs, I examined the *Serca2* gene expression level using RT-PCR and qRT-PCR and found that there was no difference in gene expression level between normal VSMCs and variant angina VSCMCs (Fig5.A,B). It was previously reported that SERCA2 activity could be modulated by post-translational modifications (PMT)[31]. According to the Hajjar group[32], SERCA2 binds to SUMO1 and to

the ubiquitin-conjugating enzyme UBC9. I examined protein expression levels of SERCA2 by western blot. The SERCA2 band intensity was increased in variant angina VSMCs (Fig5.C). It was previously reported that SERCA2 is specific for SUMO1 but not for SUMO2 or SUMO3[31]. Along with the increase in SERCA2 expression, the levels of SUMO1 and UBC9 were significantly increased in the variant angina group. However, the expression levels of UBA2 and SENP1 were the same in the normal and variant angina groups (Fig5.C). Therefore, SERCA2 posttranslational modification is likely to play an important role in variant angina. Moreover, SERCA2 expression levels were also analyzed by immunofluorescence. The SERCA2 fluorescence intensity were increased in variant angina VSMCs (Fig5. D).

To characterize the potential role of SERCA2 in variant angina VSMCs, I examined total protein of SERCA2 activity. When compared with normal VSMCs, the variant angina patient group showed higher total protein of SERCA2 activity levels (Fig5.E). I also examined high level of SERCA SUMOylation in VSMCs.

Overexpression of SERCA2 and point mutation in smooth muscle cells

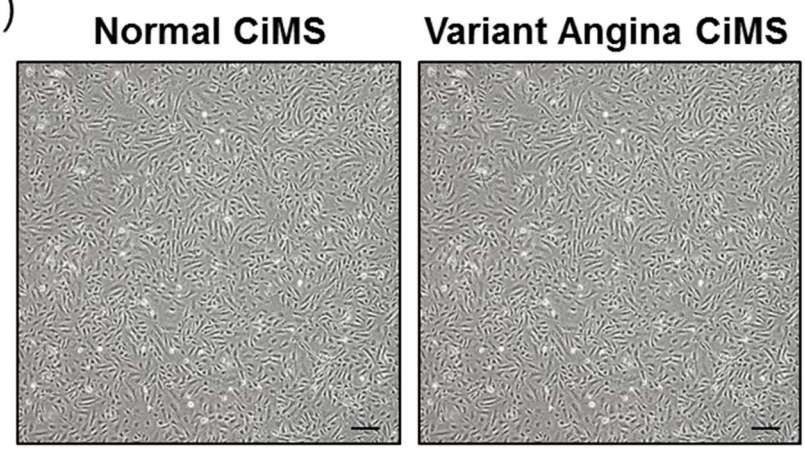
To characterize the potential function of SERCA2 in variant angina, I restored SERCA2 expression in human VSMCs by delivering the *Serca2* gene using a lentiviral vector. Transduction with pLVX-SERCA2 resulted an increase in *Serca2* expression in the hVSMCs after two weeks (Fig6.A). Next, I measured calcium efflux using the FLUO4 intracellular calcium fluorescence dye. In *Serca2*-overexpressing hVSMCs, intracellular calcium efflux was two times higher than the control group and patient specific calcium efflux were observed in variant angina group (Fig6.B). Next, I disrupted expression of *Serca2*, using a sh-pLK0.1 lentiviral vector in variant angina VSMCs. After knocking down the expression of *Serca2*, I examined intracellular calcium efflux. Surprisingly, intracellular calcium and patient specific calcium efflux were restored in the variant angina group (Fig6.C). I examined SERCA SUMOylation in VSMCs. Moreover, total SERCA2 and SUMO1 protein levels were increased in variant angina group (Fig7. A) Furthermore, the SUMOylated SERCA2 band was very strong in the variant angina group (Fig7.B). These data suggest that SUMOylated

SERCA levels significantly increase in variant angina, a possible mechanism causing hypercontraction in vascular smooth muscle cells.

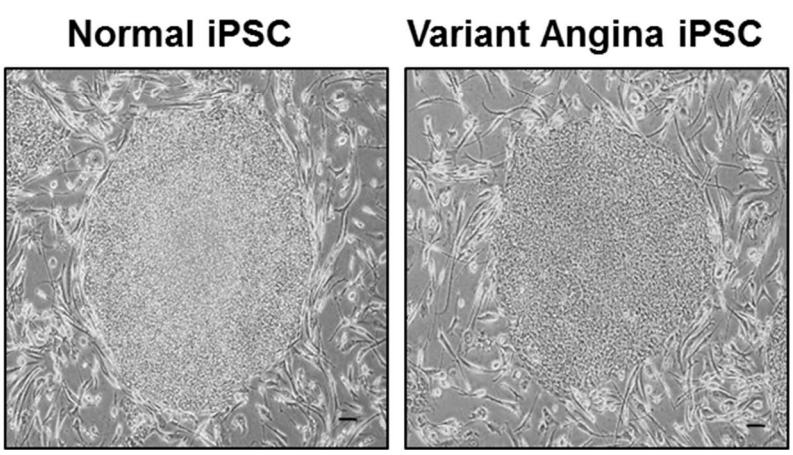
The Hajjar group previously reported that SERCA2 is SUMOylated at lysines 480 and 585. These residues are conserved in mouse, rat, pig and human *Serca2* [32]. Therefore, I generated two *Serca2* variants with mutations in these lysine in lentiviral expression vectors. To analyze the effects of these point mutations on cellular function, I delivered each lentivirus into variant angina derived VSMCs. I examined intracellular calcium levels and patient specific calcium efflux (Fig7.C). To development of therapeutic medicine, I used Ginkgolic acid, a known chemical that inhibits Sumolyated E1 molecule. Consequentially, intracellular calcium and patient specific calcium efflux were restored in the variant angina group (Fig7.D). Also, total protein of SERCA2 activity were decreased when ginkgolic acid was treated (Fig7.E). These data indicate that SUMOylation increases the ATPase activity in total protein of SERCA2. Thus, I demonstrated for the first time to my knowledge, that *Serca2* expression levels and SUMOylated-SERCA2 are the most important factors in regulating intracellular

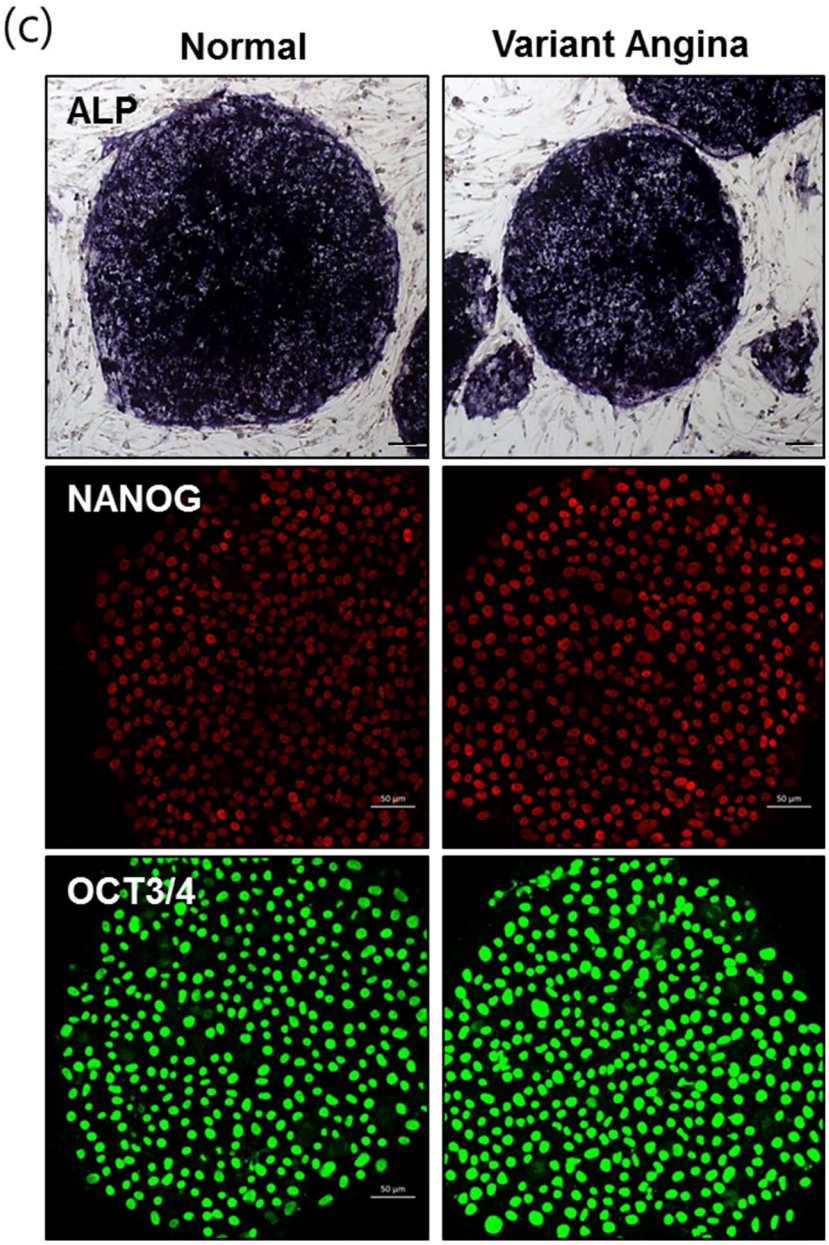
calcium levels and hyper contraction of VSMCs. These findings provide evidence that PTMs of SERCA2 may play a role in the cause of this illness.

