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Effect of estradiol on Ca^{2+} handling proteins in hypertrophied H9c2-derived myotubes

Disertación presentada por:

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Dedication

Esta tesis es fruto de trabajo y dedicación personal, que fue lograda gracias al apoyo de las personas que tuvieron confianza en mí. De manera especial, agradezco a mi asesor de tesis, el Dr. Julio Altamirano Barrera, por asesorarme, acompañarme en este proceso de aprendizaje, compartirme su conocimiento e impulsar en mí un pensamiento crítico que incrementa mi interés en la ciencia. Agradezco al Dr. Gerardo García Rivas por apoyarme, asesorarme, y fomentar en mí la importancia del análisis. Al Dr. Martín Rogelio Ramos, a mis compañeras Paulina y Mónica, a mis compañeros de la maestría y a los miembros del grupo de cardiología les agradezco por brindarme las herramientas necesarias para desarrollar mi conocimiento en el trabajo de laboratorio. Agradezco a los miembros de mi comité de tesis, Dra. Ana Laura Isabel de la Garza Hernández, Dra. Norma Leticia Gómez Viquez y Dra. Rebeca García Varela, por sus comentarios y aportaciones para la revisión de este proyecto de tesis. Importantemente, agradezco a mis papás y a mis hermanas por impulsarme a ser mejor cada día. Finalmente agradezco a Darío y Yuliana, quienes siempre estuvieron a mi lado dándome sus palabras de aliento cuando el camino se ponía difícil.

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Effect of estradiol on Ca^{2+} handling proteins in hypertrophied H9c2-derived myotubes

By

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Summary

Cardiac hypertrophy is characterized by abnormal growth of muscle mass, accompanied by dysregulations in cell structure and function, and can lead to heart failure. Some of the main changes that hypertrophic cells undergo are sarcomere disorganization, loss of T tubules, alterations in Ca^{2+} management, and cardiac contractility. There is a higher prevalence of cardiovascular diseases in young and middle-aged men compared to women, which is why female sex hormones (being 17β-Estradiol the main circulating female sex hormone) are considered to play a cardioprotective role. Cardiac hypertrophy, its molecular mechanisms and therapeutic strategies have traditionally been studied in different *in vivo* and *in vitro* models. Each model has advantages and disadvantages, such as challenging maintenance in primary cells or lack of complete cardiac phenotype in immortalized cell lines. The H9c2 cell line can be differentiated into myotubes, using retinoic acid as the inducing agent, to have a phenotype more cardiac-like. In this project it was standardized a model of hypertrophied H9c2-derived myotubes induced by angiotensin II, and the model was used to assess the cardioprotective role of pretreatment with 17β-Estradiol in terms of inflammation markers, gene expression of Ca^{2+} regulatory proteins, assessed by qPCR, and cell hypertrophy assessed by confocal microscopy. It

was observed an attenuation of hypertrophic phenotype in differentiated myotubes with 17β-Estradiol pretreatment.

Content

Chapter 1- Problem statement

1.1 Problem statement

The World Health Organization (WHO) defines cardiovascular diseases (CVD) as: "a set of disorders of the heart and blood vessels." According to WHO, CVDs are classified into: arterial hypertension, coronary heart disease, cerebrovascular disease, peripheral vascular disease, heart failure, rheumatic heart disease, congenital heart disease and cardiomyopathies (WHO 2019), and despite the scientific and technological improvements, CVDs are still a public health issue.

People with risk of CVDs may also have increased blood pressure, glucose, lipids and adipose tissue (WHO 2017). One of the major causes of death is atherosclerotic CVD, which is a chronic disease that might have its roots in the formation of atheroma plaques, as a result of alterations in lipid metabolism, lipid deposition and chronic inflammation in endothelial cells (Van Camp 2014; Zhu et al. 2018). Catecholamines mediate the normal cardiovascular effects of the sympathetic nervous system by stimulating adrenergic receptors, generating inotropic, chronotropic and lusitropic effects. However, there is an association between high levels of catecholamines and CVDs (Bastos, Gomes, and Ribeiro 2017; Sun et al. 2012).

High levels of catecholamines have been observed during stress, ischemia reperfusion injury and heart failure. As a result of these high levels, heart can suffer hypertensive crisis, tachycardia, ischemia, and arrhythmia. Catecholamine excess have harmful effects on cardiomyocytes in which reactive oxygen species (ROS) are a byproduct of catecholamines degradation and increased mitochondrial metabolism (Costa et al. 2011).

An important final consequence of CVDs is heart failure, which can be developed from hypertension and cardiac hypertrophy (Tanai and Frantz 2015). Hypertension is a chronic degenerative disease, diagnosed in about one billion people worldwide. The causes of hypertension include volume overload, pressure overload and genetic factors. The renin-angiotensin system (RAS) activation is implicated in the physiopathology of hypertension, where angiotensin II (Ang II) plays an important role. Ang II over-stimulation can evoke cardiac hypertrophy as a consequence of hypertension (Crowley et al. 2006). Chronic hypertension leads to structural and functional changes in the cardiovascular system, such as ventricular hypertrophy, atrial enlargement or diastolic dysfunction (Shenasa and Shenasa 2017). Cardiac hypertrophy is the enlargement or thickening of the heart muscle, which predispose to heart failure. Heart failure is considered as an epidemic disease, whose abnormalities preclude the heart to pump enough blood to meet the metabolic needs of the body (NIH 2020b).

Ca^{2+} is an ubiquitous second messenger in eukaryote cells, controlling a variety of vital an specialized cellular functions (Berridge, Bootman, and Roderick 2003). In ventricular cells it is the mediator of the excitation-contraction coupling (ECC), which allows the heart to contract and consequently pump blood. Therefore, adequate handling of cytosolic Ca^{2+} in the heart is highly important (Bers 2002). Ca^{2+} alterations underlies multiple pathologies, such as cardiac hypertrophy and arrythmia (van Berlo, Maillet, and Molkentin 2013). Multiple therapeutic strategies have been developed to attenuate the consequences due to pathological cardiac remodeling observed during hypertrophy. A strategy envisioned in the past decades is the use of estrogens, which might contribute to changes in Ca^{2+} handling protein expression (Jiao et al. 2020). In the present study, a model of angiotensin II-induced

hypertrophic myotubes was standardized, and it was assessed the effect of 17-β estradiol pretreatment in the expression of Ca^{2+} handling proteins, pathological remodeling markers and hypertrophy. However, more studies are needed to demonstrate whether the 17-β estradiol pretreatment is useful to ameliorate the changes in expression of Ca^{2+} handling proteins.

1.2 Background

1.2.1 Models to study cardiac hypertrophy

Strategies for an effective management of cardiac hypertrophy are needed. To develop these strategies to prevent, reduce or revert the pathological ventricle remodeling and associated complications, it is required the use of a variety of experimental models, both, *in vivo* and *in vitro*.

Animal models of cardiac hypertrophy can be developed based on pressure/volume overload, neurohumoral agents, gene editing, ischemia/reperfusion or electrophysiological manipulation (Berry et al. 2007). The use of animals has been extremely important; however, it requires greater amount of time compared to cell lines and the sacrifice of the animals at the end of the experiment. In order to increase the understanding of the main mechanisms before using animal models, it would be useful to develop an *in vitro* reliable model that replicates the main signaling pathways, gene expression patterns, and structural and functional changes occurring during hypertrophy. This would allow, on one hand to reduce the use of animals, and in the other hand, it would provide a robust, rapid, and less expensive model to assess pharmaceutical strategies aimed to counteract hypertrophy.

To work with differentiated cardiomyocytes requires the chronic development of an animal model and the sacrifice of laboratory animals. Nevertheless, adult cardiomyocytes cannot replicate, therefore, in primary culture they dedifferentiate and die within days. Thus they are fragile and difficult to maintain (Sambrano et al. 2002).

In vitro models of cardiac hypertrophy include primary cultures of differentiated adult cardiomyocytes and neonatal cardiomyocytes (PNCAM), as well as immortalized cell lines that proliferate indefinitely. PNCAM retain their cardiac phenotype and can be maintained for longer periods of time compared to adult cardiomyocytes and can be used to assess hypertrophy. Nevertheless, obtaining PNCAM still requires animal sacrifice and cells passing is not recommended, becoming a disadvantage compared with the multiple passes that can be performed in immortalized clonal cell lines (Au - Ehler, Au - Moore-Morris, and Au - Lange 2013). The use of cell lines is a well-accepted option in pre-clinical investigation due to its multiple advantages. Besides the almost unlimited working material due to multiple passes, immortalized cell lines are relatively easy to use and avoid long approval periods and ethical concerns related to the use of animals or human cell tissue (Kaur and Dufour 2012). Some of the most widely cell lines used to study cardiovascular system are C2C12 myoblasts (skeletal muscle derived cell line) and H9c2 myoblasts (derived from LV myoblasts of embryonic rat hearts) (Berry et al. 2007).

Cardiac hypertrophy has been induced in H9c2 using a variety of conditions such as high glucose, high cholesterol, endothelin-1, angiotensin II or catecholamines (e.g., isoproterenol) (K.C. Cheng et al. 2019; Lee et al. 2016). Angiotensin II is a well-known hypertrophy-inducing agent in H9c2 cells. A comparison between PNCAM and H9C2 cells treated with 200 nM Ang II showed that after 48 hours both cell types developed

hypertrophy and increased F-actin, α-cardiac actinin and brain natriuretic peptide (BNP). This validates the use of H9c2 cells to study hypertrophy, which allows to assess therapeutic strategies to inhibit or revert its development (Watkins, Borthwick, and Arthur 2011).

1.2.2 H9c2 cell line and its differentiation process

H9c2 cell line is isolated from embryonic BDIX ventricular rat heart. BDIX is a rat strain with a cell cycle mutation, used to study cancer. H9c2 propagates as mononucleated myoblasts, and when reaching confluency they begin to form multinucleated tubular structures (Kimes and Brandt 1976). H9c2 can differentiate to myotubes upon exposure to retinoic acid (RA) and reduced serum, presenting some aspects of the cardiac muscle phenotype (Branco et al. 2015).

RA is derived from the fat-soluble retinol (vitamin A). Since RA is liposoluble, it acts as an intracellular ligand, it translocates to the nucleus as RA directly or upon derivation from retinol (figure 1). When binding to retinoic acid receptors (RAR), it contributes to a conformational change switching them from repressors to activators of gene expression, controlling different target genes that participate in the differentiation process (figure 1). During the synthesis of RA, it is crucial the oxidation of retinaldehyde, which is carried out by retinaldehyde dehydrogenases (RALDHs) (Rhinn and Dolle 2012). From all the RALDHs (RALDH1, RALDH2 and RALDH3), RALDH2 is the earliest to be expressed, which can be found in mesodermal cells, somatic and lateral mesoderm, posterior heart tube and rostral forebrain (Niederreither et al. 1997). RA is implicated in the transcription of genes such as growth hormone, adrenergic receptor β1 or protein kinase Cα (Rhinn and Dolle 2012).

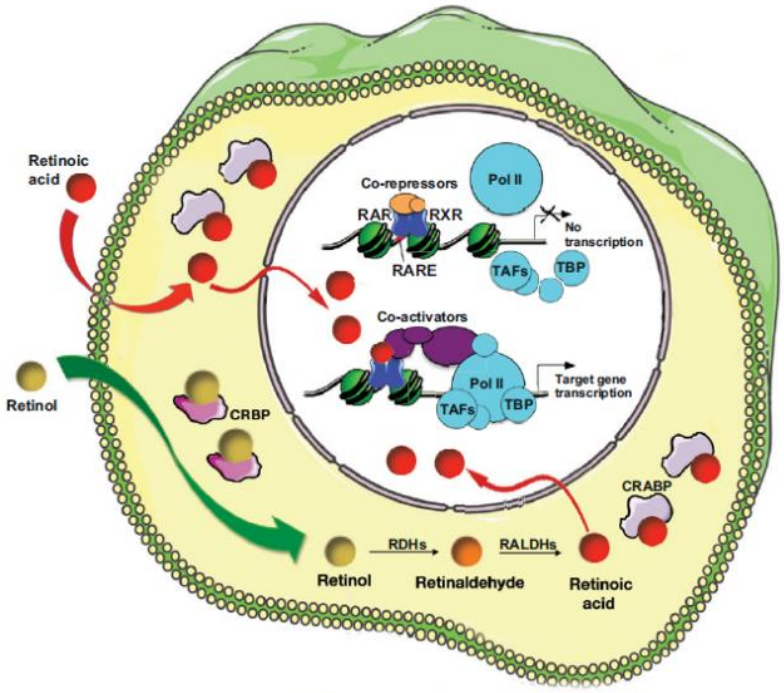

Figure 1. *RA signaling pathway* (Rhinn and Dolle 2012).

The characterization of morphological features and protein expression have been performed on H9c2 cells upon the differentiation process (Menard et al. 1999; Branco et al. 2015; Pereira et al. 2011). Undifferentiated H9c2 cells present a mononucleated, spindle-to-stellate shape. RA treated H9c2 cells presented cell fusion and formation of larger multinucleated cells, forming mitochondrial networks, and preserving cell viability. Differentiated myotubes also present a reduction in cell proliferation accompanied with higher content of cardiac troponin T, troponin I, cardiac calsequestrin and sarco/endoplasmic reticulum Ca^{2+} ATPase2 (SERCA2) (Branco et al. 2015). RA also stimulates the expression of the cardiac subunit α1 of the L-type Ca^{2+} channel, while attenuating the expression of the skeletal subunit α1 in H9c2, achieving the largest changes at day 10 after initiating the differentiation. This supports the idea that RA is involved in cardiac specific expression of Ca^{2+} handling proteins (Menard et al. 1999).

Ca^{2+} signaling is characteristic on each cell type. Myotubes show Ca^{2+} signaling resembling more to cardiomyocytes than H9c2 cells. As shown in figure 2, ryanodine receptor (RyR) and inositol 1,4,5-trisphosphate receptor (IP_3R) release Ca^{2+} from intracellular Ca^{2+} stores. Predominance of Ca^{2+} liberation from the sarcoplasmic reticulum (SR) is characteristic from cardiac cells. H9c2 cells lack RyR activation with caffeine, whereas vasopressin (VP) induces a large cytosolic Ca^{2+} spike, due to the activation of IP_3R. In contrast, differentiated myotubes with RA, have a large response to caffeine by directly activating the RyR, while VP response decreases (Szalai et al. 2000).