(a)

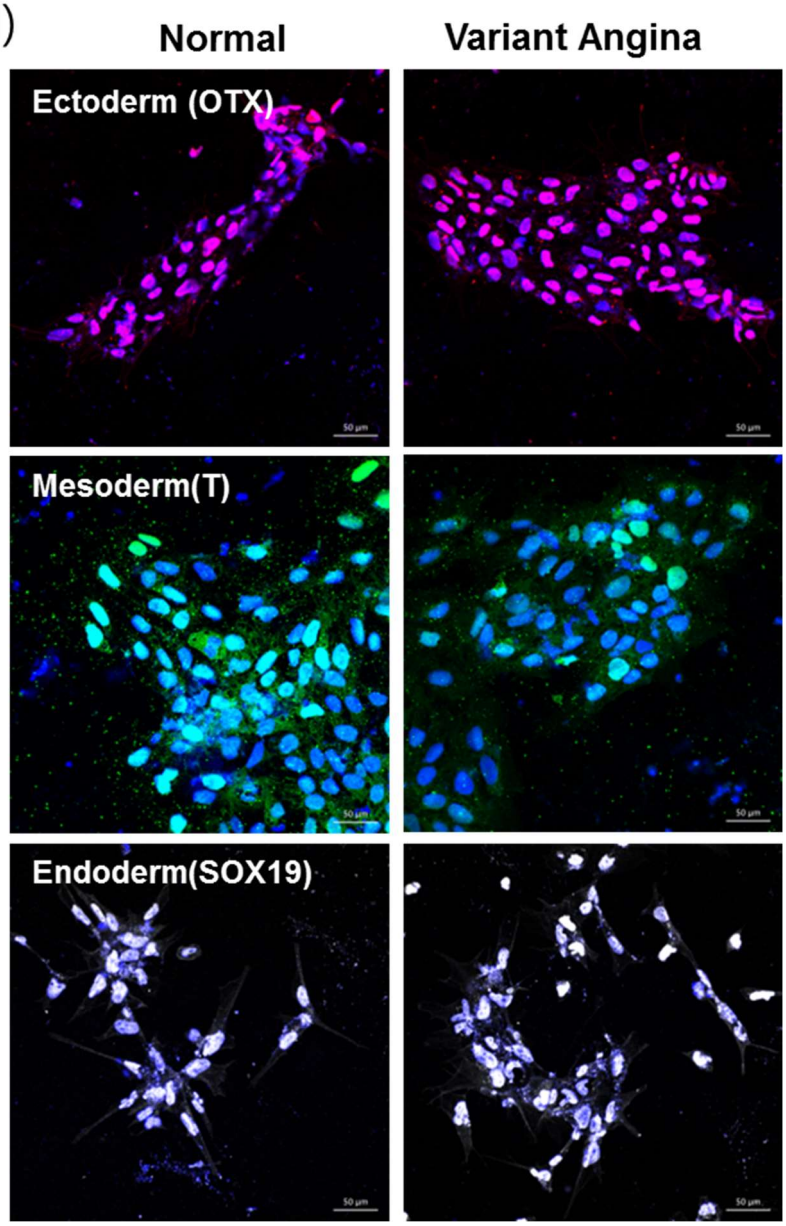

Normal CiMS
Variant Angina CiMS

(b)

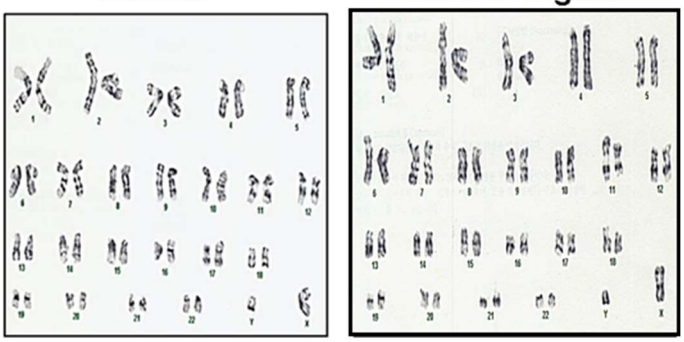

Normal iPSC
Variant Angina iPSC


(c)
Normal
Variant Angina
ALP
NANOG
OCT3/4

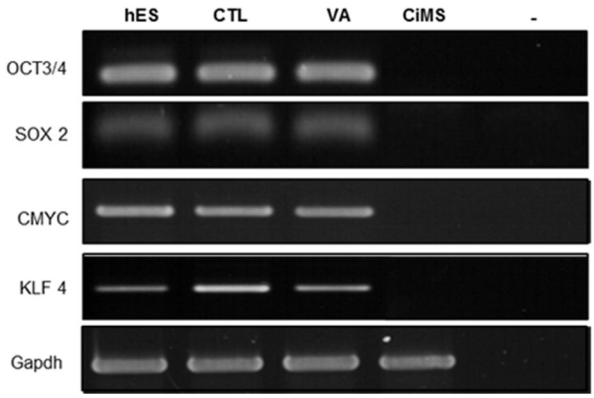
(d)


Normal
Variant Angina
Ectoderm (OTX)
Mesoderm(T)
Endoderm(SOX19)

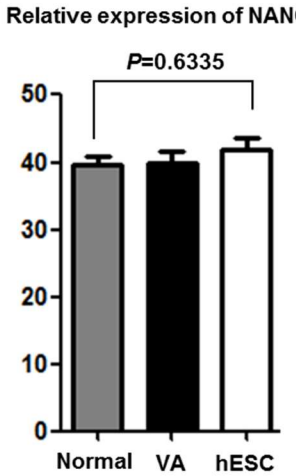
(e)

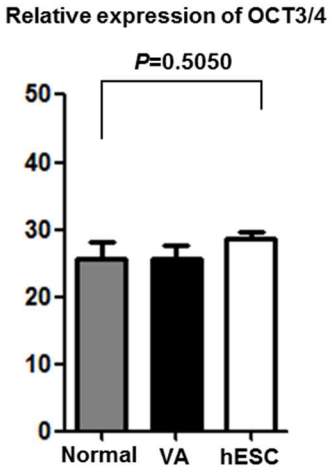

Normal
Variant Angina

(f)


hES
CTL
VA
CiMS
-
OCT3/4
SOX 2
CMYC
KLF 4
Gapdh

(g)


Relative expression of NANOG
P=0.6335
50
40
30
20
10
0
Normal
VA
hESC


Relative expression of OCT3/4
P=0.5050
50
40
30
20
10
0
Normal
VA
hESC

(h)

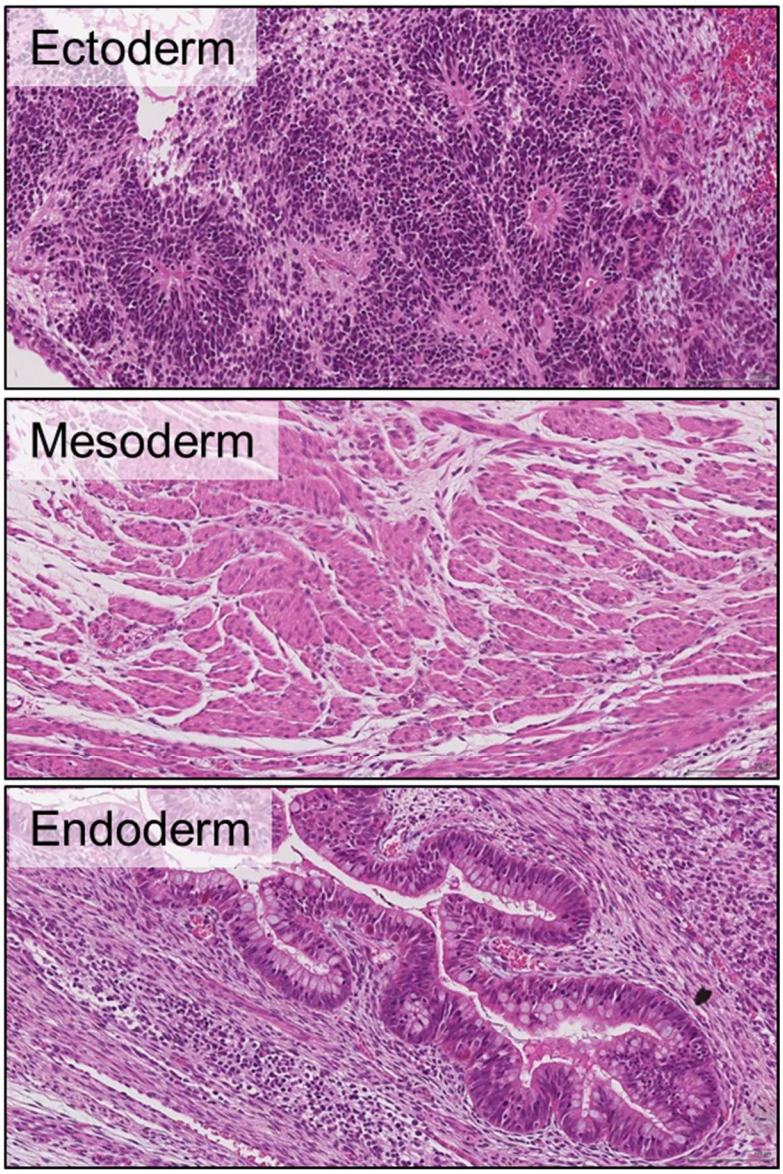


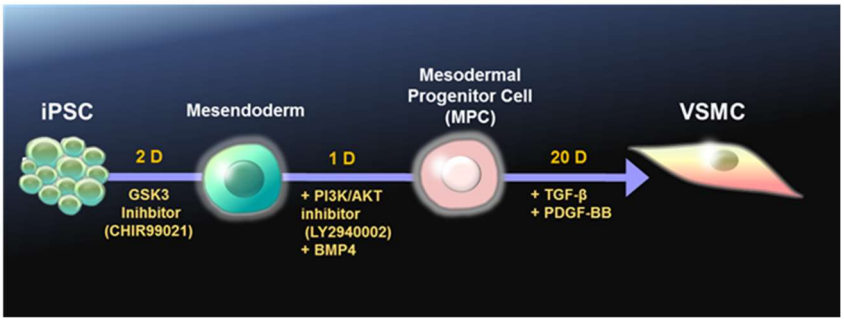
Figure1. Generation of Normal and Variant angina CiMS derived iPSC generation and characterization.