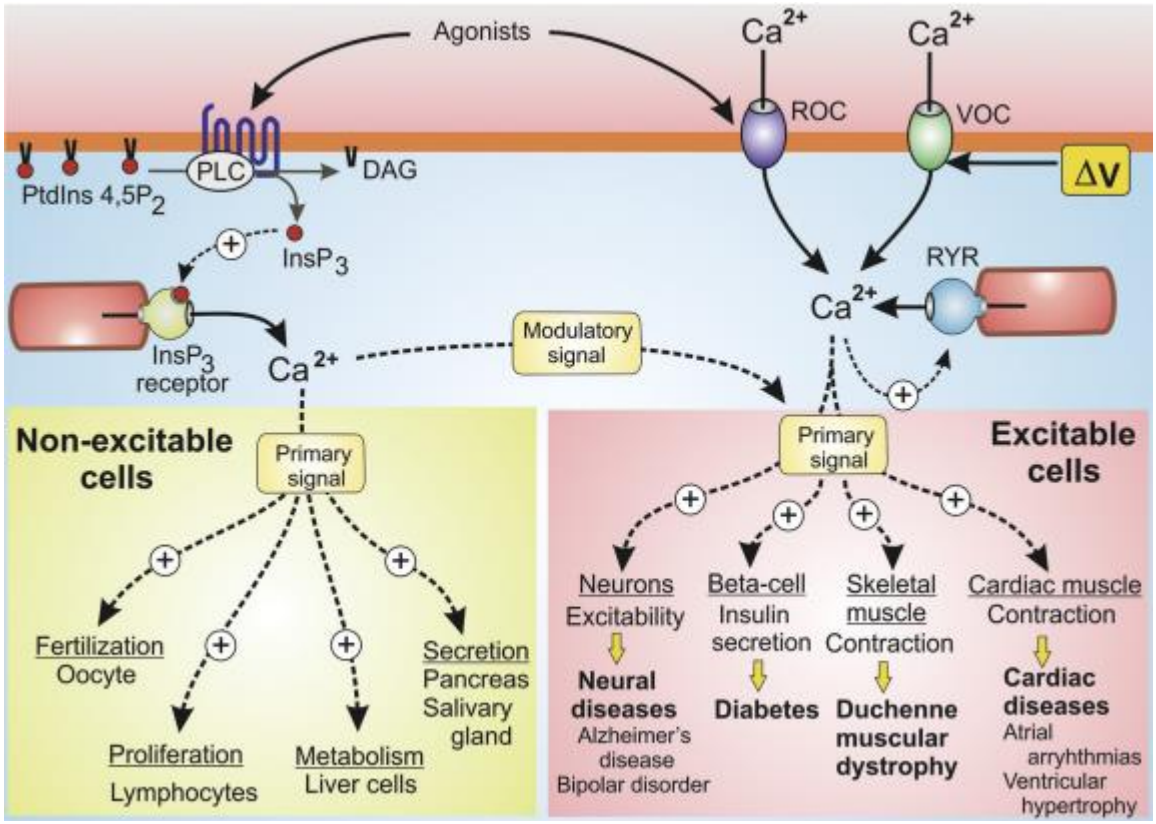

Figure 2. *Ca^{2+} signaling in excitable cells and non-excitable cells* (Berridge 2016).

1.2.3 Hormone replacement therapy and cardioprotection

Hormone replacement therapy (HRT) is the act of supplementing females with hormones that they have lost during menopause transition (Harper-Harrison and Shanahan 2019). HRT has shown to induce a reduction in coronary heart disease and mortality (Lobo 2017). After menopause there is a reduction of estradiol (E2) levels, increasing the prevalence in CVD in post-menopausal women. For this reason, HRT in post-menopausal women is a possible adjuvant treatment during CVDs. Beneficial cardiac effects have been observed upon female sex hormones supplementation in animal models and women. A study on ovariectomized rats with an ischemic precondition determined a cardioprotective effect on daidzein (a phytoestrogen). Ventricles from a murine model treated with 0.2 mg/kg of daidzein restored the cardioprotective effect in rats with ovariectomy (OVX) by down regulation of caveolin. Cardioprotection was attenuated by the eNOS (endothelial nitric oxide synthase) inhibitor, l-nitroarginine methyl ester (Goyal, Semwal, and Yadav 2016). Caveolin is a plasma membrane that mediates endocytosis and mechanotransduction. Interestingly, it has been reported that caveolin regulates eNOS signaling cascade by direct binding that inhibits eNOS activity with a subsequent decreased nitrogen oxide (NO) production and imbalance between endothelial cells and NO (Oliveira and Minshall 2018; Z. Chen, S, et al. 2018).

It has been observed in different clinical trials that E2 contributes to decrease blood pressure and inflammation, preventing endothelial dysfunction and protecting against cardiovascular tissue remodeling. During the Kronos Early Estrogen Prevention Study (KEEPS), HRT of conjugated equine estrogens 0.45 μg/day or transdermal 17β-Estradiol 50 μg/day in postmenopausal women (42-58 years of age) attenuated atherosclerosis progression by increasing HDL and reducing LDL, triglycerides, and IL-6 (Harman et al.

2014). A window of intervention in HRT was confirmed during Early versus Late Intervention Trial with Estradiol (ELITE). HRT (oral micronized 17β-estradiol 1 mg/day) in women <60 years old attenuated progression of thickness of the carotid arteries, LDL, CVD incidence and mortality. These changes were not observed during late intervention (>10 years since menopause, or >60 years old women) (Hodis et al. 2015). Finally, in a cohort study of 1604 post-menopausal women < 60 years old, it was observed a reduction in LV and right ventricle (RV) during HRT, with no association to adverse cardiac effects (Sanghvi et al. 2018).

1.3 Research question

Does 17 β estradiol prevent or reduce the molecular and functional changes, at the level of Ca^{2+} handling proteins, generated by angiotensin II-induced hypertrophy in myotubes?

1.4 Hypothesis

Since estrogens display cardioprotection in a variety of experimental models, as well as in some clinical trials; and they regulate the expression of Ca^{2+} handling proteins, then 17-β estradiol treatment will diminish the alterations induced by angiotensin II in hypertrophic H9c2-derived myotubes; trough modulation of Ca^{2+} handling proteins profile.

1.5 Objectives

1.5.1 General objective

To assess the effect of 17-β estradiol and correlate it with expression of Ca^{2+} handling proteins on

1.5.2 Specific objectives

- To standardize H9c2 cells myotube differentiation conditions.
- To standardize a model of hypertrophy in myotubes induced by angiotensin II.
- To determine changes in expression of the main Ca^{2+} handling proteins (RyR2, SERCA2, PLB, NCX, MCU cardiac LCC α1 subunit, and Micu1), pathological remodeling markers (IL6, Col1, BNP) in hypertrophied myotubes, in the presence and absence of 17-β estradiol.

1.6 Justification

One of the final consequences of pathological heart hypertrophy is heart failure (HF). HF is a condition in which the heart is unable to pump enough blood to meet the metabolic body needs. This can be either, concentric hypertrophy, in which the heart cannot fill with enough blood; or eccentric hypertrophy, in which the heart is unable to pump blood with enough force (NIH 2020b).

According to WHO, in 2016 there were 17.9 million deaths due to CVD, which represents 31% globally (WHO 2017).The Instituto Nacional de Estadística y Geografía (INEGI) in 2017 reported 703,047 deaths in Mexico, and the first place was occupied by CVD, with 141,619 deaths representing the 20% (INEGI 2018).

From the 17.9 million of deaths related to CVD, 80% occur in low- and medium-income countries (Ferreira et al. 2019). Worldwide, there are approximately 26 million

cases of HF. This pathology has a high mortality rate of 7% in ambulatory patients and 17% in hospitalized patients within 1 year of diagnosis. It is estimated that, in the adult population, 1-2% of all hospitalizations correspond to HF (Ambrosy et al. 2014; Ferreira et al. 2019).

In Mexico, 750,000 people live with HF. It is estimated that from all the population with HF in Mexico, only 25% of men and 38% of women will be alive after a five-year period (ACC 2016). CVD in Mexico have a prevalence of 26%, which represents a total cost of 6,100 million dollars per year, representing a 4% of the total expenditure in health (WHF 2016). Furthermore, according to INEGI (INEGI 2018), from the total deaths due to CVDs in Mexico, most of the cases occur in men except for the age group of >65 years. One possible reason is that post-menopausal women decrease their levels of ovarian sex hormones, which earlier had provided them with cardio protection.

1.7 Scope and limitations of study

On this study it is assessed the cardioprotective effect of estradiol at the level of expression changes in Ca^{2+} handling proteins. It is optimized an *in vitro* model of H9c2 differentiated myotubes. A limitation of the study is that, in our experimental conditions H9c2-derived myotubes lack the interaction with other cell types, such as fibroblasts, pericytes, smooth muscle cells and endothelial cells, and the local environment within the heart, and it is a chemically-induced model, without mechanical stress, which is also a main determinant of hypertrophy *in vivo*. Nevertheless, this model will provide a cost-efficient platform to study cardiac hypertrophy, and potential pharmacological interventions to prevent or reduce pathological damage present in this pathology.

Furthermore, it is less time consuming when compared to a chronically-induced hypertrophy animal model, and closer to cardiac muscle phenotype when compared to undifferentiated H9c2 myoblasts.

Chapter 2. Theoretical framework

2.1 Ventricular myocytes overview

Ventricular myocytes play a crucial role to have an adequate cardiac function. Their contractile role is essential for heart pumping, and any dysfunction will lead to a cardiac disease (Peter, Bjerke, and Leinwand 2016).

Its contractile unit is the sarcomere, containing the force-generating myofilaments (figure 3). Myofilaments are composed of titin filaments (anchor proteins) and repeated units of thin and thick filaments. The thick filaments contain myosin and accessory proteins. Myosin heavy chains form rod domains, and globular heads or cross bridges protrude from the rod domains. Thin filaments consist of two polymers, forming a double helix, of the globular protein actin, closely associated with the regulatory proteins tropomyosin and the troponin complex (Golob, Moss, and Chesler 2014). The troponin complex contains three components: a Ca^{2+} binding site (troponin C; TnC), an ATPase inhibitory subunit (troponin I; TnI) and an anchoring protein (troponin T; TnT) (M.X. Li and Hwang 2015).

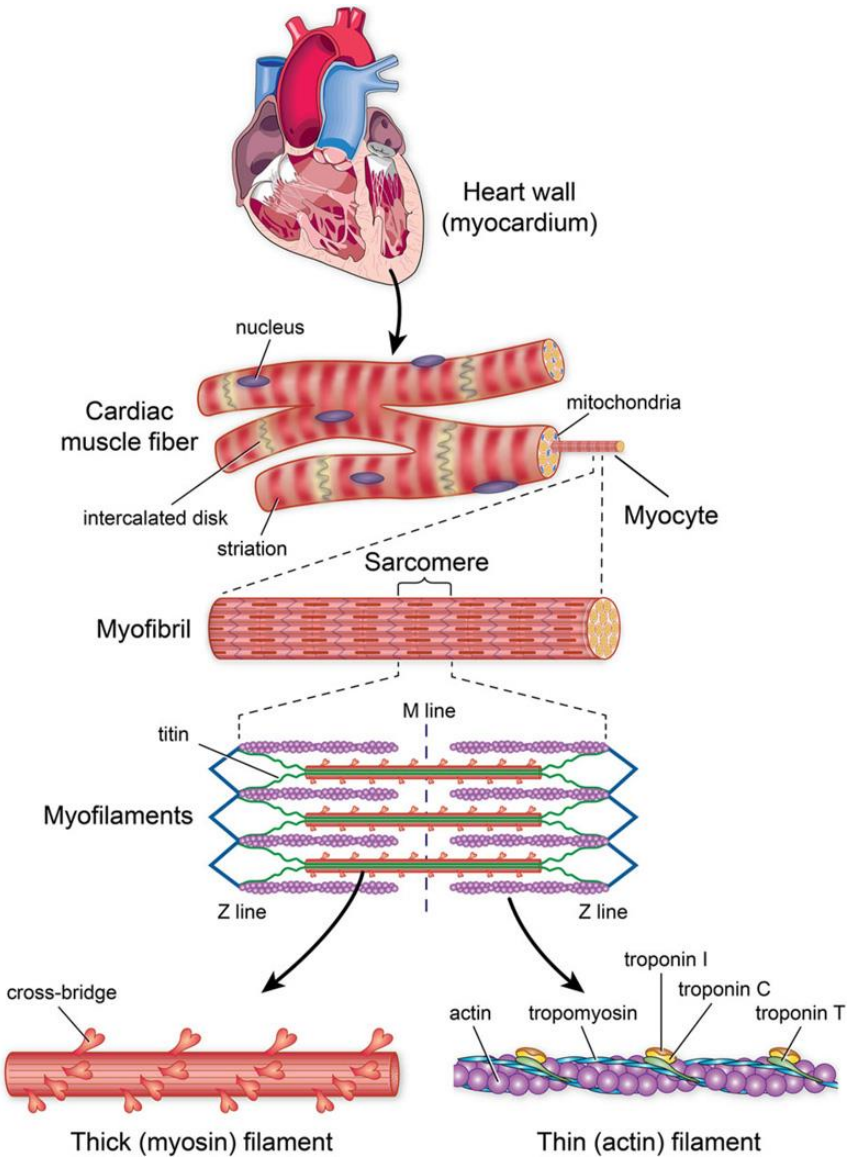

Figure 3. *Sarcomere structure* (Golob, Moss, and Chesler 2014).

Independent from the connective tissue that surrounds myocardial fiber, myocytes have their own membrane called sarcolemma. It was first described in Ramón y Cajal's publication: "Texture of the heart muscle fiber" in 1888. On this publication, Ramón y Cajal performed experiments with gold chloride and described 3 main parts in muscle fiber: wide transverse bands, narrow transverse bands and longitudinal striations (de Fuentes Sagaz 2001).

The sarcolemma has important structural specializations: Transverse tubules (T tubules). T tubules are invaginations of the sarcolemma forming an interconnected network, which contains extracellular fluid. These membrane organelles contain microdomains to compartmentalize ion handling proteins and signaling receptors (Hong and Shaw 2017) and are important to synchronize the excitation-contraction coupling (ECC) at the whole cell level.

2.2 Main proteins for calcium regulation

2.2.1 L-type calcium channels (LTCC)

Ion channels are needed to trigger the cyclic transient increase in cytosolic Ca^{2+} concentration, which in turn regulates the cardiac contraction. L-type calcium channels (LTCC), also called dihydropyridine receptors, are voltage-gated sarcolemma channels. LTCCs are mostly localized on T tubules. These protein complexes have four subunits: a 190-250 kDa α1 subunit, a transmembrane disulfide linked α2δ subunit, an intracellular β subunit, and a γ subunit has also been found on skeletal muscle, with related subunits expressed in the heart and brain. There are three major families of voltage gated Ca^{2+} channels (Ca_v1, Ca_v2 and Ca_v3). The Ca_v1 family is further subdivided in $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$, and $Ca_v1.4$ (Ertel et al. 2000), and the $Ca_v1.2$ isoform (α1c or cardiac isoform) mediates Ca^{2+} current (I_{Ca}) in the heart.

LTCC are activated upon membrane depolarization, allowing a small Ca^{2+} influx, allowing sarcoplasmic reticulum Ca^{2+} release, which in turn causes cell contraction (Hofmann et al. 2014). On ventricular cells, once LTCCs are activated, it occurs a mechanism called Ca^{2+} induced, Ca^{2+} release (CICR). In CICR, Ca^{2+} enters via activated

LTCC, which intracellularly activates RyR generating Ca^{2+} release (Berridge, Bootman, and Roderick 2003). Figure 4.

2.2.2 Ryanodine receptor (RyR)

RyR is an intracellular Ca^{2+} channel located in the membrane of the SR (Berridge, Bootman, and Roderick 2003). This protein exists as tetramer forming a channel which also functions as a scaffolding protein for numerous other signaling proteins. In mammals, there are 3 RyR isoforms: RyR1 is found mainly in skeletal muscle, RyR2 in heart muscle but also in other type of cells, and RyR3 does not predominate in any particular tissue (Bers 2004).

In heart myocytes, the RyR2s are organized in clusters near the sarcolemmal-SR junction, where LTCCs are located (figure 4). The opening of one LTCC in each couplon (arrays that constitute a functional Ca^{2+} release complex of LTCC and RyR) is enough for activating the local SR Ca^{2+} release process by RyRs. To ensure that each couplon is normally activated, there are approximately 10-25 LTCCs per 100 RyRs in each couplon. This arrangement contributes to the CICR process, where Ca^{2+} entry induces Ca^{2+} release from the SR, a key event for cell contraction (Bers 2002).

2.2.3 Sarcoplasmic reticulum calcium-ATPase (SERCA)

SERCA is a protein located at the SR membrane (Stammers et al. 2015). The main function of this P-ATPase is the active transport of Ca^{2+} into the SR using energy from ATP hydrolysis, thereby returning cytosolic Ca^{2+} to the low basal levels during diastole and promoting cell relaxation (figure 4). The isoform that predominates in the cardiac tissue

is SERCA2a (Periasamy and Kalyanasundaram 2007; Bhupathy, Babu, and Periasamy 2007).

SERCA2a has a molecular mass of 110 kDa, and is composed by three regions: cytoplasmic head, transmembrane domain with two Ca^{2+} binding sites, and luminal loops. SERCA has an actuator domain, a phosphorylation domain, and a nucleotide domain (Stammers et al. 2015; Periasamy and Kalyanasundaram 2007).

The extent of SERCA pumping activity depends on its expression levels and of its functional regulator, the SR membrane protein phospholamban (PLB). Nevertheless, SERCA function can also be affected by translational modifications such as oxidation by reactive oxygen species (ROS) and ATP availability (Qin et al.).

2.2.4 Phospholamban (PLB)

PLB is a 52 amino acid phosphoprotein located in the SR membrane that exists as pentamers and monomers (Periasamy, Bhupathy, and Babu 2008). It is composed of three domains: domain IA, domain IB, and domain II. The domain IA is at the cytoplasm side and consists of residues from1 to 20, of which the first 16 are in α-helix conformation (Bhupathy, Babu, and Periasamy 2007).

When PLB is in its dephosphorylated form, it interacts with SERCA decreasing its affinity for Ca^{2+}, and thereby decreasing SERCA performance under basal conditions (Stammers et al. 2015; Gorski, Ceholski, and Young 2017; Periasamy, Bhupathy, and Babu 2008). Nevertheless, upon phosphorylation in Ser-16, PLB detaches from SERCA, releasing it from its inhibition, enhancing Ca^{2+} transport to SR due to a four-fold increase of SERCA activity. Thereby, PLB is a primary mediator of the β-adrenergic response in

the heart. A major effector of PLB phosphorylation is the protein kinase A (PKA), a holoenzyme (composed of a regulatory subunit dimer and two catalytic subunits) which phosphorylates protein targets to regulate their activity in response to increased levels of the second messenger cAMP in response to β-adrenergic stimulation (Turnham and Scott 2016; Law et al. 2017).

2.2.5 Sodium-calcium exchanger (NCX)

The sodium calcium exchanger (NCX) is a protein located in the sarcolemma, which performs secondary active transport allowing the movement of three Na^+ ions to the intracellular space per one Ca^{2+} ion to the extracellular space (figure 4) (Bers 2002). In mammals, there are three isoforms of this exchanger: NCX1 is highly expressed in cardiac tissue, NCX2 in skeletal muscle and NCX3 in the brain (Linck et al. 1998).

NCX1 main purpose is to extrude Ca^{2+} from cells (i.e., Ca^{2+} that entered upon LCC opening) performing a fundamental role in Ca^{2+} homeostasis (Linck et al. 1998; Lariccia and Amoroso 2018). Given the electrogenic role of the NCX, its deregulation is associated with pathological processes such as arrhythmias (Lariccia and Amoroso 2018). Exacerbated function of NCX1 might cause delayed afterdepolarizations, which are mayor factor of diastolic dysfunction and lethal arrhythmias in conditions of Ca^{2+} overload (Javidanpour et al. 2018; Menick et al. 2007).

2.3 Excitation-contraction coupling and mitochondrial Ca^{2+} homeostasis

In heart, the ECC is a process mediated by CIRC, in which an electrical signal stimulates cardiomyocytes to contract in a synchronized way. This electrical impulse is known as action potential (AP). A fundamental component in this process is the second

messenger Ca^{2+}. The contraction force of the heart depends on the Ca^{2+} influx and total amount of Ca^{2+} released from the SR (figure 4) (Bers 2002).

In cardiac muscle, ECC initiates by an AP that promotes the opening of LTCCs in the T tubules membrane (Stammers et al. 2015). T tubules are essential for ECC, due to their high concentration of LTCCs, and their proximity to RyR2. This allows Ca^{2+} to enter the cytosol and induce Ca^{2+} release from internal storage, resulting from RyR2 activation (Hong and Shaw 2017).

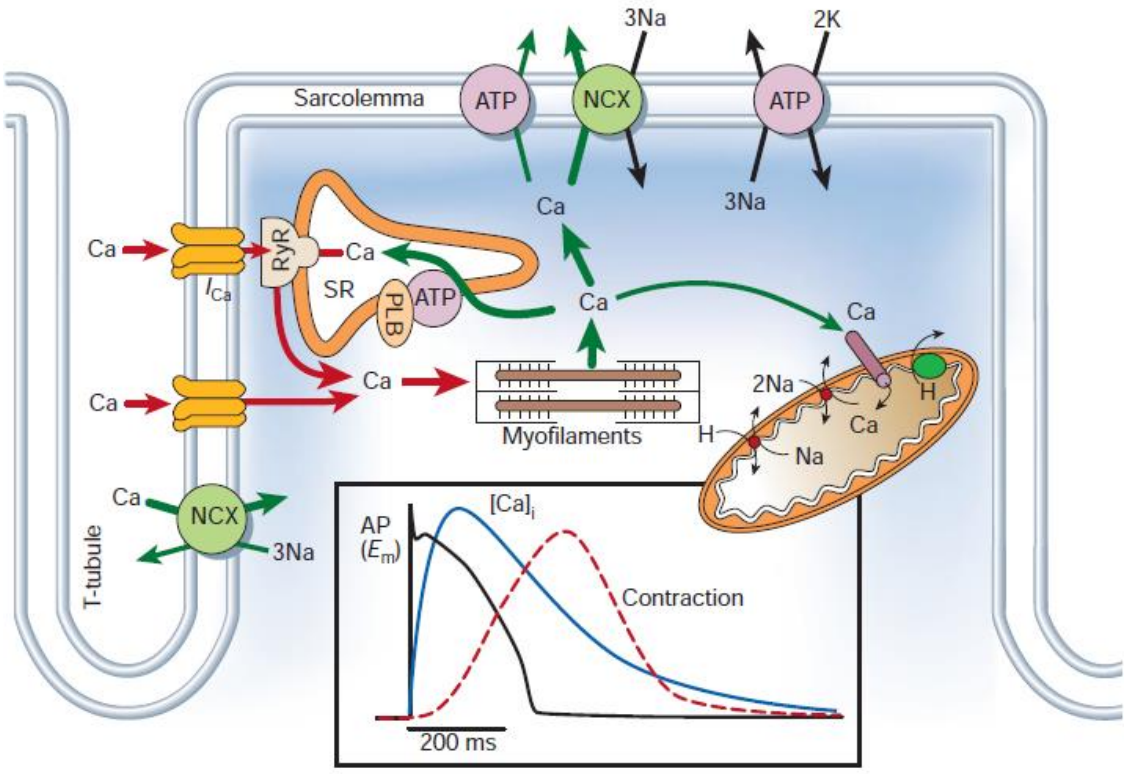

Figure 4. *Ca^{2+} transport in ventricular myocytes* (Bers 2002).

A key event for contraction is the binding of cytosolic Ca^{2+} to TnC. This will remove the inhibitory region of TnI from the actin-binding site inducing a conformational change of tropomyosin (M.X. Li and Hwang 2015). This tropomyosin change will expose

the myosin binding sites for actin, forming the actin-myosin cross-bridges when ATP is hydrolyzed to ADP and P_i, causing myosin head to attach the actin-binding site (Y. Cheng and Regnier 2016). The cross-bridges of the myosin filaments attached to the actin filaments force them to slide over each other generating cell contraction. Afterwards, in the presence of ATP and Ca^{2+}, the myosin is released from actin, dissociating the cross-bridge which allows relaxation. This cycle is repeated, and myosin heads suffer a conformational change from being at 45° to 90°, where it can rebind to actin (figure 5) (Squire 2016; Gordon et al. 2013).

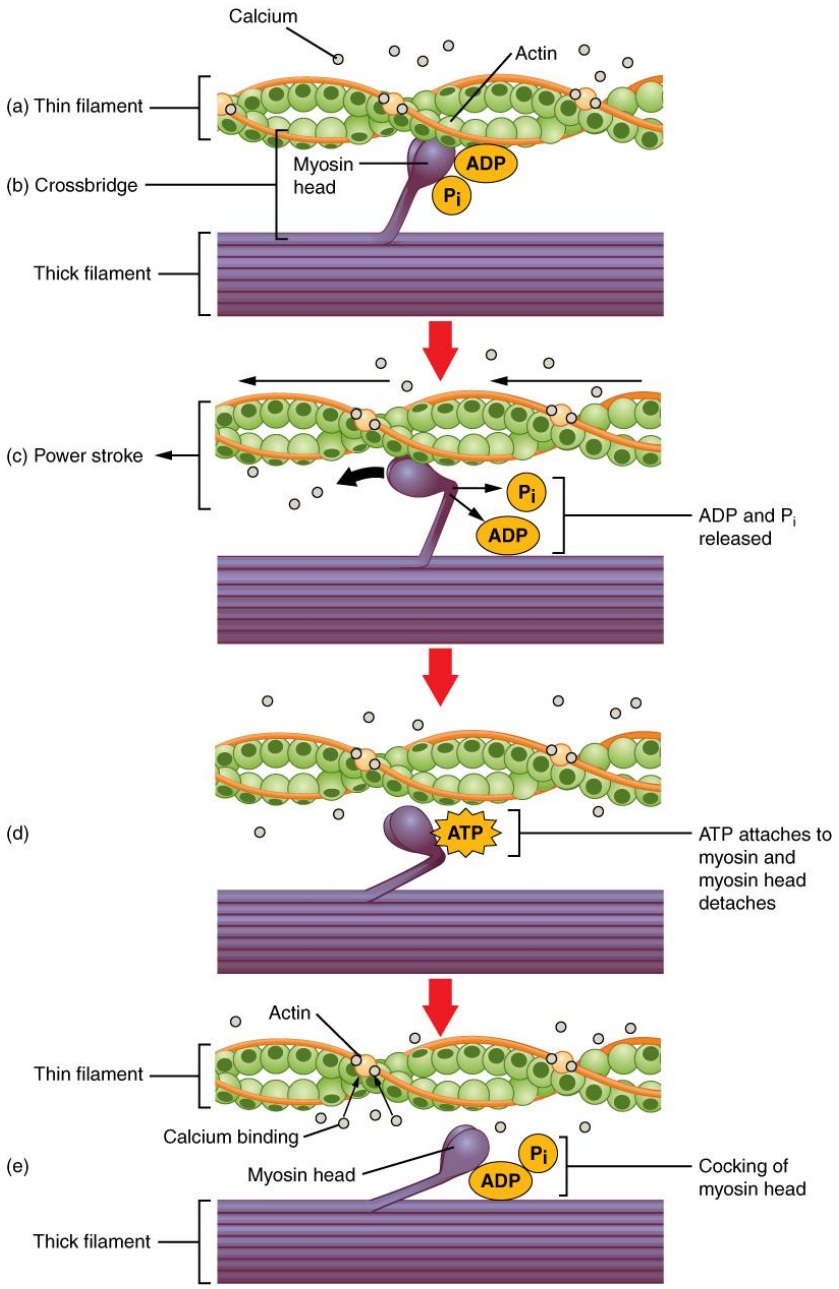

Figure 5. *Muscle contraction* (Gordon et al. 2013)

For cardiac relaxation, it is necessary to decrease cytosolic Ca^{2+} and restore the SR Ca^{2+} content for the next ECC cycle. This will also allow heart chambers to refill with blood (Bers 2002). When Ca^{2+} dissociates from TnC, a new ATP molecule binds to the myosin head detaching from actin; and the cytosol returns to the low basal levels of Ca^{2+} with the help of SERCA and NCX (Hong and Shaw 2017; M.X. Li and Hwang 2015; Y.

Cheng and Regnier 2016). A small fraction of cytosolic Ca^{2+} can be introduced into the mitochondria, by the Ca^{2+} uniporter (MCU) (Aoi et al. 2016), and it will be important to regulate mitochondrial ATP synthesis.

Cardiac ECC is mediated by the activation of thousands of couplons, yielding independent microscopic Ca^{2+} release events known as Ca^{2+} sparks. The spatial and temporal summation of thousands of Ca^{2+} sparks cause the transitory increase of Ca^{2+} concentration in the whole cell known as the Ca^{2+} transient (Fowler et al. 2018). At diastole, opening of RyR2 is not common, and spontaneous diastolic Ca^{2+} sparks are infrequent (Sobie, Song, and Lederer 2005). However, under pathological conditions, spontaneous Ca^{2+} sparks, resulting from Ca^{2+} mishandling, may be exacerbated and underlie cardiac dysfunction (Fowler et al. 2018).