(a) Normal volunteer and Variant angina CiMS were isolated on PBMCs (scale bar=20um).(b) Normal and Variant angina derived iPSC colonies on bright phase (scale bar=20m) (C) Immunohistology of iPSC clones. Phase contrast with alkaline phosphatase (AP) staining and immunofluorescence staining of iPSC for the following pluripotent markers: NANOG, OCT3/4. Three iPSC clones were analyzed for each lines (AP staining Scale bar-20um, immunofluorescence Scale bar=50μm) (D) Verification of Germ layer markers in differentiated pluripotent stem cells. Ectoderm differentiated cells were simultaneously stained with OTX2-Alexa-anti mouse-555 (red). Mesoderm differentiated cells were stained with Brachyury (T)- Alexa anti-mouse 488 (green). Endoderm differentiated cells were stained with SOX17 -Alexa anti-mouse 647 (grey). All nuclei were counterstained with DAPI (blue)

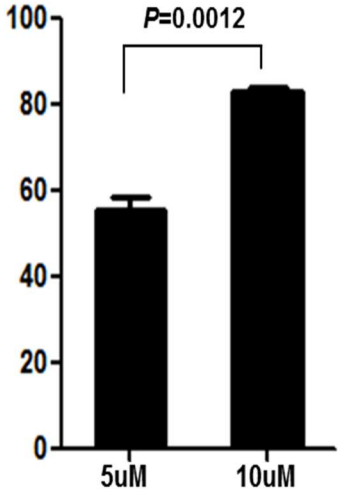
(E) In normal hiPSC karyotype was obtained 46 XY, and characteristic of a chromosomally normal male. In variant angina iPSC karyotype was obtained 46 XY and characteristic of a chromosomally normal male. (F) RT-PCR for pluripotency

markers: OCT3/4, SOX2, CMYC,KLF4 (G) NANOG (n=3 each of goup) and OCT3/4(n=3 each of group) expression levels of Normal, Variant angina iPSC and hESC were determined by qRT-PCR. Data represent mean SEM. (H) Teratoma formation analyses of immunodeficient NOD SCID mice. The tissue sections were derived from teratoma and stained with hematoxylin and eosin. Three germ lineages are in each tissue. Scale bars are 50 μm.

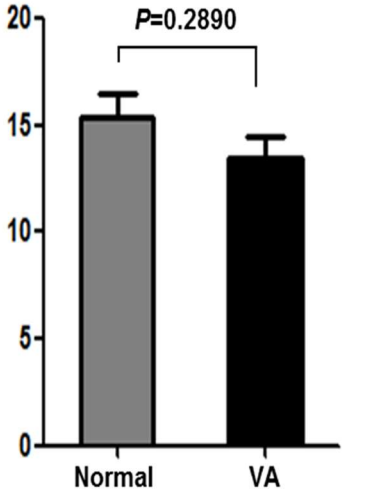
(a)

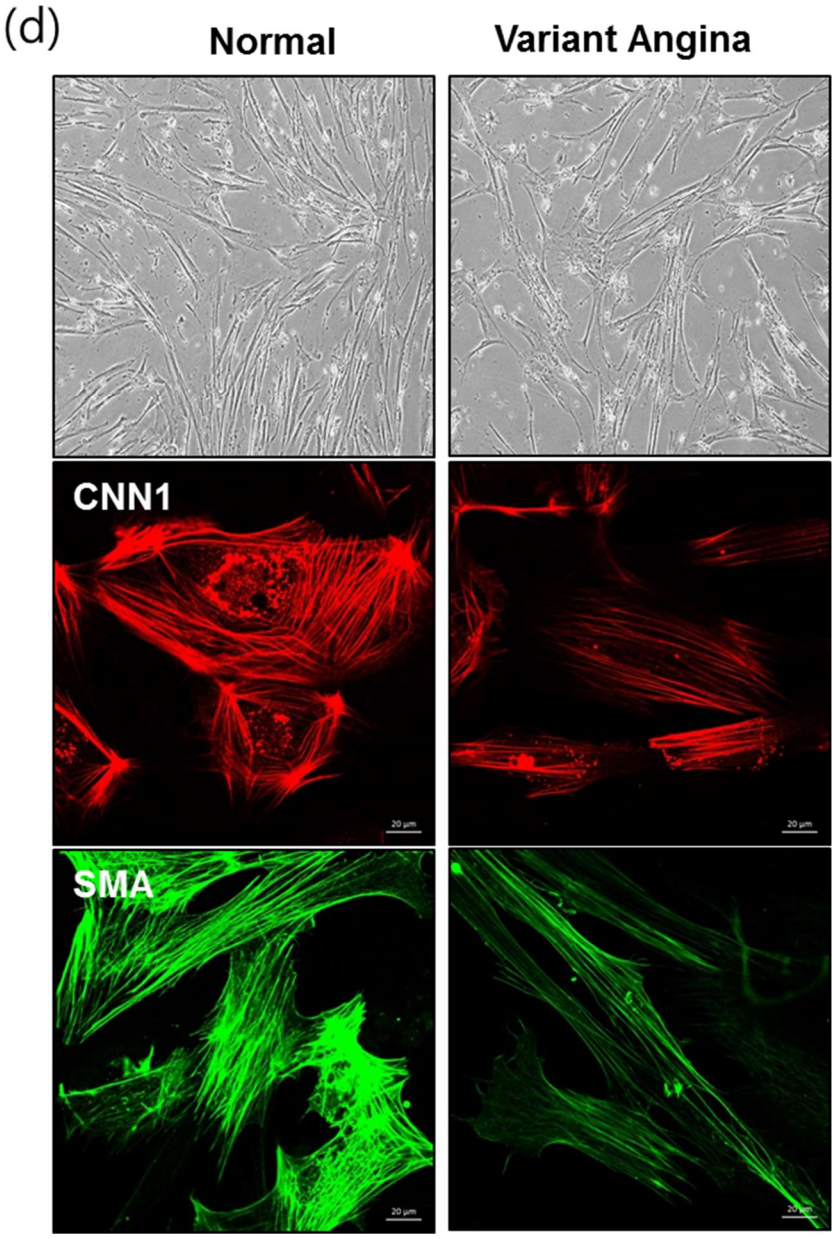

iPSC
Mesendoderm
Mesodermal
Progenitor Cell
(MPC)
VSMC
2 D
GSK3
Inihbitor
(CHIR99021)
1 D
+ PI3K/AKT
inhibitor
(LY2940002)
+ BMP4
20 D
+ TGF-β
+ PDGF-BB

(b)

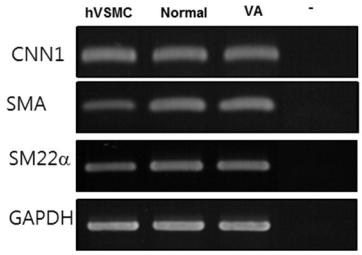

Brachyury T (%)
P=0.0012
100
80
60
40
20
0
5uM
10uM

(c)

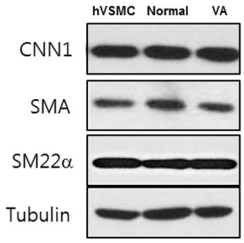

Relative expression of Brachyury T
P=0.2890
20
15
10
5
0
Normal
VA


(d)
Normal
Variant Angina
CNN1
SMA
20 μm
20 μm
20 μm

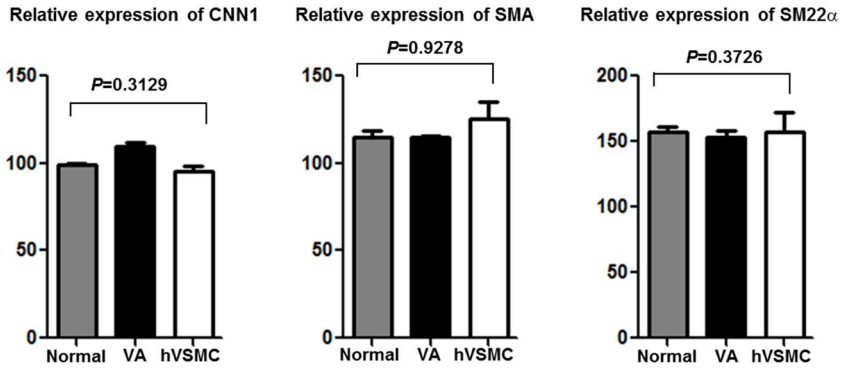
(e)


hVSMC
Normal
VA
-
CNN1
SMA
SM22α
GAPDH

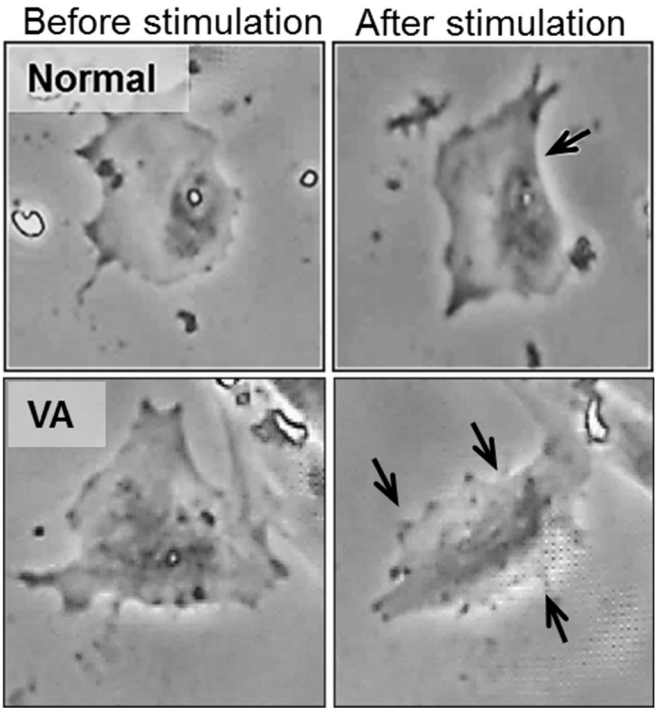
(f)


hVSMC
Normal
VA
CNN1
SMA
SM22α
Tubulin

(g)