Cardiac dysfunction can also be modulated trough mitochondrial Ca^{2+} homeostasis. MCU is responsible for Ca^{2+} influx to mitochondria. In pathological conditions, an excessive Ca^{2+} influx trigger the formation of mitochondrial permeability transition pore (mPTP), which can result in a pro-apoptotic response (Recchia, Gorgodze, and Gabisonia 2019). Micu 1 and Micu 2 interact in MCU complex regulating its function by having a stimulatory role and acting as gatekeeper, respectively (Patron et al. 2014). Interestingly, an increase in Micu1/MCU ratio contribute to decrease the probability of mPTP opening, which might contribute in CVDs damage tolerance (Chapoy-Villanueva et al. 2019).

2.4 Cardiac hypertrophy

The heart muscle is capable to respond and remodel upon numerous stimuli. This cardiac plasticity enables the heart to adapt itself against environmental demands. These

stimuli might be physiological or pathological, and promote the heart mass growth, or cardiac hypertrophy (Hill and Olson 2008). Afterbirth, cardiomyocytes exit the cell cycle and become differentiated; thus, any increase in muscle mass is due to hypertrophy (cell enlargement), rather than hyperplasia (increased number of cells). As the heart grows during normal human development, individual cardiomyocytes enlarge, but do not divide, in order to reduce wall stress and maintain heart function due to the increased workload (Nakamura and Sadoshima 2018). According to Laplace's law, left ventricle (LV) wall stress is inversely proportional to LV wall thickness. During hypertrophy, it is observed an increased LV wall thickness in order to decrease LV wall stress (Gjesdal, Bluemke, and Lima 2011).

Heart muscle pathological remodeling can be due to concentric hypertrophy, or eccentric hypertrophy (figure 6). Each classification is associated with specific structural and cellular changes. During concentric cardiac hypertrophy, it is characteristic a lateral cardiomyocytes growth that increases the wall thickness accompanied with parallel addition of sarcomeres. This type can develop in response to hypertension and aortic stenosis. Eccentric cardiac hypertrophy is related to addition of sarcomeres in series and longitudinal cardiomyocytes growth that increases chamber volume. It can develop after valvular insufficiency (Hill and Olson 2008; Gjesdal, Bluemke, and Lima 2011).

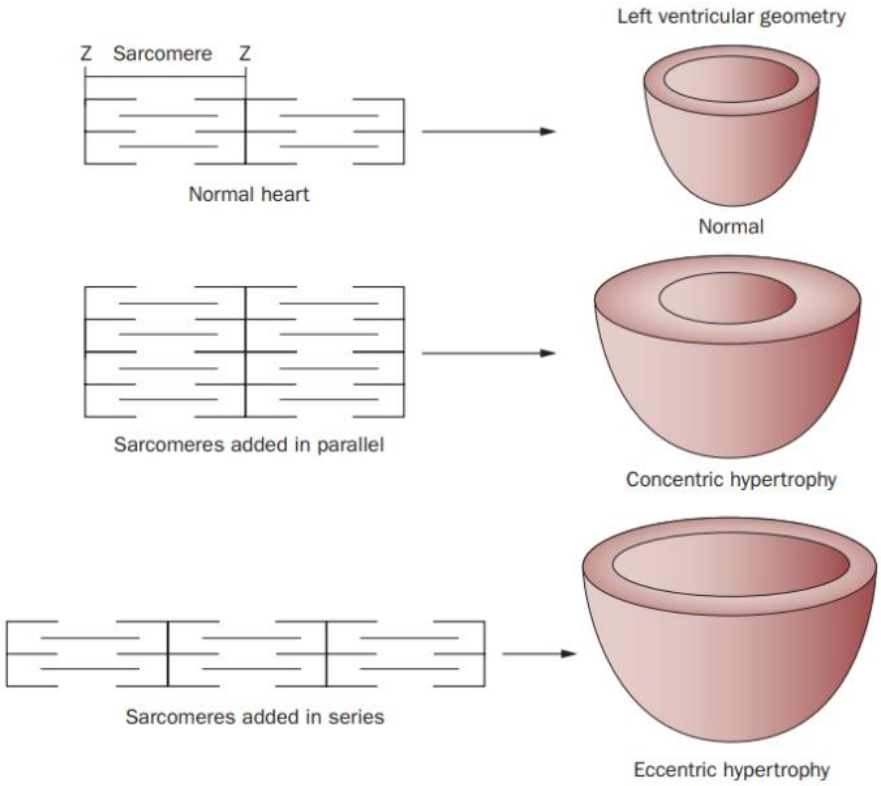

Figure 6. *Concentric and eccentric hypertrophy* (Gjesdal, Bluemke, and Lima 2011).

Physiological hypertrophy results from exercise or pregnancy, it can be distinguished from pathological hypertrophy because the changes in wall thickness and ventricle dilatation are mild and/or reversible (Samak et al. 2016). Pathological cardiac hypertrophy is a chronic degenerative process with abnormal increase in heart muscle mass and altered cellular structure and function (van Berlo, Maillet, and Molkentin 2013). It is triggered by numerous stimuli such as hemodynamic load (pressure or volume), neurohumoral (angiotensin II, endothelin or catecholamines), cardiac injury (ischemia/ reperfusion), congenital cardiac defects (familial hypertrophic cardiomyopathy with mutation on genes encoding for myosin or troponin (MYH7, MYBPC3, TNNT2, or TNNI3) or metabolic diseases such as obesity or metabolic syndrome) (NIH 2020a; van Berlo, Maillet, and Molkentin 2013).

Disorganization of the sarcomere, alterations in Ca^{2+} handling and contractility, and loss of cardiomyocytes with fibrotic replacement (with a characteristic increase in collagen type one (Col1) and fibroblast activation(Hinderer and Schenke-Layland 2019)), are important changes that occur in pathological cardiac hypertrophy (Hill and Olson 2008). During cardiac hypertrophy development, it is also characteristic a low-grade inflammation. It has been reported during hypertrophy that the immune system releases pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6). Patients with hypertrophy have increased TNF-α and IL-6 levels in plasma compared to healthy controls (L. Fang et al. 2017). Cardiac hypertrophy is adaptive at early stages, however, when the stimuli is chronic, in the long-term it transitions to a progressively deteriorated structural and functional state (Akki, Smith, and Seymour 2008). The adrenergic nervous system and the renin-angiotensin system (RAS) are initially activated to increase contractility and survival in early phases upon stress. However, with chronic stress it becomes a pathological condition (Shimizu and Minamino 2016).

In multiple pathologies such as hypertension, arrhythmia, atherosclerosis, aortic aneurysm, or cardiac hypertrophy RAS activation is involved (figure 7). RAS regulation of blood pressure and extracellular volume involves renin, angiotensinogen (AGT), angiotensin-converting enzyme (ACE), angiotensin I (Ang I), Ang II and Ang II receptors (AT1 and AT2). Upon hypovolemia, Na^{+} excretion or increase in sympathetic tone, renin is liberated. Renin cleavages AGT to produce Ang I, which is cleaved by ACE to produce Ang II. Ang II acts by binding to its receptor (AT1 or AT2), evoking a cellular response (Wu et al. 2018; de Almeida and Coimbra 2019). Figure 8.

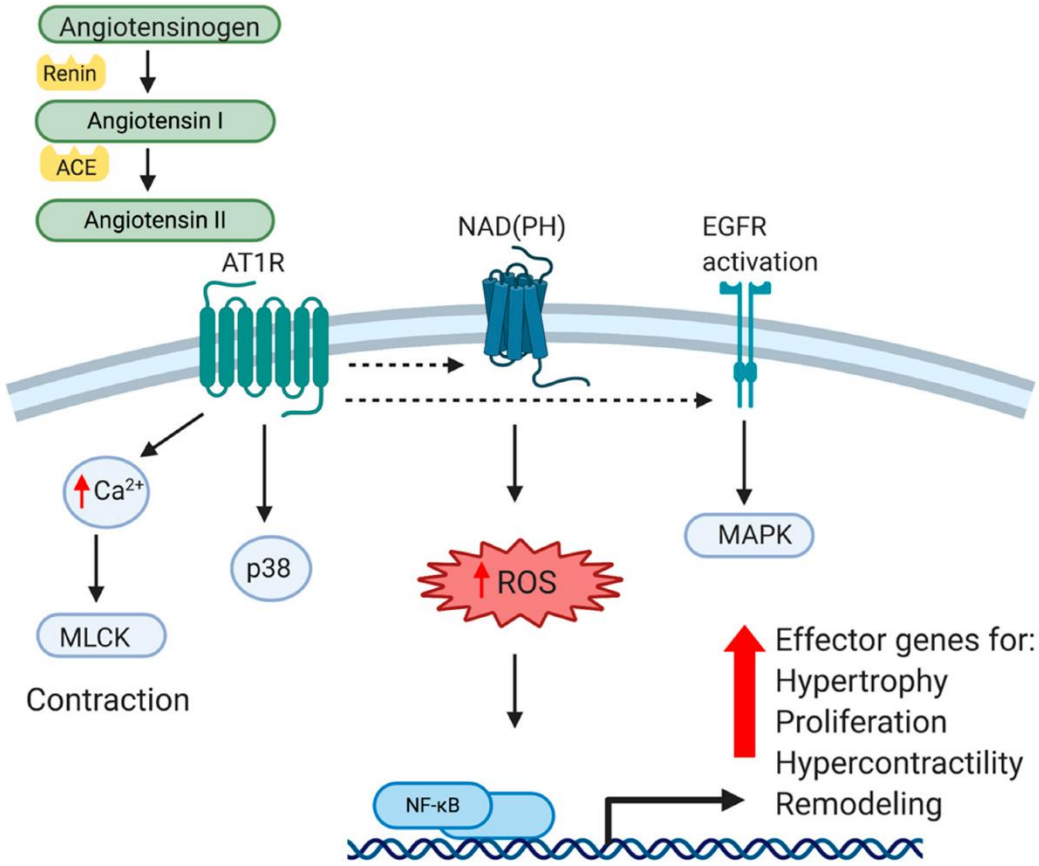

Figure 7. *Cellular and molecular pathological effects of Ang II* (St. Paul et al. 2020).

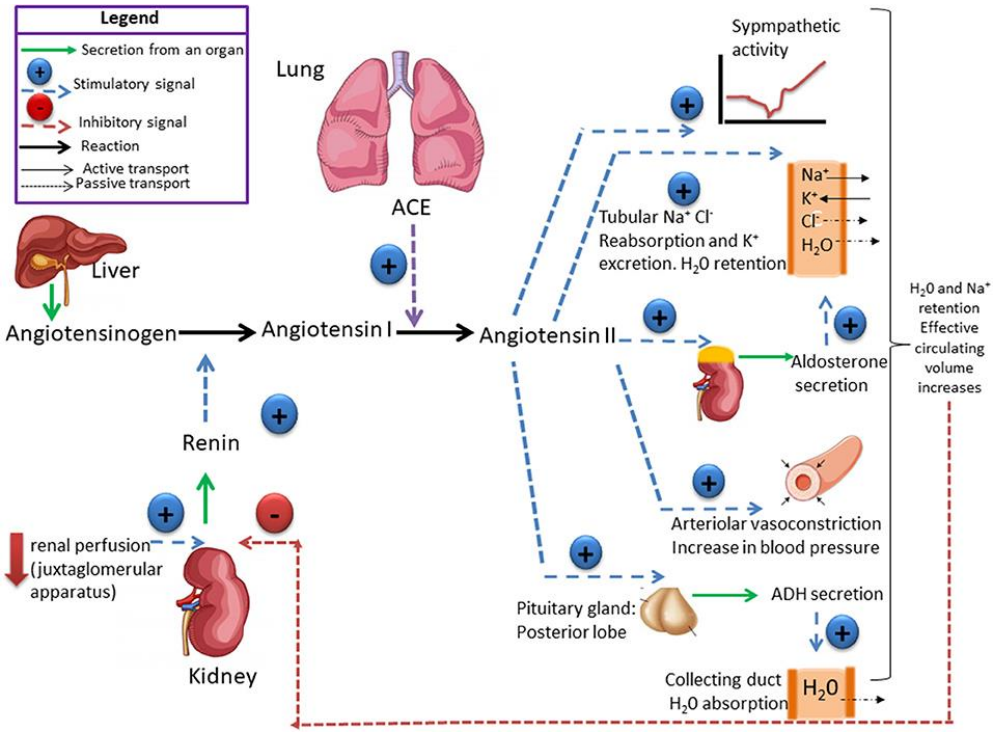

Figure 8. *The renin-angiotensin system* (de Almeida and Coimbra 2019).

Ang II is an octapeptide known to trigger cardiac hypertrophy acting though the inositol triphosphate (IP_3)-mediated Ca^{2+} signaling pathway. Ang II activates AT_1 receptors (Gq-protein coupled receptors expressed in vascular smooth cells, heart, lungs, kidney among others) (Wassmann and Nickenig 2004). When activated, it induces the activation of phospholipase C, producing IP_3 and diacylglycerol (DAG), which are intermediates in phospholipid biosynthesis and act as signaling molecules. Increased IP_3 levels along with Ca^{2+} activate nuclear Inositol triphosphate receptors (IP_3R), inducing a nuclear Ca^{2+} signaling which in turns modulates gene transcription (Berridge 2016). Figure 9.

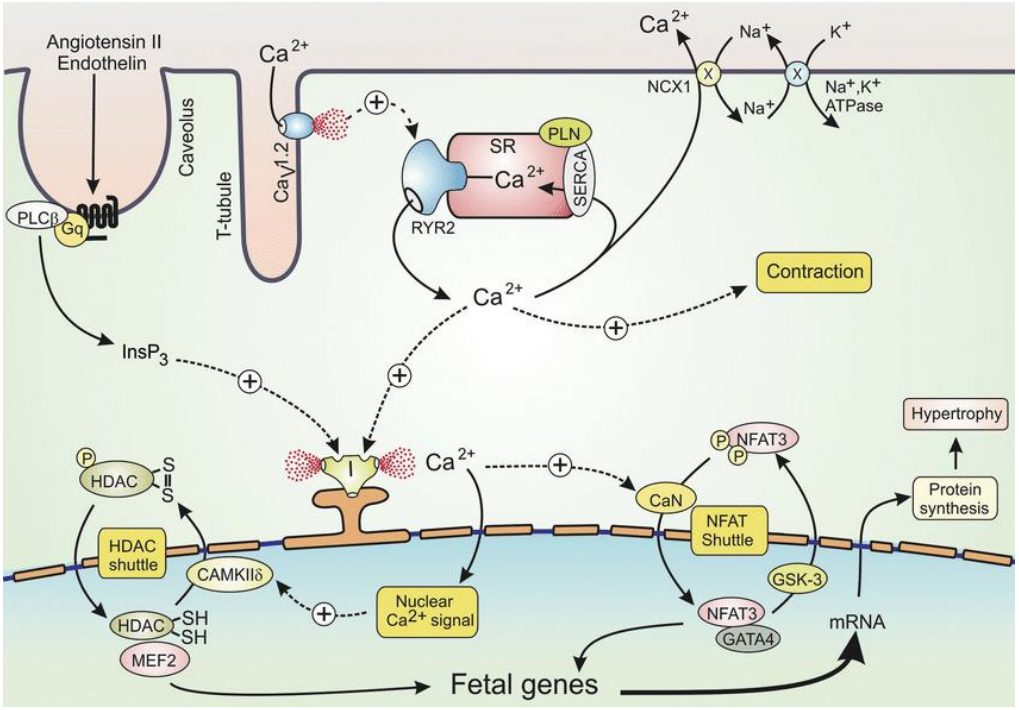

Figure 9. *Angiotensin II hypertrophy pathway* (Berridge 2016).

This nuclear Ca^{2+} signaling modulates calcineurin/nuclear factor of activated T cells (NFAT) and Ca^{2+}/calmodulin-dependent kinase II (CaMKII)– histone deacetylase (HDAC) pathways (figure 9) (Shimizu and Minamino 2016). On cardiomyocytes, the density of RyR is higher than IP_3R. However, during cardiac hypertrophy IP_3R expression and function is increased, resulting in increased protein synthesis, gene reprogramming and promoting necrosis (Garcia and Boehning 2017; Go et al. 1995).

Furthermore, activation of AT_1 receptors is known to increase the production and release of ROS, which is mainly linked to the activation of NAD(P)H oxidase system (Wassmann and Nickenig 2004). NAD(P)H oxidase (NOX) is an enzyme that catalyzes the oxygen reduction by transferring one electron from NADPH. Its main product is superoxide anion that act as signaling molecule and protects against bacteria in physiological conditions. Cardiomyocytes express NOX2 and NOX4, its overactivation is

implicated in CVDs. NOX2 is up regulated in myocardial infarction and cardiac hypertrophy, an increase of NOX2 activity will increase RyR2 S-glutathionylation, leading to Ca^{2+} leak form the SR (figure 10) (Donoso et al. 2011). NOX2 has been involved in Ang II left ventricle hypertrophy. Murine models with NOX2 knockout gene ($NOX2^{-/-}$) did not present Ang II dose-dependent increased NADPH oxidase activity; which was observed in wild type animal models (Cave et al. 2006).

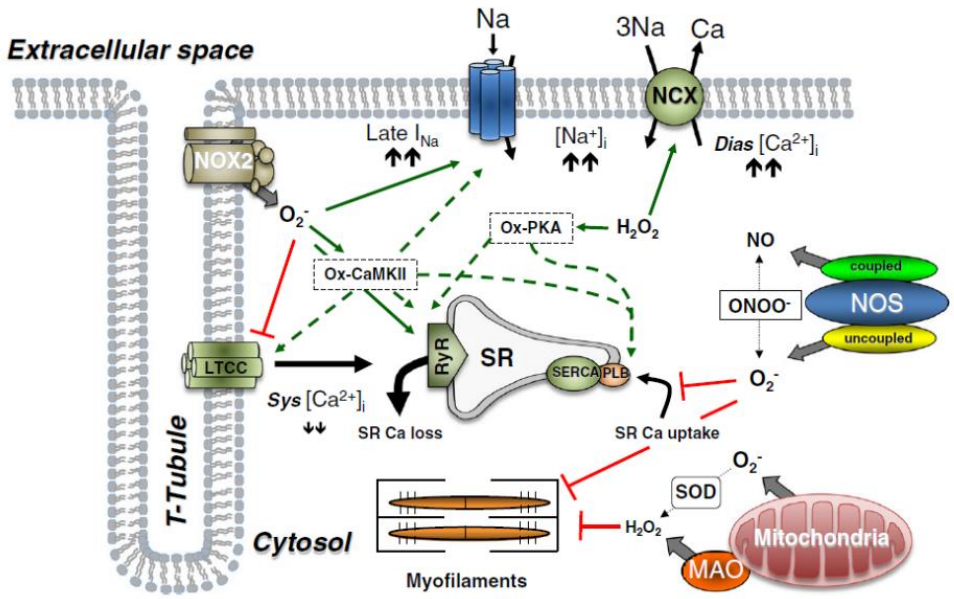

Figure 10. *ROS regulation in hypertrophied cardiomyocyte* (Sag, Santos, and Shah 2014).

Increased ROS production alters ECC process by activating Ca^{2+} handling regulators such as PKA and CaMKII. PKA modulates β-adrenergic stimulation by stimulating adenylyl cyclase, increasing the production of cAMP and activation of PKA which phosphorylates PLB, LTCC, RyR2 and TnI (Bers 2002). β-adrenergic overstimulation can occur in pathological conditions, leading to Ca^{2+} overload and contractile dysfunction. Moreover, when ROS oxidizes CaMKII at methionine residues in

its regulatory domain, which enhances basal CaMKII activity. CaMKII constant activity is implicated in SR Ca^{2+} leak due to RyR2 activation (among other targets). ROS can activate RyR2 directly by irreversibly oxidizing its cysteine residues (figure 10) (Sag, Santos, and Shah 2014).

Ca^{2+} released through IP_3R activates the Ca^{2+}-activated serine-threonine protein phosphatase, calcineurin. In the cytoplasm, calcineurin dephosphorylates NFAT inducing its nuclear translocation resulting in increased protein synthesis and fetal isoform expression, as will be mentioned in the following paragraphs. These changes contribute to pathological cardiac hypertrophy development (Shimizu and Minamino 2016). Ca^{2+} signaling also activates CaMKII, isoforms CaMKIIδ and CaMKIIγ are expressed in the heart (Dewenter et al. 2017). CaMKII can also be activated after Ca^{2+} increase upon catecholamines binding to adrenergic receptors. An increase in β-adrenergic activity is related to cardiac hypertrophy, by activating adenylyl cyclase activity and subsequent increase in PKA activity, which is a downstream activator of CaMKII. α-adrenergic receptors, couple with protein kinase C (PKC) and mitogen-activated protein kinase (MAPK), promoting cardiac hypertrophy (Shimizu and Minamino 2016; Grimm and Brown 2010). MAPK regulates cell growth among other processes. In response to α-adrenergic stimulation, increased ROS signaling modifies Cys 118 in RAS (Sag, Santos, and Shah 2014).

CaMKII interacts with class II HDAC in the nucleus, phosphorylating HDAC4 and HDAC5, which regulates the myocyte enhancer factor 2 (MEF2). HDAC4 act as a repressor of MEF2. When CaMKIIδB phosphorylates HDAC, it promotes its nuclear export, thus diminishing the repression of MEF2 (Berridge 2016). It is also possible ROS-

mediated oxidation at Cys 247/ 276, generating HDAC4 nuclear export and subsequent MEF-2 activation (Sag, Santos, and Shah 2014). Enhanced MEF activity has been implicated in cardiac remodeling, fetal gene reprogramming and inflammation (Dewenter et al. 2017).

An important characteristic of pathological heart hypertrophy is a switch to a fetal expression profile. There is a change from adult metabolism, characterized by the oxidation of fatty acids, to an increase of carbohydrate energy use. This is also accompanied with the expression of fetal isoforms to optimize the use of lower energy availability (Taegtmeyer, Sen, and Vela 2010). Maladaptive gene expression includes an up regulation of atrial natriuretic peptide (ANP), BNP, cardiac myosin heavy chain-β (MHC-β), and skeletal α actin (SKA) (Chien et al. 1993; Taegtmeyer, Sen, and Vela 2010).

In cardiomyocytes isolated from hypertrophic hearts, there is an increase of ANP and BNP. ANP and BNP promote cGMP production by activating guanylyl cyclase A (GC-A) receptor. cGMP subsequently activates cGMP-dependent protein kinase (PKG), which modulates cell growth and contraction (Kuhn 2015). It has been demonstrated that either enhancing cGMP/PKG signaling pathway or blocking cGMP hydrolysis suppresses calcineurin/ NFAT activity and pathological cardiac remodeling. Transient receptor potential canonical 6 (TRPC6) inhibition or enhancement of PDE5 reduces calcineurin/ NFAT activity, thus suppressing the expression of fetal isoforms in cardiomyocytes (Koitabashi et al. 2010).

In adult humans, MHC-α is predominantly expressed in ventricle, however, in failing heart, a decrease in both MHC-β and MHC-α is observed. Nevertheless, the increase in MHC-β/ MHC-α ratio is enough to alter contractile function with a decreased filament

sliding velocity (Razeghi et al. 2001). This change in MHC isoforms is an adaptive response to preserve energy, however, the depressed contractile function promotes the progression of heart failure (Hunter and Chien 1999).

Another important event of pathological hypertrophy is an increase of myocyte death and fibrotic remodeling, contributing to systolic and diastolic dysfunction (Shimizu and Minamino 2016). An important component of diastolic dysfunction is cardiac fibrosis activated by mechanical stress as well as humoral mediators. The increased fibrotic replacement might be induced by Ang II, which activates the JAK/STAT pathway, increasing fibroblast proliferation and collagen in the extracellular space. In hypertrophic cells, there is a gene up regulation of fibronectin and transforming growth factor (TGF-β1), a potent inducer of cardiac fibrosis (Yasunari et al. 2005). Fibroblast growth factor-2 (FGF-2) expressed by cardiac fibroblasts is also known to induce cardiac hypertrophy via MAPK signaling pathway (Shimizu and Minamino 2016).

Structural changes that occur during cardiac hypertrophy include SR and T tubules remodeling that appear during compensated hypertrophy and worsen in uncompensated hypertrophy and heart failure. This disarrangement contributes to Ca^{2+} mishandling due to the abnormal activation of RyR (Perez-Trevino et al. 2015; Vitale et al. 2020; Guo et al. 2013).

It has been demonstrated a SERCA down regulation during cardiac hypertrophy development. Increase on the expression of Na^{+}/Ca^{2+} exchanger as a compensatory process of SERCA down regulation has also been observed. At initial stages of cardiac hypertrophy, Ca^{2+} transport handling is not affected. However, upon repetitive and rapid stimuli, as when heart rhythm increases, it will diminish the Ca^{2+} transient due to limited

increase of SR filling, because SERCA cannot pump Ca^{2+} as normally required (Inesi, Prasad, and Pilankatta 2008).

Furthermore, an up regulation of PLB is also observed on human failing hearts accompanied with an increase in protein phosphatase 1 (PP1), which dephosphorylates PLB. Type 1 PP1 activity is inhibited by heat shock protein 20 (Hsp20), suggesting that Hsp20 modulates contraction by regulating PP1/PLB axis. S100A1 is also implicated in Ca^{2+} handling; it is a protein prevalent in cardiac cells that contain Ca^{2+}-binding motifs acting as Ca^{2+} sensor. It affects Ca^{2+} uptake and release by interacting with SERCA2a, PLB and RyR. S100A1 is down regulated at final stages of heart failure (Kranias and Hajjar 2012).

2.5 Therapies used against cardiac hypertrophy

To attenuate the dysfunction that occurs during pathological cardiac hypertrophy development, there are several strategies used nowadays. Some of the most frequent strategies to reduce LV mass are beta-adrenergic receptor (β-AR) blockers, ACE inhibitors and angiotensin II receptor blockers (ARBs) (Shimizu and Minamino 2016).

β -blockers are antagonists of β-ARs, which are implicated in several physiological processes as well as cardiac hypertrophy development. Three generations of drugs have been developed: nonselective β-blockers, cardio selective β-blockers, and vasodilating β-blockers. All β-blockers have affinity for binding to β-ARs without evoking a physiological response, thus competing for the binding site (Oliver, Mayor, and D'Ocon 2019). Some examples of β –blockers that have been useful against cardiac hypertrophy are bisoprolol, carvedilol, metoprolol and nebivolol. Interestingly, other drugs such as bucindolol and

xamoterol have demonstrated no benefit or increased mortality by increasing β2-adrenoreceptors in patients with respiratory diseases (Baker, Hill, and Summers 2011).

As previously mentioned, ACE is a carboxylase predominantly expressed in the lungs that converts inactive angiotensin I into its active peptide angiotensin II. ACE inhibitors inhibit the activity of ACE, reducing blood pressure and thereby abolishing hypertension. Some examples of ACE inhibitors are trandolapril, perindopril, temocapril, captopril and enalapril (Takai et al. 2004). One of the risks of using ACE inhibitors include acute renal failure and/or hyperkalemia. Which is related to a decrease in mean arterial pressure levels, thus, renal perfusion pressure cannot be sustained (Schoolwerth et al. 2001).

ARBs are non-peptide compounds derived from imidazole-5-acetic acid with a tetrazolo-biphenyl structure. ARBs directly block the action of Ang II by a blockade of AT_1 receptors. However, a co-stimulation of AT_2 receptors can occur due to increased levels of Ang II, causing an increase in nitric oxide (Yasunari et al. 2005). Among the most used ARBs are candesartan, olmesartan, losartan, telmisartan and valsartan, which attenuate the development of cardiac hypertrophy (L. Li et al. 2010).

Strategies used against cardiac hypertrophy will depend on the stimuli that initiated the pathological process. Other strategies that have been explored include the use of diuretics, Ca^{2+} channel blockers, gene therapy, antioxidants, and sex hormones among others (Rosenbaugh et al. 2013; Klingbeil et al. 2003). It has been demonstrated an interaction between sex hormones and RAS pathway in cardiovascular function, thus, sex hormones may be a key factor in differences of cardiac function among men and women (Dalpiaz et al. 2015).

2.6 Sex hormones

Sex hormones (androgens, estrogens and progesterone) are endogenous signaling molecules in charge of modulating cellular processes via gene regulation and protein modification (Regitz-Zagrosek and Kararigas 2016). Its circulating levels on biological systems have an important impact on the regulation of the cardiovascular system (Mauvais-Jarvis, Clegg, and Hevener 2013).

2.6.1 Estrogens

On the human body, the most common endogen estrogens are estrone (E1), estradiol (E2) and estriol (E3); we can also find estetrol (E4) during pregnancy. Estradiol is a hormone produced in the ovaries that mediates its effect through estrogen receptors (ER) in genomic and non-genomic pathways (Thomas and Potter 2013). From the existing ER, ERα and Erβ, in general terms, share protein structure and mechanisms of action, and vascular and cardiac cells express both. These proteins dimerize and translocate to the nucleus, where they can activate or inhibit gene expression by binding to the estrogen response element (ERE) (Eyster 2016). However, G protein-coupled estrogen receptor (GPER, formerly known as GPR30) is located in the cellular membrane. After E2 binding to GPER, it activates signaling pathways such as ERK/MAPK, PI3K (increasing cell survival) and cAMP conferring a vasodilatation effect in vascular endothelial cells when inducing eNOS expression. GPER can also activate PKA, increasing Ca^{2+} influx (figure 11) (Clark et al. 2014; Iorga et al. 2017). It has been reported a relation between genomic and non-genomic pathways in which GPER can phosphorylate ERα, inducing its stabilization and further nuclear translocation. Furthermore, both ERα and ERβ bound to

cell membrane are also able to act in a non-genomic way, similar to GPER (Clark et al. 2014). 17β estradiol (17β-E2) is the major feminine hormone circulating, which besides being produced by the ovaries, it can also be produced by the conversion of testosterone by aromatase in multiple tissues such as vascular, endothelial and muscle cells (Lucas-Herald et al. 2017; Nathan et al. 2001).