Relative expression of CNN1
P=0.3129
150
100
50
0
Normal
VA
hVSMC
Relative expression of SMA
P=0.9278
150
100
50
0
Normal
VA
hVSMC
Relative expression of SM22α
P=0.3726
200
150
100
50
0
Normal
VA
hVSMC

(h)


Before stimulation
After stimulation
Normal
VA

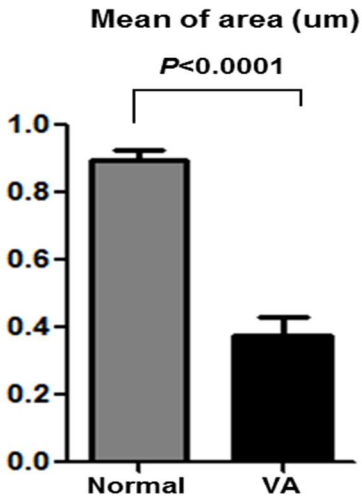
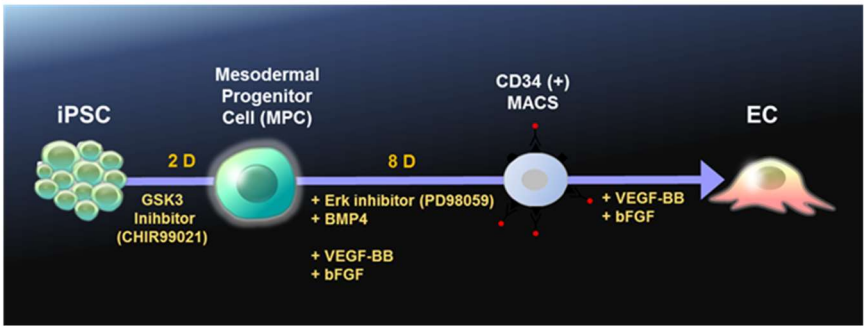

Mean of area (um)
P<0.0001
1.0
0.8
0.6
0.4
0.2
0.0
Normal
VA

Figure2. Normal and Variant angina iPSC derived Vascular smooth muscle differentiation and characterization.

(A) Schematic figure which outlines the conditions for deriving origin specifis vascular smooth muscel cells from hiPSC. (B) Efficent CHIR concentration were measured by Flow cytemotric analysis demonstrated that the mesendoderm marker Brachyury T were highly expressed at CHIR 10μM. (C) qRT-PCR demonstrated increasing expression of mesendoderm markers in normal and variant angina during CHIR treatment . Data represent means SEM (n=4 each of group). (D) Immunohistology of iPSC derived vascular smooth muscle cells. Phase contrast and immunofluorescence staining of vascular smooth muscle cells for the following smooth muscle markers: CNN1, SMA. Six vascular smooth muscle clones were analyzed for each line. (phase bright Scale bar=20μm, immunofluorescence Scale bar=50μm) (E) RT-PCR demonstrated expression of smooth muscle marker in the iPSC derived vascular smooth muscle and human vascular smooth muscle (F) Western blot analysis confirmed the presence of the mature SMC proteins, CNN1, SMA and SM22α in the hiPSC-derived VSMC and human -VSMC. (G) qRT-PCR demonstrated the presence of vascular smooth muscle

maturation markers, CNN1, SMA and SM22α, in iPSC derived VSMCs and human VSMC. Data represent means SEM (n=5 each of group). (H) Contraction analysis confirmed the function of mature vascular smooth muscle cells. Data represent means SEM (n=3 each of group, scale bar=20μm)

(a)


iPSC
Mesodermal
Progenitor
Cell (MPC)
CD34 (+)
MACS
EC
2 D
8 D
GSK3
Inihbitor
(CHIR99021)
+ Erk inhibitor (PD98059)
+ BMP4
+ VEGF-BB
+ bFGF
+ VEGF-BB
+ bFGF

(b)

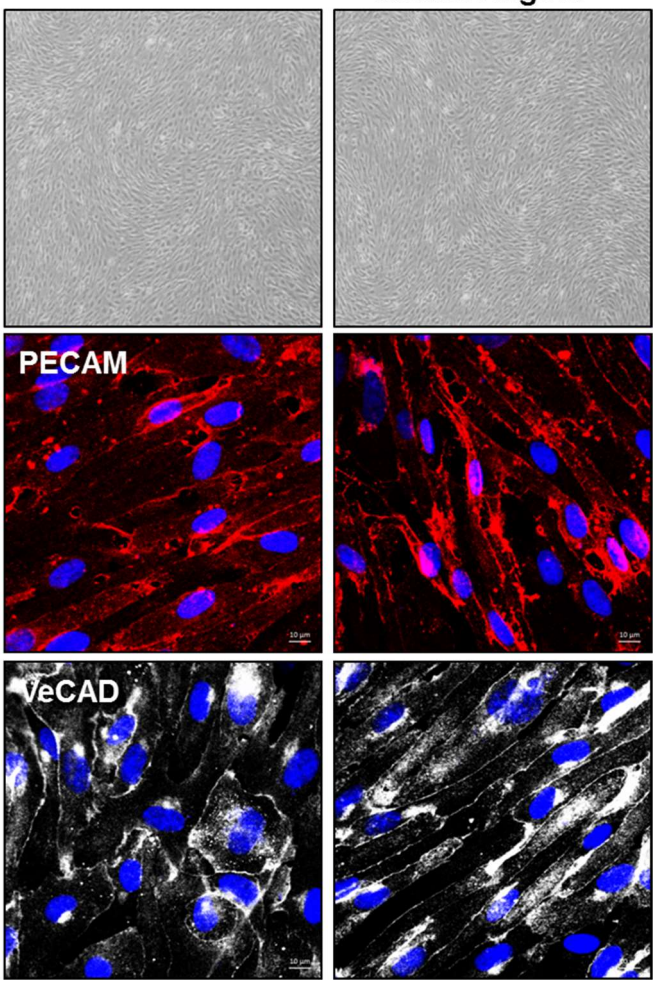
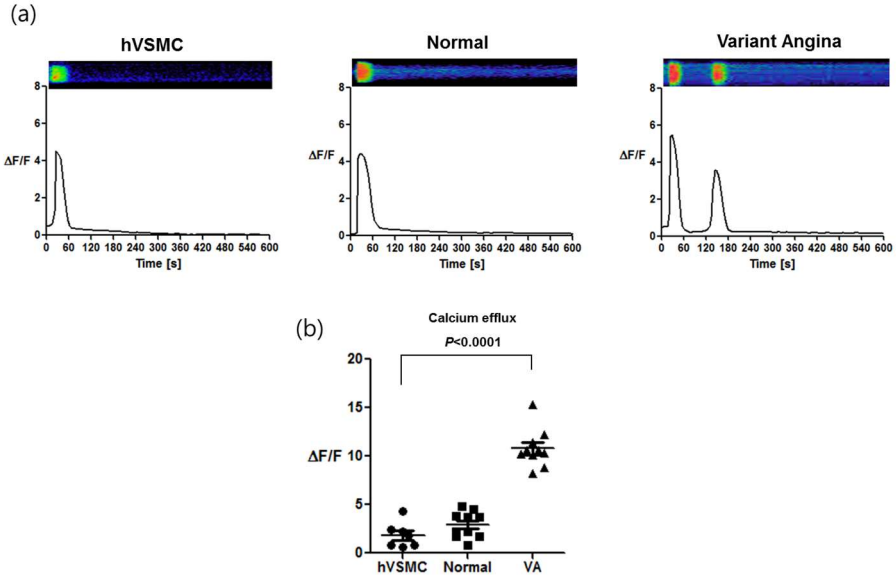
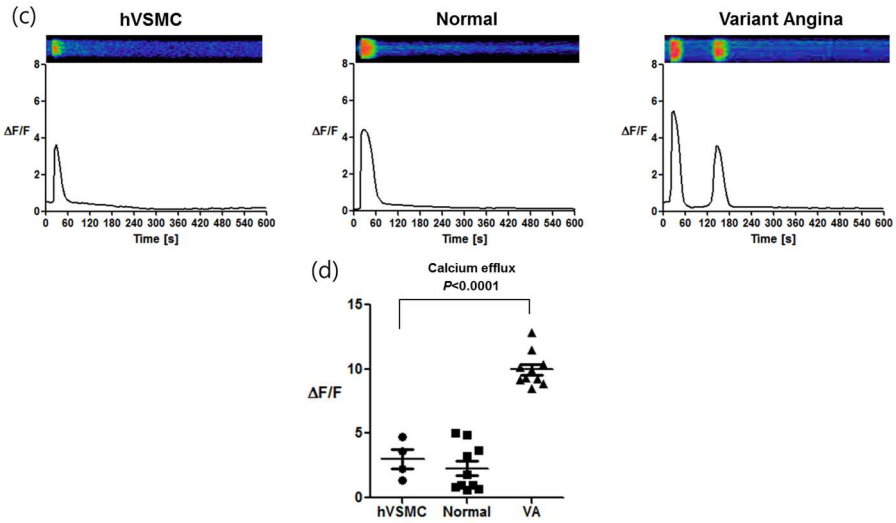