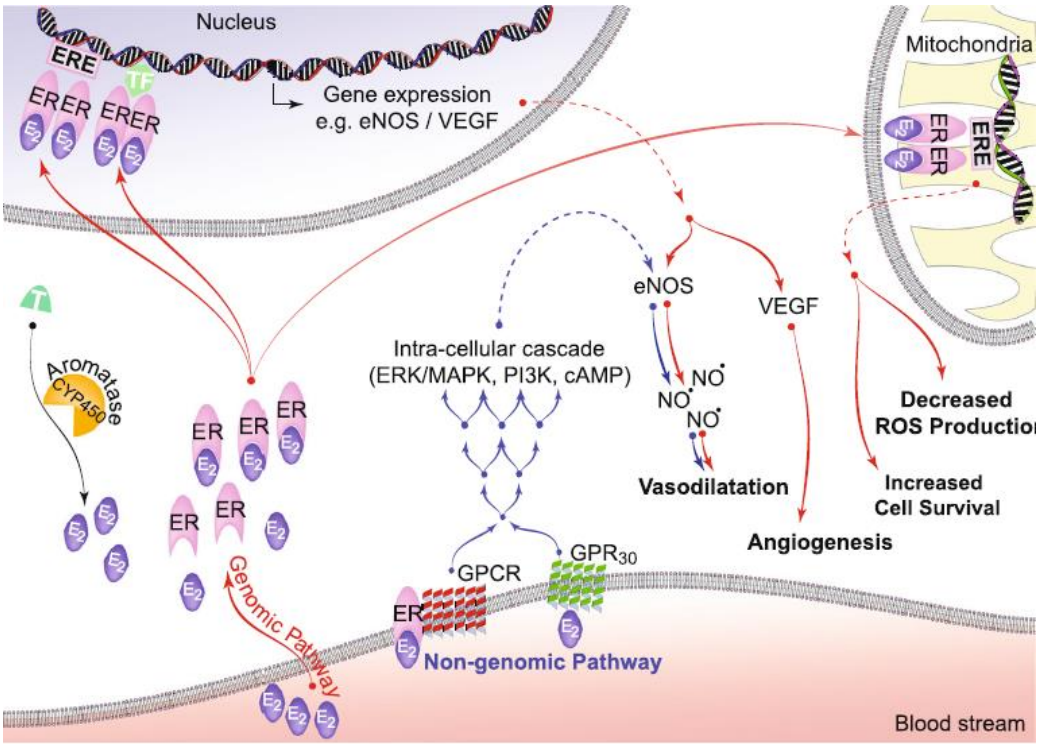

Figure 11. *Genomic and non-genomic effects of 17β-E2* (Iorga et al. 2017).

As mentioned before, female sex hormones protect heart in a genomic and non-genomic way. A genomic way of acting is by promoting angiogenesis. Angiogenesis is the process of blood vessels formation, and it is necessary for oxygen and nutrient supply to the heart that is a high-energy consuming organ. It has been demonstrated that estradiol up-regulates vascular endothelial growth factor (VEGF), thus, promoting angiogenesis in cardiac muscle (Iorga et al. 2017). On cardiac microvascular endothelial cells, a 0.01

μmol/L 17β-E2 dose showed to increase cell migration as well as proliferation, suggesting that 17β-E2 contributes to revascularization and capillary structure maintenance (Liu et al. 2018).

Cardioprotective effects of estrogens have been reported in vascular endothelium. Besides the effects mentioned before, E2 exert an up-regulation of endothelial NO synthase (eNOS), increasing NO which act as a vasodilator (Sader and Celermajer 2002). There are also important non-genomic cardioprotective effects of E2, which contribute to increase NO endothelium secretion and vasodilatation (Mendelsohn and Karas 1999). At the transcriptional level, 2-methoxyestradiol induces the synthesis of eNOS (W. Chen et al. 2015).

Furthermore, ERα and ERβ attenuate extracellular matrix remodeling induced by neurohumoral agents, by decreasing fibronectin, collagen I and III levels. During cardiac hypertrophy induced by Ang II and endothelin-1, increased TGFβ-1 is observed in fibroblasts. On an in-vitro model, dipropylnitrile, an ERβ agonist, prevented fibroblast transition to myofibroblast; thus, reducing collagen I and collagen III production (Pedram et al. 2010). On a pressure-overload mice model, OVX 17β-E2 down-regulate mRNA and protein levels of collagen I and collagen III by ERα binding to collagen I and III promoter (Dworatzek et al. 2016).

Importantly, E2 also modulates the expression of the main Ca^{2+} handling proteins. It has been observed that E2 increase SERCA2/PLB ratio as well as a reduction in cardiac LTCC expression, these changes will be explained with more detail later in this document (Groban et al. 2019). Moreover, 17β-E2 induce a down-regulation of CAMKII levels. RyR phosphorylation by CAMKII modulates RyR Ca^{2+} release activity among other targets,

which would explain the decreased Ca^{2+} sparks frequency observed in intact female rats with basal E2 levels (Ma et al. 2009; Fares et al. 2012).

17β-E2 binds equally to ERα and ERβ (Regitz-Zagrosek and Kararigas 2016). Typical circulating levels of 17β-E2 fluctuate between 0.36 nM (follicular phase) and 2.8 nM (mid-cycle) depending on the feminine reproductive cycle. In post-menopausal woman, 17β-E2 levels are low (0.14-0.21 nM), which is similar to 17β-E2 levels in men (Mendelsohn 2000; S.C.Yen , Jaffe, and J.W.onsiewicz 1978). There are differences among males and females, which influence rhythmic heart activity. In rat ventricular myocytes, 17β-E2 induce the blockage of L-type I_{Ca} probably mediated by GPER due to the short 17β-E2 exposition (25 minutes)(Korte and Grohe 2006; Philp et al. 2006).

2.6.2 Androgens

Testosterone, along with 5α-dihydrotestosterone, are the principal steroidal androgens circulating in human blood. Androgens act in genomic or non-genomic ways by binding to its androgen receptors (AR). AR might modulate gene transcription by translocating to the nucleus, dimerizing and binding to androgen response element (ARE). About 0.5% of testosterone can be metabolized to estrogen via cytochrome P450 aromatase (Lucas-Herald et al. 2017).

There are marked differences on men and women sex hormone levels. Testosterone levels in women (0.6 - 2.5 nM) are lower than men (10 - 30 nM) levels (Regitz-Zagrosek and Kararigas 2016; Lucas-Herald et al. 2017). Since men have higher testosterone levels and increased CVDs incidence than women, it is assumed that; testosterone contributes to CVDs (N. Li et al. 2019). However, testosterone has beneficial effects on cardiac muscle,

inhibit atheroma and promotes vasodilatation (Worboys et al. 2001). Low testosterone level (<2.6 x10^{-9} M) is correlated with increased risk in CVD in post-menopausal women with an existing cardiac disease (Malkin et al. 2010). Furthermore, it was explored E2 effect in males in a male transgenic model overexpressing aromatase. Transgenic males experienced reduced ischemia reperfusion damage compared to wild type. Given that aromatase is involved in the conversion of testosterone to E2, it is supported the idea that E2 has cardioprotective effects in both male and females (Bell et al. 2014).

2.7 Female sex hormones and cardioprotection

There are differences in Ca^{2+} handling between male and female, that affects force development and relaxation. Given that it is uncommon the study of normal Ca^{2+} signaling in human cells, rodent models are frequently used (Curl, Wendt, and Kotsanas 2001). Female rodents have smaller Ca^{2+} transients and shortening, less SR Ca^{2+} load (upon β-adrenergic stimulation) compared to males (J. Chen et al. 2003).

There is evidence suggesting preservation of myocardial contractility in women compared to men after the age of 50 years (Claessens et al. 2007). Myocardial contractility and expression of PLB and SERCA in a rat model with ovariectomy (OVX) were preserved in the early phase (7, 15 and 30 days after OVX) and reduced at long-term (60 days after OVX). Contractility and expression of Ca^{2+} handling proteins were restored upon E2 replacement (Paigel et al. 2011).

Age contributes to alterations and CVDs development in both sexes, being more marked in male who demonstrated reduced number of ventricular myocytes and fibroblast proliferation, which is proportional to age (Keller and Howlett 2016). This has also been

assessed in rodent models, demonstrating prominent age-associated changes in ECC in ventricular myocytes and a decline in contractility, which is greater in male rodents (the ECC process efficacy decline with age up to ~56 %) (Howlett 2009; Keller and Howlett 2016). Furthermore, fractional shortening and Ca^{2+} transients were smaller in young females compared to young males. With a decline with age of ~7 to ~2% fractional shortening in males and peak Ca^{2+} transient ~48 to ~28 nM (Howlett 2009).

Estrogens have been shown to attenuate catecholamine-induced vasoconstriction and exert a decrease in sympathetic response to mental stress (Ghadri et al. 2018). It was assessed the regulatory role of ovarian sex hormones in a rat model, showing a down regulation of SERCA mRNA levels after OVX. It was also observed a diminished SERCA-mediated SR Ca^{2+} uptake in OVX rats compared to controls (Bupha-Intr and Wattanapermpool 2006).

Furthermore, it was assessed ACE and ACE2 activity in spontaneously hypertensive rats, comparing male rats, gonadectomized male rats, female rats and OVX female rats. Male rats exhibited higher hypertrophy and ACE activity compared to female rats. OVX increased hypertrophy and ACE2 activity as well as decreased SERCA2a/PLB ratio. No change in SERCA2a/PLB ratio was observed in male rats. This implies that feminine sex hormones have a beneficial effect against cardiac hypertrophy (Dalpiaz et al. 2015).

Cardioprotection by sex hormones have been tested in a variety of experimental models. H9c2 cells pre-treatment with 10^{-8} M 17β-E2 inhibited ISO-induced increase in cell size, diminished Ca^{2+} mishandling and cytosolic Ca^{2+} accumulation. E2 has protective

effects against pathological cardiac changes by suppressing PP1 expression and enhancing phosphorylated-PLB levels, which allows better Ca^{2+} handling by the SR (H.Y. Fang et al. 2018).

These cardioprotective effects are related to the changes that have been observed in the expression of Ca^{2+} handling proteins in different models of E2 supplementation (figure 12). Female rats showed an increase in the LTCC subunit α1c during OVX. These changes were markedly reduced with hormonal restitution of 16 μg/kg estradiol benzoate for 2 weeks (Chu et al. 2006). Similar results were obtained with a 60-day release pellet subcutaneously implanted to female rats. The pellet contains 1.5 mg of 17β-E2. This hormone therapy attenuated the increased RyR Ca^{2+} release that was observed during OVX. This, along with reduced LTCC subunit α1c expression during OVX, is a possible explanation of the reduced Ca^{2+} transients observed in female (Kravtsov et al. 2007). SERCA2a protein levels and SERCA2a/PLB ratio have shown a significant decrease with OVX. These differences returned to basal levels with 40 μg/kg 17β-E2 during 6 weeks in female mice and 40 μg/kg 17β-E2 during 8 weeks in female rats, respectively (Turdi et al. 2015; Ren et al. 2003). Moreover, NCX protein levels seem to decrease after OVX, and return to levels compared to intact female rats with 16 μg/kg estradiol benzoate for 2 weeks; contributing to cytosolic Ca^{2+} removal and diminishing pathological effect during Ca^{2+} overload (Chu et al. 2006).

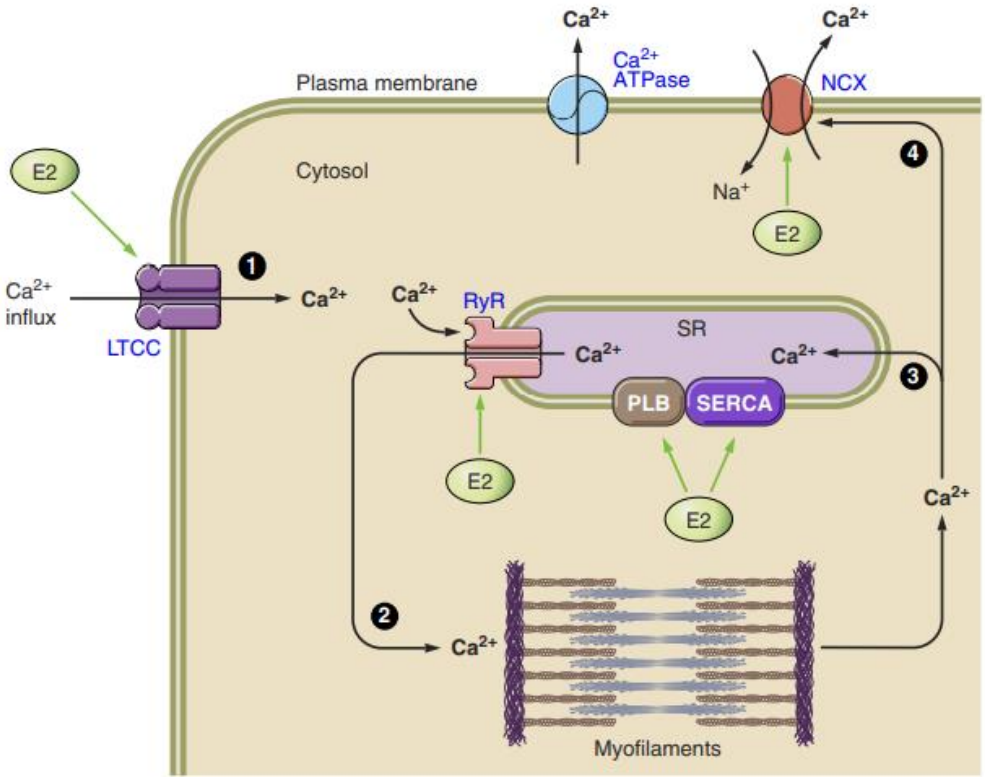

Figure 12. *E2 effect on main Ca^{2+} handling proteins* (Jiao et al. 2020).

Chapter 3- Materials and methods

3.1 H9c2 cells differentiation

H9c2 cell line was obtained from ATCC (Manassas, VA, USA). At day 0, H9c2 cells were plated at low density (35,000 cells/ml in a T-25 cell culture flask for RNA extraction or 25,000 cells/coverslip in 6-well plates for confocal imaging) in Dulbecco′s Modified Eagle′s Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin (100 IU/ml) and streptomycin (100 µg/ml) under standard culture conditions 37 °C, humidified atmosphere of 5% CO_2. Penicillin and streptomycin were previously tempered to avoid cells stress. To seed cells in 6-well plates, volume of cells and medium was adjusted to 300 µl, and placed at the center of the well, only in the coverslip spot. 6-well plate with cells was incubated during 30 minutes, and afterwards added 700 µl of DMEM. Cells from T-25 culture flask and 6-well plate were places in an incubator during 24 hours for attachment. After 24 hours, at day one, a change in the medium was performed, replacing it with DMEM supplemented with 1% FBS previously tempered. For the differentiation process, at day one it was added 1 µM RA daily in dark conditions to avoid degradation. The culture medium was replaced every two days. Before adding RA each day, cell morphology was monitored on an inverted microscope. RA was prepared in dimethyl sulfoxide 1 mM (DMSO) and stored at -20 °C. Differentiated myotubes were characterized at day seven. (Menard et al. 1999; Branco et al. 2015).

3.2 Myotubes hypertrophy and E2 treatment

Five groups were established as shown in figure 13, group one of differentiated myotubes, following the differentiation process previously established. Group two of myotubes treated with 17β-E2 , group three of hypertrophied myotubes, group four of hypertrophied myotubes with 17β-E2 pre-treatment and group five of hypertrophied myotubes with 17β-E2 pre-treatment and ICI -182,780 (ICI) also known as Fulvestrant, an ER down regulator (Robertson 2001). As phenol red has weak estrogenic activity, phenol red-free medium was used in all the groups (Welshons et al. 1988).

Groups two to five will be explained in this section. Once myotubes were differentiated, at day four groups two, four and five were pre-treated with 17β-E2 for 72 hours. A preliminary treatment study was performed to determine the appropriate dose to use between a range of 1 to 100 nM. 17β-E2 was dissolved in ethanol and stored at -20 °C. 0.1% ethanol was applied to the rest of the groups (Watkins, Borthwick, and Arthur 2011). The groups three, four and five at day five and six were added 1 μM Ang II for 48 to obtain hypertrophied myotubes (H.Y. Fang et al. 2018; Murphy et al. 2009). Additionally, group five was treated with a reported dose of 1 μM ICI 182,780 since day three (Robertson 2001).

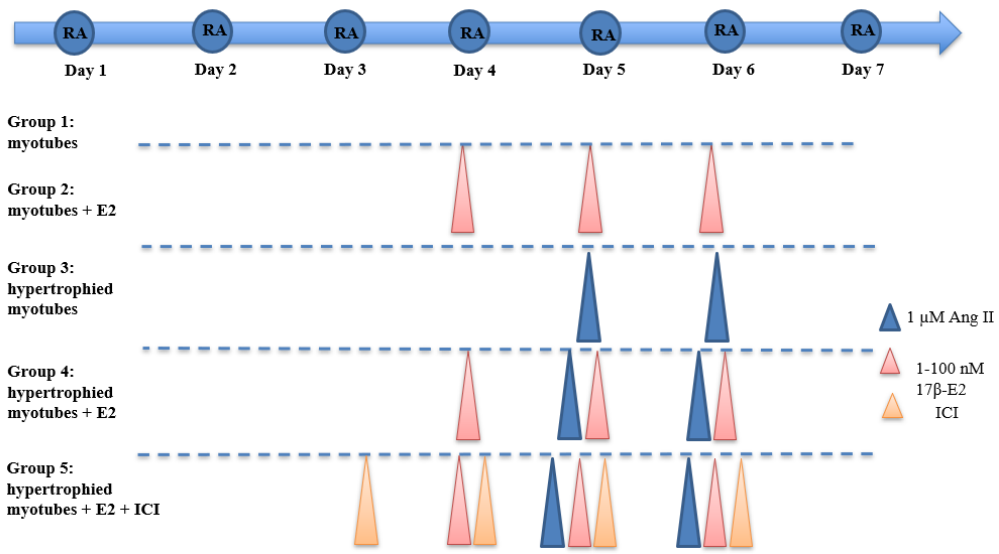

Figure 13. *Myotubes treatments.*

3.3 Confocal microscopy

At day 7, cell area was assessed in all groups, as an index of cell size to quantify hypertrophy. To determine cell area, cells were incubated with 1 μM of calcein AM for 15 minutes. Afterwards, cells were incubated with 1 μM of DRAQ5 TM, immediately thereafter, each coverslip was transferred to the confocal microscope stage (Leica Microsystems, Wetzlar, Germany). An argon laser was used to excite, and emission was collected at 500-530 nm for calcein AM and 690-790 nm for DRAQ5 TM. The image acquisition was performed with 1024 × 1024 pixels, with a frame average of two, and 600 μM pinhole. Images were exported and analysis was performed using ImageJ to determine hypertrophy parameters. Calcein AM helps to identify the boundaries of the cell and determine cell area, and DRAQ5 TM helps to identify nuclei and distinguish individual cells.

3.4 Change in expression of genes by qPCR

Changes in expression of genes were assessed by qPCR. RNA was extracted from all five cell groups using TRIzol (Invitrogen) reagent, if the sample was not processed the same day, RNALater (Invitrogen) was used to preserve samples. Samples with RNALater were stored at 4°C and used next day, and to store for longer than 24 hours, samples were kept at -80°C. RNA integrity was determined in agarose gels, observing 28S and 18S rRNA bands. Purity was also assessed by absorbance at 260/280 nm, which should be >1.8, when no contaminants are present. Samples were adjusted to 1000 ng in 15 μL H_2O, converted to cDNA and qPCR analysis was performed. cDNA and PCR mixes were used to assess expression of RyR2, SERCA2, PLB, NCX, MCU cardiac LCC α1 subunit, IL6, Col1 and BNP were measured. Real time PCR was performed with 40 cycles, followed by data analysis (Rio et al. 2010; Branco et al. 2015).

3.5 Analysis

The data process was performed using Microsoft Excel and GraphPad Prism 8.0. Data was considered statistically significant when $P < 0.05$. GraphPad Prism 8.0 was used to generate graphs to present results. One-way ANOVA with Tukey's multiple comparison test. Data was expressed as means ± SEM for the number of experiments indicated in the legends.

Chapter 4- Results

H9c2 cells were observed in an inverted microscope, noting at day five a complete change towards an elongated morphology, suggesting that myotubes formation was completed. Initially, we evaluated a 14-day differentiation protocol followed in ITESM Cardiovascular Research Group. The protocol was based on previously reported conditions in T-75 flasks, using 35,000 cells/ml in a T-25 cell culture flask (Branco et al. 2015). The results can be observed in figure 14 A-C. We tried to induce differentiation with the same seeding number in 6-well plates, however, the elongated morphology was lost, as observed in figure 14 D-E. Furthermore, in T-25 flasks, cells reached full confluence at day eight, but at day 14 there were zones where the cells began to die in some T-25 flasks leaving blank spaces (figure 14 C), which could possibly affect the final qPCR measurements and makes difficult to compare results in both seeding conditions (T-25 flasks and 6-well plate). Figure 14.

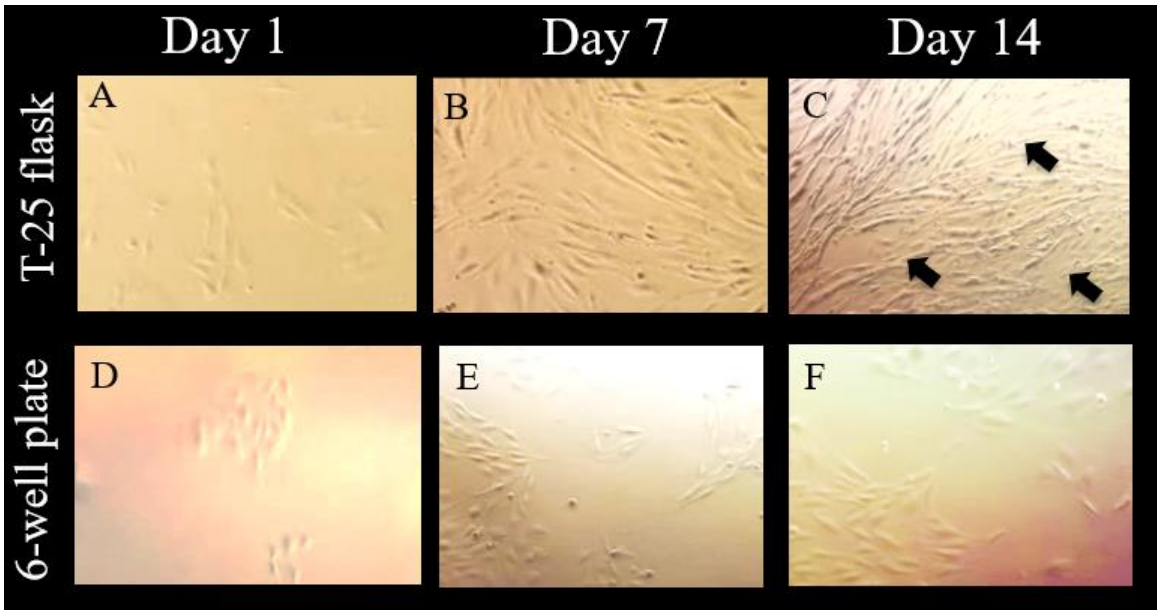

Figure 14. *H9c2 cells 14-day differentiation protocol.*

Several seeding conditions in 6-well plates were tested as observed in figure 15. Increased cell seeding to the whole well did not promotes differentiation, showed in figure 15 A-H. Given that the purpose of using 6-well plates was to grow cells over a coverslip in order to work with them at the confocal microscope, it was tested a condition covering an area in the center of the well, similar to the area occupied by the coverslip. Afterwards it was tested the same conditions with the actual coverslip, obtaining similar results. Ideal conditions on 6-well plates were 25,000 cells per coverslip as observed in figure 15 I-L.

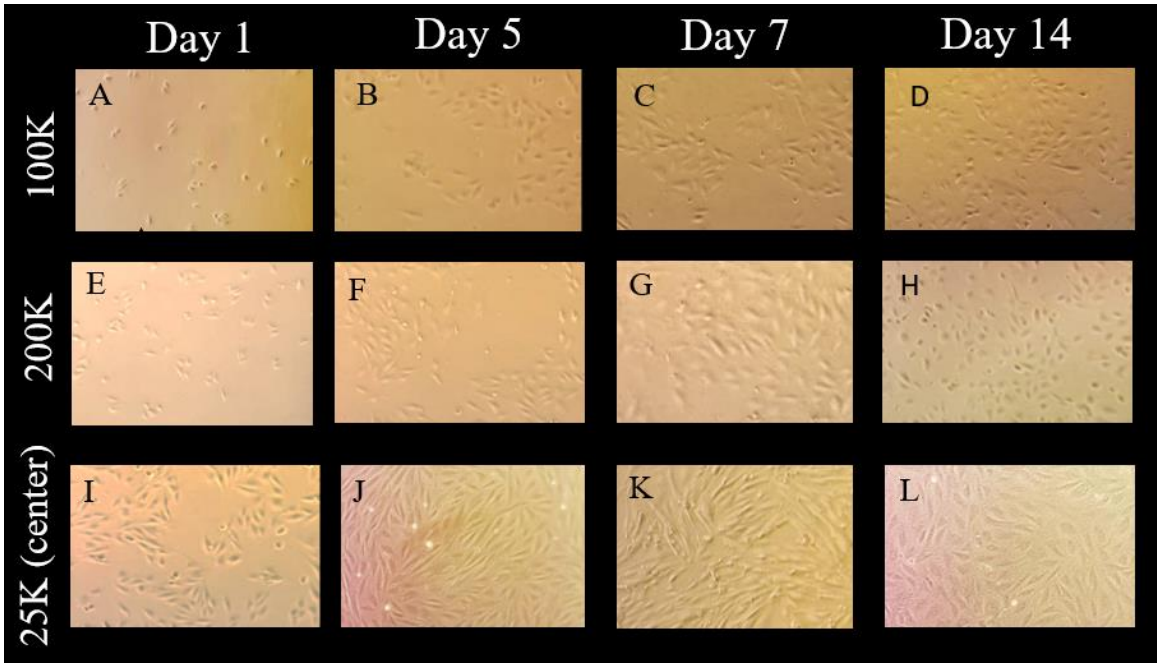

Figure 15. *H9c2 cells differentiation conditions in 6-well plate.*

Pharmacological treatments were performed on H9c2 cells following the differentiation process at day 14. As shown in figure 16 B, upon treatment with 1 μM Ang II, H9c2 cells at day 14 increased cell area by 64% compared to control, and this was significantly reduced when treated with 17β-E2. However, in figure 16 A it is observed

that cell morphology was not in optimal conditions, as cells were not elongated. Therefore, we tested the possibility to reduce the model to fewer days, where morphology of myotubes could be maintained. The time course of changes in morphology of the cells was followed, noting that myotubes formation started at day 5 (figure 17 B) keeping the morphology up to day 7 (figure 17 D). Therefore, it was decided to follow a 7-day differentiation process and assess the pharmacological treatments effect.