Normal
Variant Angina
PECAM
VeCAD

Figure3. Normal and Variant angina iPSC derived endothelial cells and characterization. (A) Schematic figure which outlines the conditions for deriving origin specifis Endothelial cells from hiPSC. (b) Immunohistology of iPSC derived endothelial cells. Phase contrast and immunofluorescence staining of endothelial cells for the following endothelial markers: PECAM and Ve-CAD (phase bright Scale bar=20μm, immunofluorescence Scale bar=50μm)

Charbachol



Ergometrine maleate



Acetylcholine

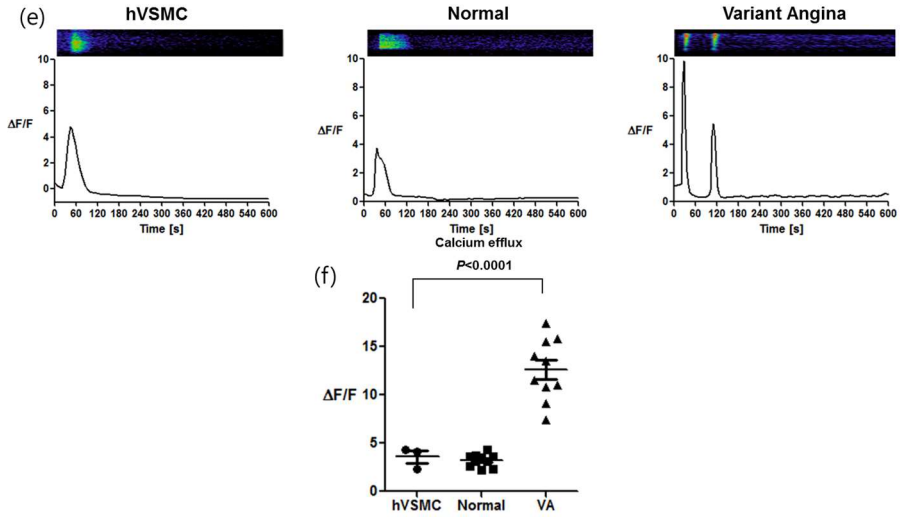
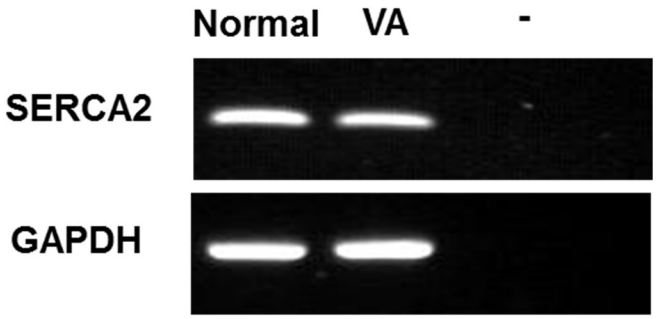


Figure4. Functional characterization of Normal and Variant angina iPSC derived Vascular smooth muscle .

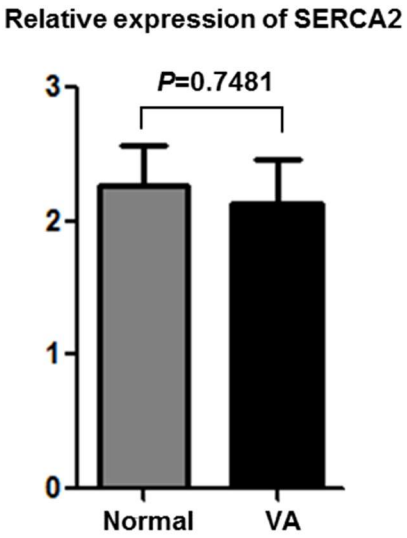
(A)calcium efflux were anaylzed with using FLUO4 calcium fluoroscence. FLUO4 were performed in response to carbachol (250μM). Change in the relative fluorescence unit (ΔF/F) of FLUO4 loaded cells was monitored by time elapse confocal (NiKon) over 10 min after the addition of carbachol, an inducer of contraction. (B)Each group of FLUO-4 intensity of calcium efflux were analyzed. Date represent means SEM (VSMC n=7, Normal n=10, Variant angina n=10) (C) calcium efflux were anaylzed with using FLUO4 calcium fluoroscence. FLUO4 were performed in response to Ergomatrine maleate (200μM). Change in the relative fluorescence unit (ΔF/F) of FLUO4 loaded cells

was monitored by time elapse confocal (NiKon) over 10 min after the addition of carbachol, an inducer of contraction. (D) Each group of FLUO4 intensity of calcium efflux were analyzed. Date represent means SEM (VSMC n=7, Normal n=10, Variant angina n=10) (F) calcium efflux and secondary calcium spark rations were anaylzed with using FLUO-4 calcium fluoroscence. FLUO4 were performed in response to Acetylcholine (250μM). Change in the relative fluorescence unit (ΔF/F) of Fluo-4 loaded cells was monitored by time elapse confocal (NiKon) over 10 min after the addition of carbachol(G)Each group of FLUO-4 intensity of calcium efflux were analyzed. Date represent meas SEM (VSMC n=7, Normal n=10,Variant angina n=10)

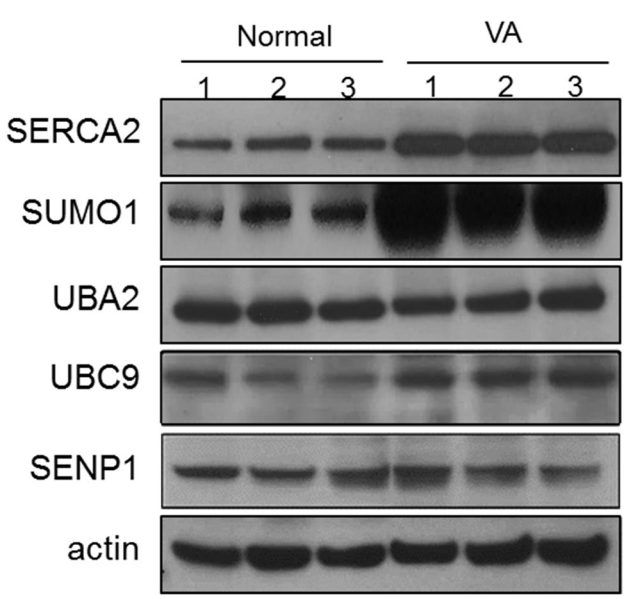
(A)

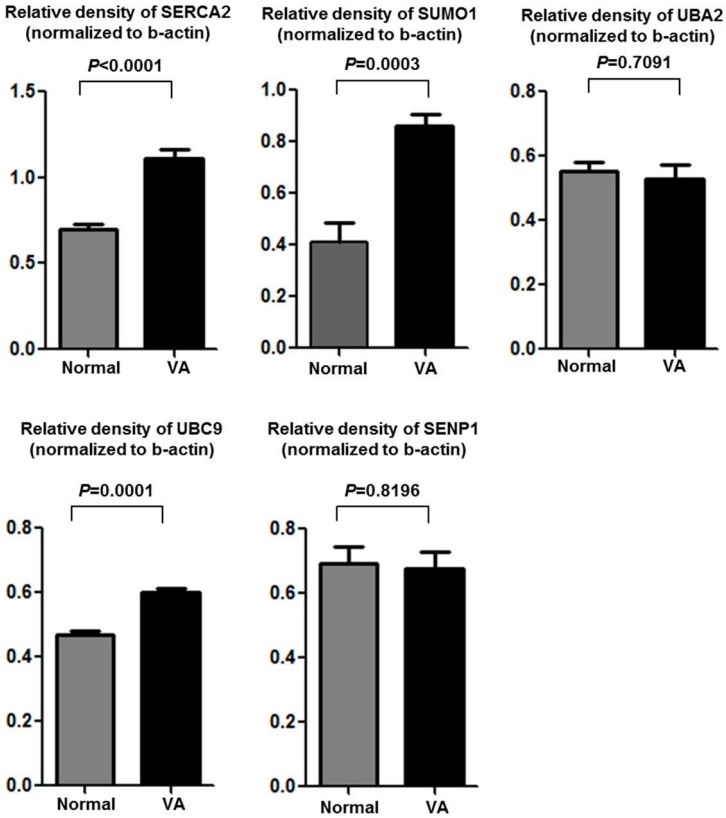

Normal
VA
-
SERCA2
GAPDH

(B)

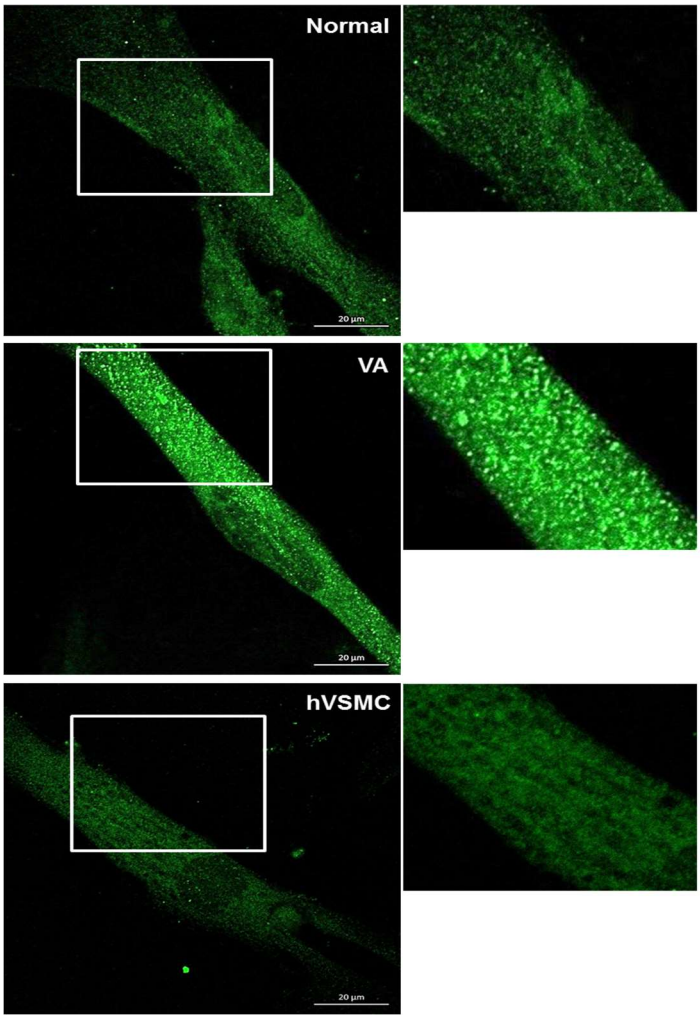

Relative expression of SERCA2
P=0.7481
3
2
1
0
Normal
VA

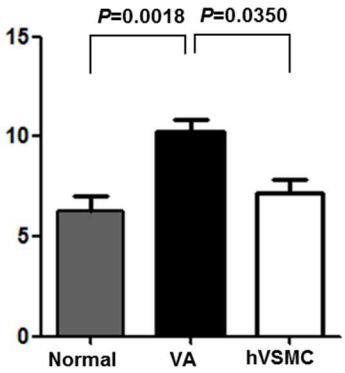
(C)


Normal
VA
1
2
3
1
2
3
SERCA2
SUMO1
UBA2
UBC9
SENP1
actin

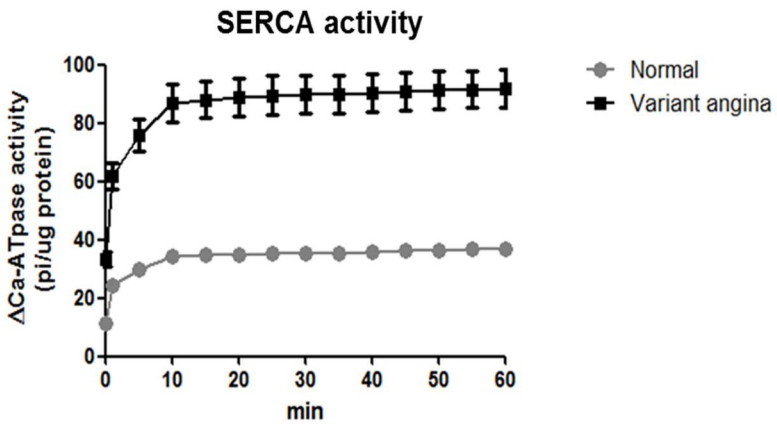

Relative density of SERCA2
(normalized to b-actin)
P<0.0001
1.5
1.0
0.5
0.0
Normal
VA
Relative density of SUMO1
(normalized to b-actin)
P=0.0003
1.0
0.8
0.6
0.4
0.2
0.0
Normal
VA
Relative density of UBA2
(normalized to b-actin)
P=0.7091
0.8
0.6
0.4
0.2
0.0
Normal
VA
Relative density of UBC9
(normalized to b-actin)
P=0.0001
0.8
0.6
0.4
0.2
0.0
Normal
VA
Relative density of SENP1
(normalized to b-actin)
P=0.8196
0.8
0.6
0.4
0.2
0.0
Normal
VA

(D)


Normal
20 μm
VA
20 μm
hVSMC
20 μm


P=0.0018
P=0.0350
15
10
5
0
Normal
VA
hVSMC

(E)


SERCA activity
ΔCa-ATpase activity
(pi/ug protein)
100
80
60
40
20
0
0
10
20
30
40
50
60
min
Normal
Variant angina

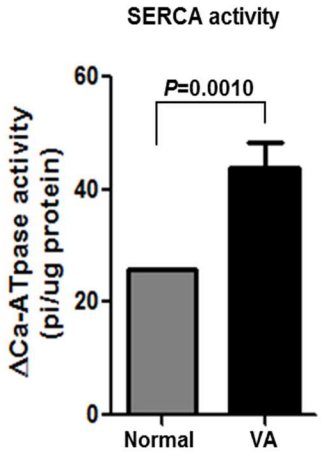
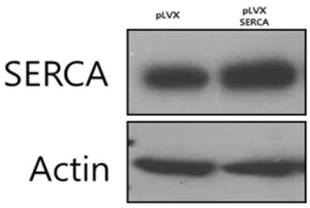
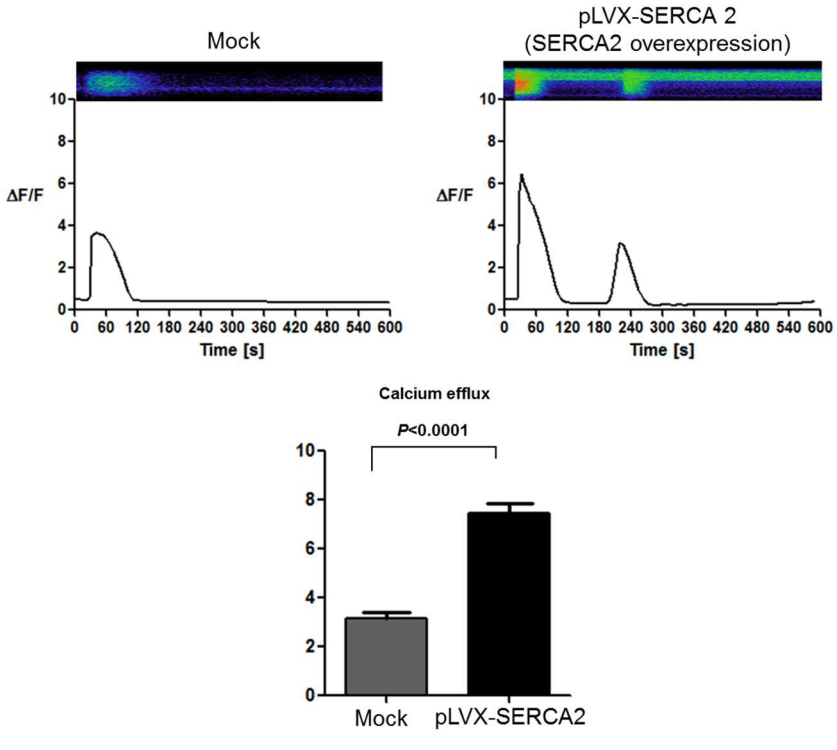

SERCA activity
ΔCa-ATpase activity
(pi/ug protein)
60
40
20
0
P=0.0010
Normal
VA

Figure5. Endogenouse SERCA2 protein expression level and activity in Normal and Variant angina derived VSMCs.(A) RT-PCR demonstrated the expression levels of SERCA2 in normal and variant anigna derived VSMCs. (B)qRT-PCR demonstrated expression of SERCA2 in normal and variant angina derived VSMCs. Data represent means SEM (n=3 each for group) (C) Immunoblot results from a representative experiment and protein quantification. All data represent the mean SEM (n =3 each for group)(D) Immunofluoroscence denomstrated expression of SERCA2 level in normal and variant angina derived VSMCs. (E) Ca^{2+}-dependent ATPase activity of normal and variant angina SERCA2 activity. This data represent three independent experiment. Data represent means SEM (n=3 each of group).

(A)


pLVX
pLVX SERCA
SERCA
Actin

(B)


Mock
pLVX-SERCA 2
(SERCA2 overexpression)
ΔF/F
Time [s]
Calcium efflux
P<0.0001
Mock
pLVX-SERCA2

(C)

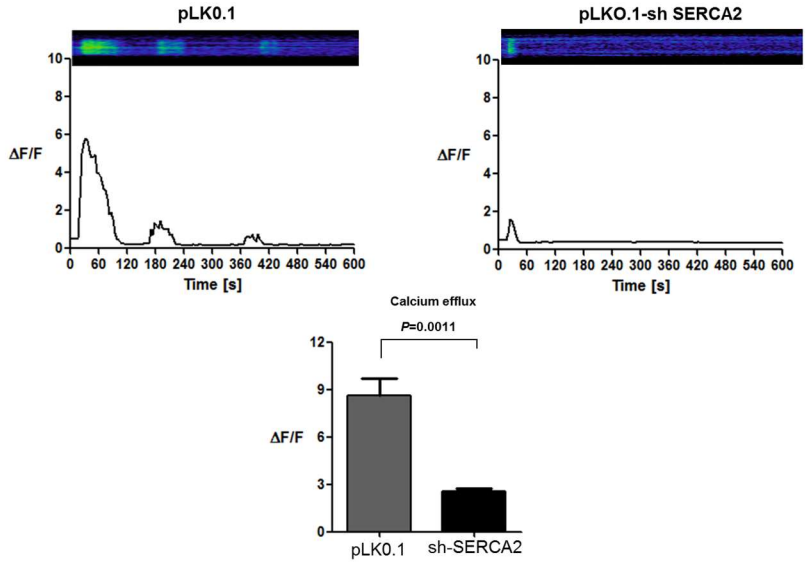

pLK0.1
pLKO.1-sh SERCA2
ΔF/F
Time [s]
0 60 120 180 240 300 360 420 480 540 600
Calcium efflux
P=0.0011
pLK0.1
sh-SERCA2

Figure6. Functional characterization of SERCA2 overexpression in Normal and Variant angina iPSC derived Vascular smooth muscle .

(A) We demonstrated overexpression level of SERCA2 in hVSMC using immunoblot.

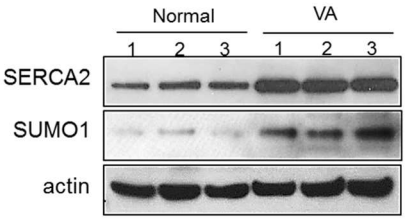
(B) The effect of SERCA2a treated in human vascular smooth muscle cells were anaylzed with using FLUO4 calcium fluoroscence. FLUO4 were performed in response to carbachol (250μM). Change in the relative fluorescence unit (ΔF/F) of Fluo-4 loaded cells was monitored by time elapse confocal (NiKon) over 10 min after the addition of carbachol, an inducer of contraction. Each group of FLUO-4 intensity of calcium efflux were anaylzed. Date represent means SEM (n=3 each for group)

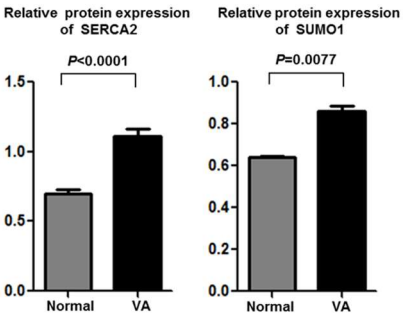
(C) The effect of knockout SERCA2 treated in variant angina groups cells were analyzed with using FLUO-4 calcium fluoroscence. FLUO4 were performed in response to carbachol (250uM). Change in the relative fluorescence unit (ΔF/F) of Fluo-4 loaded cells was monitored by time elapse confocal (NiKon) over 10 min after the addition of carbachol, an inducer of contraction. Data represent means SEM (n=3 each for group.

(H) The effect of SERCA2 point mutation viral vector were treated in variant angina derived VSMCs and intracellular calcium

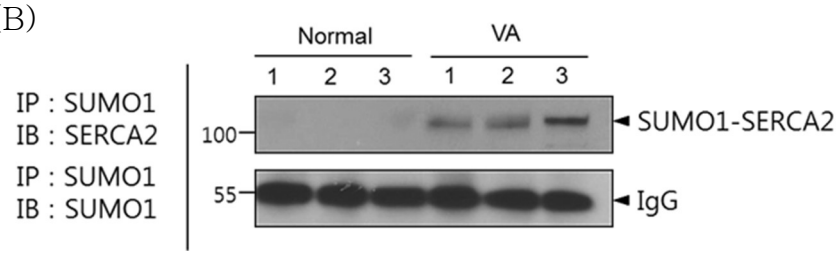
level were anaylzed using with FLUO-4 claicum fluoroscence. FLUO4 were performed in response to carbachol (250uM). Change in the relative fluorescence unit (ΔF/F) of Fluo-4 loaded cells was monitored by time elapse confocal (NiKon) over 10 min after the addition of carbachol, an inducer of contraction. Data represent means SEM. (n=3).

(A)

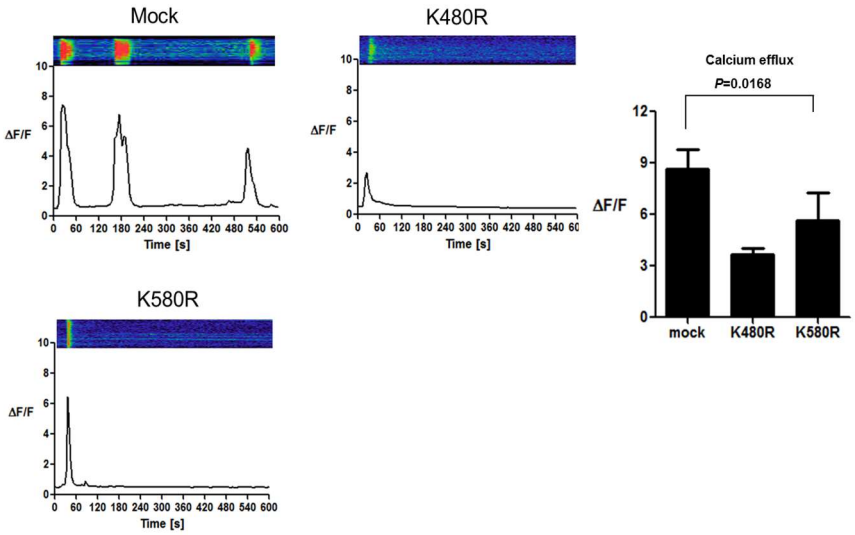

Normal
VA
1 2 3 1 2 3
SERCA2
SUMO1
actin


Relative protein expression of SERCA2
P<0.0001
1.5
1.0
0.5
0.0
Normal
VA
Relative protein expression of SUMO1
P=0.0077
1.0
0.8
0.6
0.4
0.2
0.0
Normal
VA

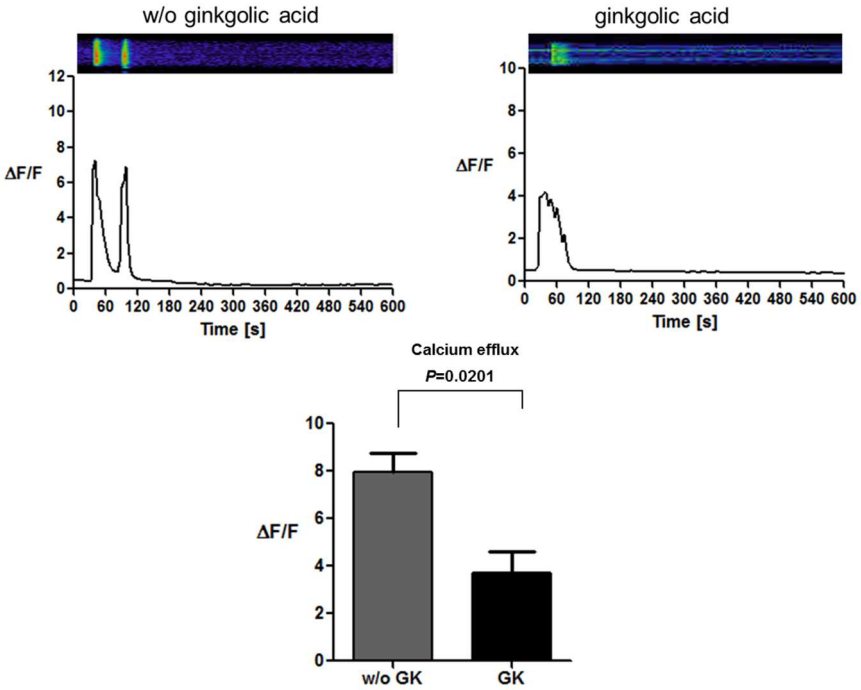
(B)


Normal
VA
1 2 3 1 2 3
IP : SUMO1
IB : SERCA2
100
SUMO1-SERCA2
IP : SUMO1
IB : SUMO1
55
IgG

(C)


Mock
K480R
K580R
ΔF/F
Time [s]
Calcium efflux
P=0.0168
mock
K480R
K580R

(D)


w/o ginkgolic acid
ΔF/F
Time [s]
ginkgolic acid
ΔF/F
Time [s]
Calcium efflux
P=0.0201
ΔF/F
w/o GK
GK

(E)

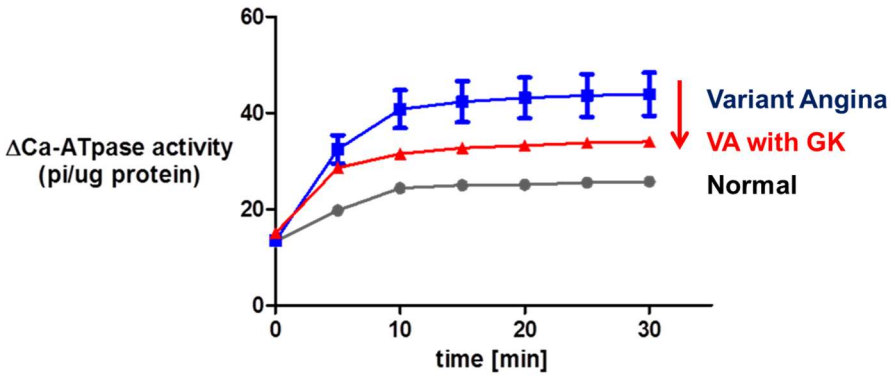

ΔCa-ATpase activity
(pi/ug protein)
time [min]
Variant Angina
VA with GK
Normal

Figure7. *Functional characterization of SERCA2 Sumoylated and point mutation in Normal and Variant angina iPSC derived Vascular smooth muscle .*

(A) We demonstrated edogeneous level of SERCA2 and SUMO1 in hVSMC using immunoblot.

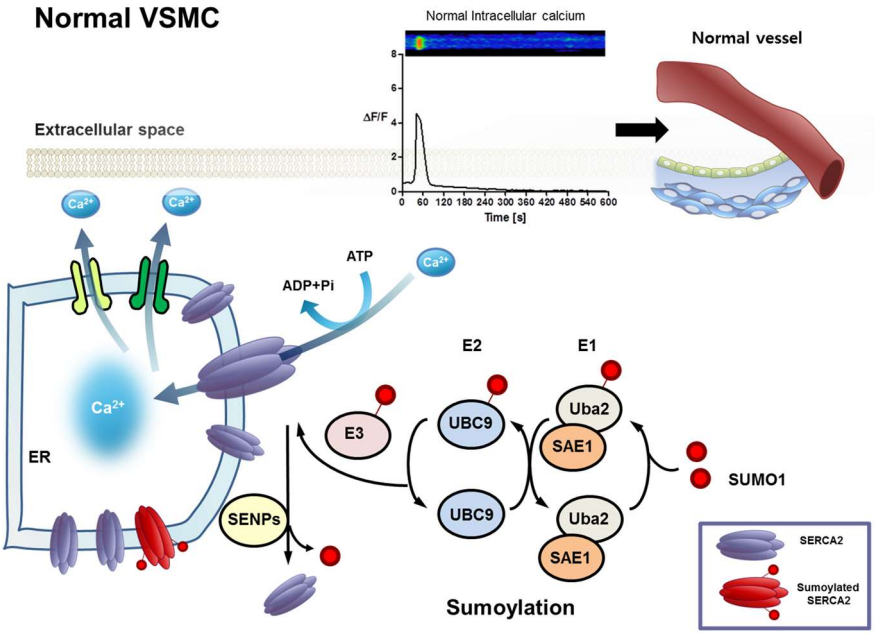
(B) Representative immunoprecipitation for SUMO1 and SERCA2a.

(C) The effect of SERCA2 point mutation viral vector were treated in variant angina derived VSMCs and intracellular calcium level were anaylzed using with FLUO-4 claicum fluoroscence. FLUO4 were performed in response to carbachol (250uM). Change in the relative fluorescence unit (ΔF/F) of Fluo-4 loaded cells was monitored by time elapse confocal (NiKon) over 10 min after the addition of carbachol, an inducer of contraction. Data represent means SEM. (n=3).

(D) The effect of Ginkgolic acid (E1 inhibitor) treated in variant angina groups cells were analyzed with using FLUO-4 calcium fluoroscence. FLUO4 were performed in response to carbachol (250uM). Change in the relative fluorescence unit (ΔF/F) of Fluo-4 loaded cells was monitored by time elapse confocal (NiKon) over 10 min after the addition of carbachol, an inducer of contraction. Data represent means SEM (n=3 each for group).

(E) Total protein Ca^{2+}-dependent ATPase activity of normal and variant angina were analyzed after treated ginkgolic acid. This data represent three independent experiment. Data represent means SEM (n=3 each of group).

(A)


Normal VSMC
Normal Intracellular calcium
Normal vessel
Extracellular space
ΔF/F
Time [s]
ATP
ADP+Pi
E2
E1
E3
UBC9
Uba2
SAE1
SUMO1
SENPs
ER
Sumoylation
SERCA2
Sumoylated SERCA2

(B)

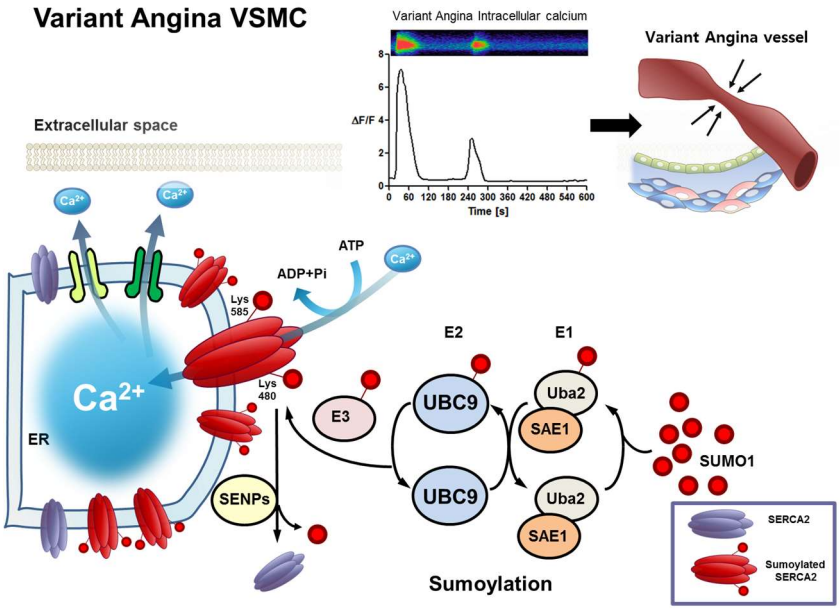

Variant Angina VSMC
Variant Angina Intracellular calcium
Variant Angina vessel
Extracellular space
ΔF/F
Time [s]
ATP
ADP+Pi
Lys 585
Lys 480
E2
E1
E3
UBC9
Uba2
SAE1
SUMO1
SENPs
ER
Sumoylation
SERCA2
Sumoylated SERCA2

Figure8. Schematic figure of *Normal and Variant angina mechanism.* Under basal conditions, SUMOylation enhances the stability of SERCA2 and its Ca21 pump function to regulate vascular smooth muscel contractility. However, increased levels of SUMOylated SERCA2 trigger impaired total protein of SERCA2 activity and induce vacular hyperconstriction in variant angina patients.

DISCUSSION

This study is the first to report generation of iPSC, derived from peripheral blood of normal and variant angina patients. Using these iPSC, I differentiated them into vascular smooth muscle and endothelial cells. This study showed the novel mechanism and therapeutic potential effect and clinical relevance in variant angina disease model.

Isolation CiMS from variant angina and generation of induced pluripotent stem cells

Various types of cells, including adult stem cells, have been used as a source of generation iPSC or stem cells therapy. However, these invasive methods, such as collecting these cells from skin, bone marrow and adipose tissues by surgical procedure is, followed with pain and limited in the clinical setting. In order to overcome these limitations, some groups demonstrated the generation of iPSC cells using T cells populated in peripheral blood and reported the protein based generation iPSC[33]. Even Fukuda groups had succeeded in the generation of iPSC cells using T-cells in the peripheral blood[34]. CiMS cells can be

isolated just from 10cc of peripheral blood and generate induced pluripotent stem cells., These methods had some limitations including high cost and complexity of the isolation process. The method I designed hence can be used to isolate these cells not only just from variant angina patients, but also patients with diverse of rare disease syndromes and by using these patient-derived cells. Furthermore, the pathological cause of diseased mechanism could be investigated and identified using this novel method.

Intracellular calcium efflux shows differential effect on variant angina derived vascular smooth muscle cells

After differentiation into vascular smooth muscle cells, I observed intracellular calcium intensity within cells treated with FLUO4 on normal and variant angina derived vascular smooth muscle cells. In this study, vasoactive agonists such as, Carbachol, Acetylcholine, and Ergometrin maleate, induced the intracellular calcium efflux intensity and increased calcium sparks in variant angina VSMCs, but not in human VSMCs and normal control VSMCs. The cause of VSMC hyperactivity also remains poorly understood. I showed that one of the reasons

might be from different activity of SERCA2 and its post translational modification. This study showed that SERCA2, which is a molecule involved in calcium uptake, its protein levels and sumoylated levels are induced at variant angina. Moreover, the amount of SUMO1 is increased in variant angina group. My study was based on strong background data that has clearly established that impaired SERCA2 is a key molecular abnormality phenotype in variant angina. The therapeutic effects of SERCA2 and SUMO1 could nearly completely recover impaired contractile function in variant angina hearts. The detailed mechanism of induced sumoylated-SERCA2 levels in variant angina requires more experiments to verify this phenomenon.

There are many mechanisms causing coronary artery spasm. The crucial role of regulation of coronary vascular tone is to, mainly release vasodilators. The pivotal factor is Nitric oxide [35]. It is important to observe Nitric oxide levels in endothelial cells. Therefore, further experiment of endothelial cell dysfunction is needed to determine the nitric oxide levels in iPSC derived endothelial cells.

In conclusion, this study demonstrated for the first time a generation induced pluripotent stem cells from variant angina

patients and reported a novel mechanism that post-translocation modification such as SUMOylation affects SERCA2 activity and contractility function in variant angina. These findings indicate that SUMOylation of SERCA2 may provide a novel therapeutic strategy for the treatment of variant angina.

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국문 초록

국내 사망 원인 중 1,2 위를 다투는 심현관 질환 중 심장혈관인 관상동맥의 심한 경련에 의해 일어나는 이형 협심증은 주로 한국인과 일본인에서 발병되는 희귀 난치성/중증 질환임. 심한 경우 심근경색, 치명적 부정맥 등으로 사망에 이르는 희귀 난치성/중증 질환이나 아직까지 그 기전이 명확하게 밝혀져 있지 않아 중증 환자의 경우 사망하는 경우가 상당이 많음. 본 연구에서는 이형 협심증 환자의 말초혈액을 이용하여 역분화 줄기세포를 제작한 후, 평활근세포와 내피세포로 분화하여 그 기전을 규명하려고 함.

역분화 줄기세포를 이용하여 분화한 평활근 세포를 이용 세포내 칼슘을 인지 할 수 있는 FLUO-4 라는 형광염색을 통해, 실시간 촬영에 의한 환자에서의 평활근 세포 내의 칼슘 변화를 관찰함. 더 나아가 역분화 줄기세포를 이용하여 분화한 내피세포를 이용하여, 혈관 형성의 유무를 관찰하여 환자에서의 내피세포의 변화를 관찰함.

이러한 세포 내의 칼슘 변화 실험을 통해 이형 협심증의 평활근세포가 정상인 군과 비교하여 과도한 칼슘 변화가 일어나서 과수축이 일어난다는 것을 증명함.

평활근 세포 수축에서의 SERCA2 가 연관 되어있다는 것을 증명하였으며, 최초로 이형 협심증의 원인을 찾게 되었음.

또한 본 연구 결과는 이형 협심증의 기전을 밝혔을 뿐만 아니라, 가장 효과적으로 치료할 수 있는 방법과 신약 개발을 할 수 있을 것으로 기대함.

주요어: 역분화 줄기세포, 이형 협심증, 분화, 평활근 세포, 내피 세포, 분화, 칼슘 변화, FLUO4, SERCA2, Sumoylation

학 번: 2009-31111