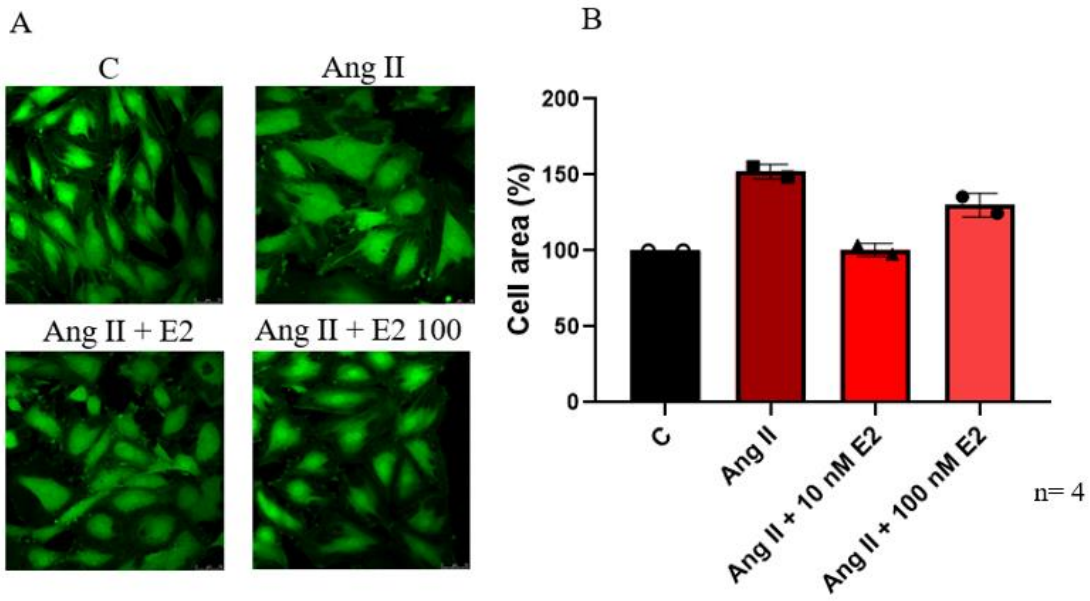

Figure 16. *H9c2 cells at day 14 Ang II hypertrophic effect and 17β-E2 modulation.*

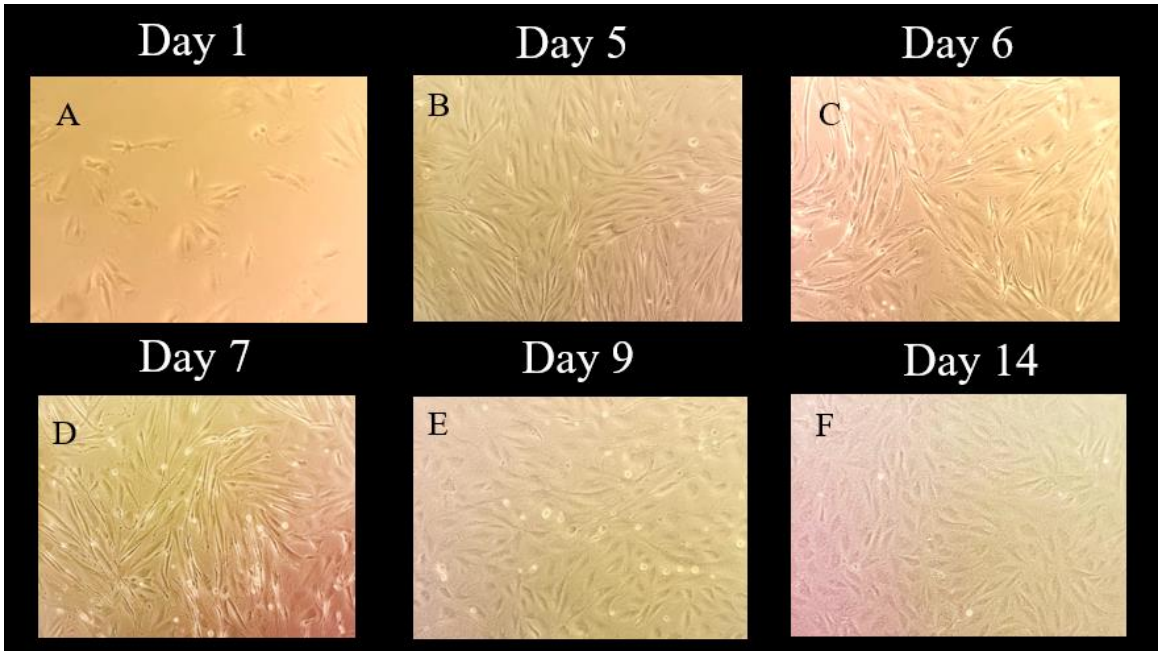

Figure 17. *Cell differentiation conditions in 6-well plates 25,000 cells/ coverslip (center).*

Treatments were given to the cells as previously described. Differentiated cells preserved the elongated morphology with the pharmacological treatments, as observed in figure 18. Confocal microscope assessment was used to determine cell area changes.

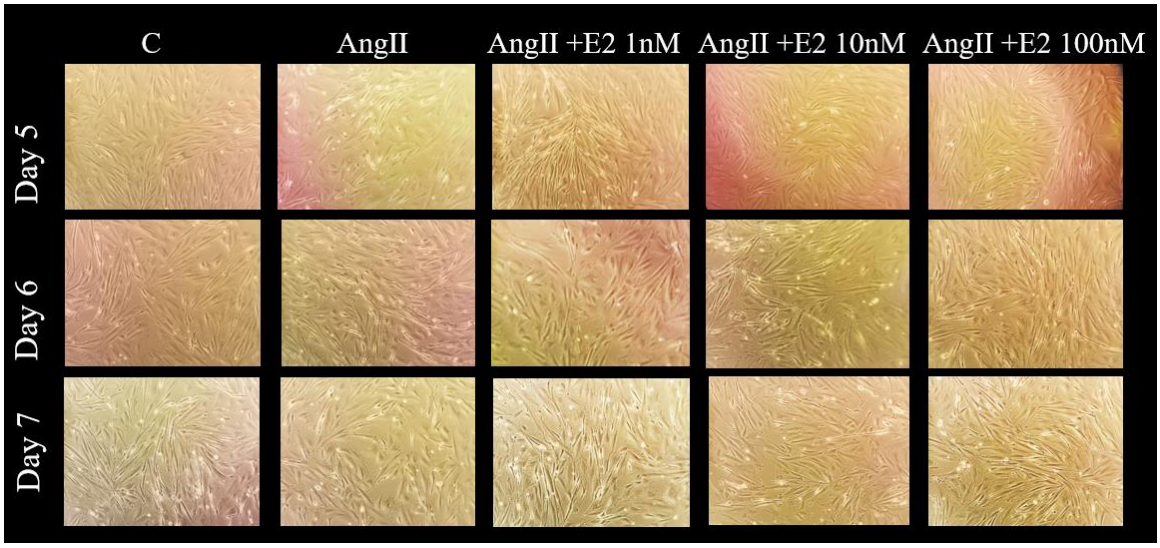

Figure 18. *Cell differentiation and treatments in 6-well plates 25,000 cells/ coverslip (center).*

The selected 7-day differentiation process and treatments were assessed. Differentiated H9c2 cells at day 7 formed elongated and multinucleated myotubes (figure 19 A), it is clearly observed the increased cell area in Ang II group (figure 19 B), which is reduced with 10 nM and 100 nM 17β-E2 (figure 19 D-E). Data analysis in randomly selected cells showed that, upon 1 μM Ang II treatment, there was a ~54% cell area increase compared to control (figure 20). However, this increase was diminished when cells were treated with 17β-E2 in a dose dependent manner. Moreover, 17β-E2 alone did not change cell area of myotubes (figure 19 F). In figure 19 G it can be appreciated that treatment with ER inhibitor abolishes the protective effect of E2 pretreatment.

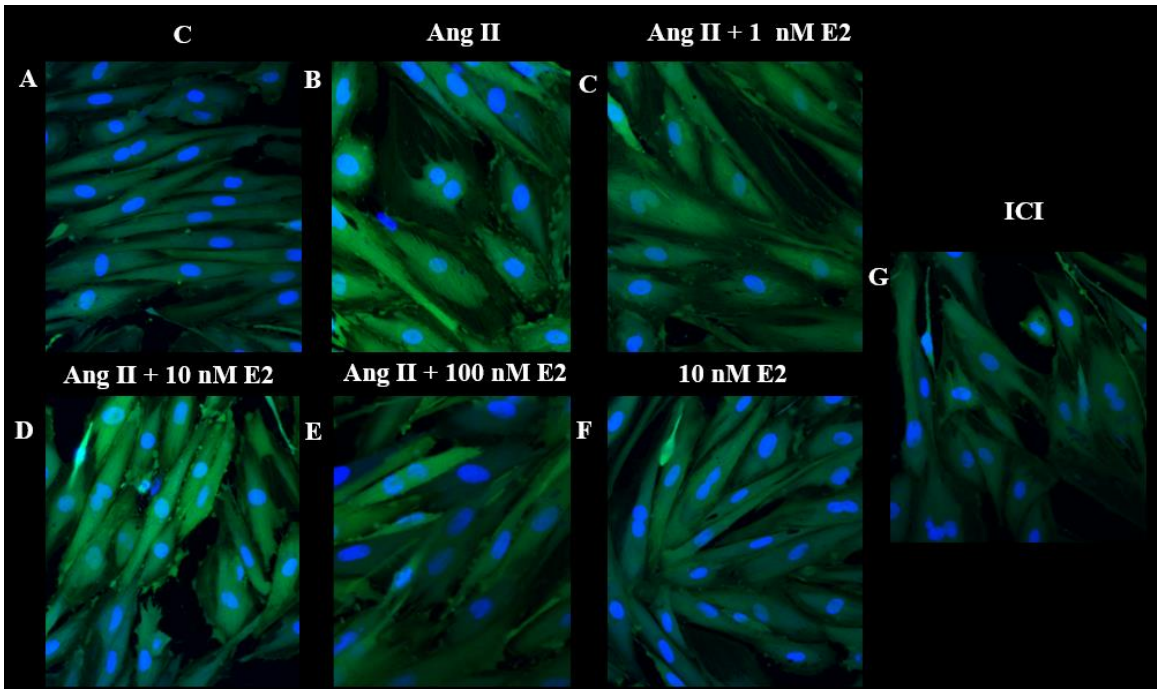

Figure 19. *Ang II and* 17β-E2 treatment effect in H9c2 myotubes.

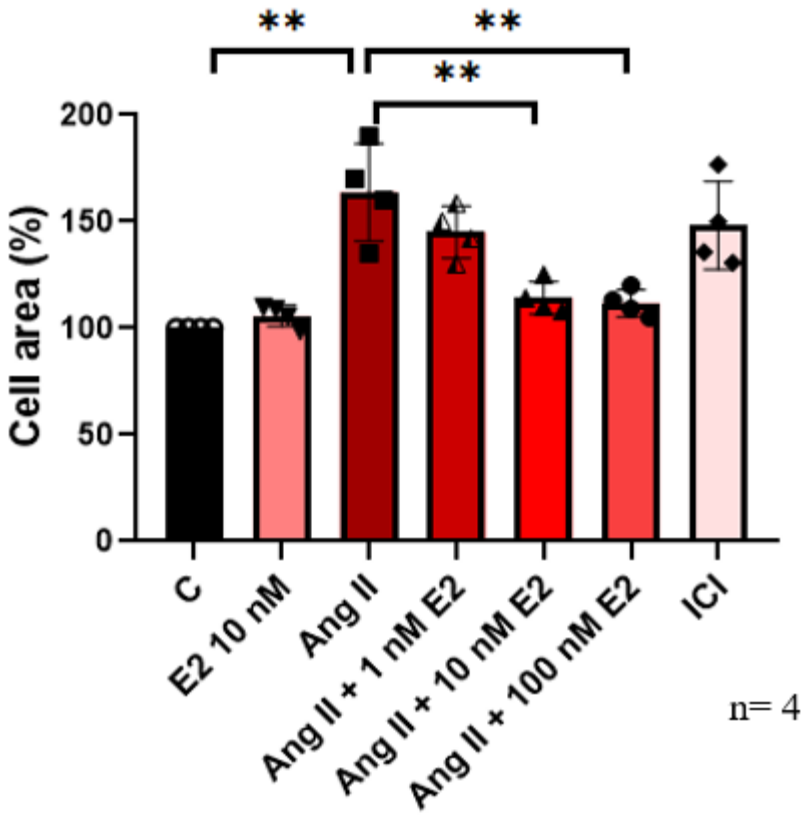

Figure 20. *Cell area changes with Ang II and 17β-E2 treatments in H9c2 myotubes.*

All cells assessed had an elongated morphology and were randomly selected, however, the number of cell nuclei varied; >20% of cells were multinucleated (2-4 nuclei) in all groups. Figure 21 A-G. In Table 1, it is described the different groups characteristics in cell area and nuclei number.

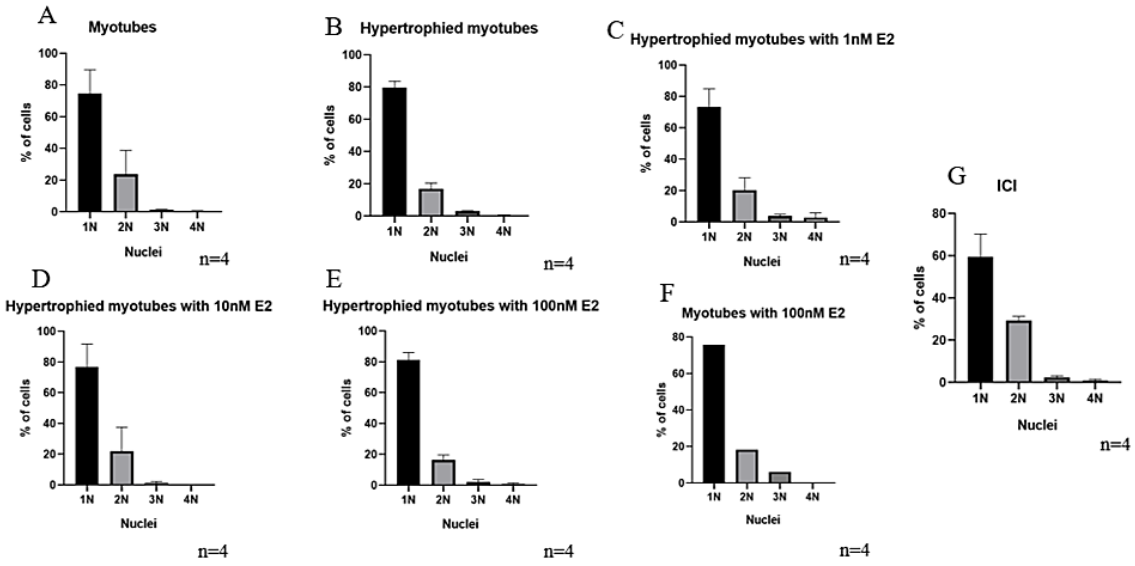

Figure 21. *Nuclei number between groups.*

Group	C	Ang II 1 µM	Ang II+1nME2	Ang II+10nME2	Ang II+100nME2	10nME2	ICI
Cell area (µm)	4958±303	8165±333	7627±258	5506±336	5314±279	5511±246	8245±263
≥2 nuclei (%)	26±11	26±10	31±4	23±9	20±3	33±5	33±3

Table 1. *Myotubes cell area and nuclei numbers.*

It was not observed a significant change in the expression of most genes of Ca^{2+} handling proteins assessed (figure 22 and figure 23). The exception was PLB and LTCC subunit α1c, as shown in figure 22 B and E. It is observed in figure 22 E an increase in LTCC subunit α1c in cells pre-treated with E2 compared to Ang II group. PLB gene expression was significantly increased in Ang II group compared to control. However, it did not decrease with E2 treatment. Importantly, a reduction in SERCA2a/PLB ratio is observed in figure 22 C upon Ang II treatment. RyR2 and NCX gene expression had no change compared to control (figure 22 D, F, and figure 23 D, F). There was no change in E2 group compared to control. It is observed in figure 23 A-E that ICI-182,780 tended to

increase PLB and RyR2 mRNA levels compared to control. However, this is expected as in this group it is abolished the E2 protection.

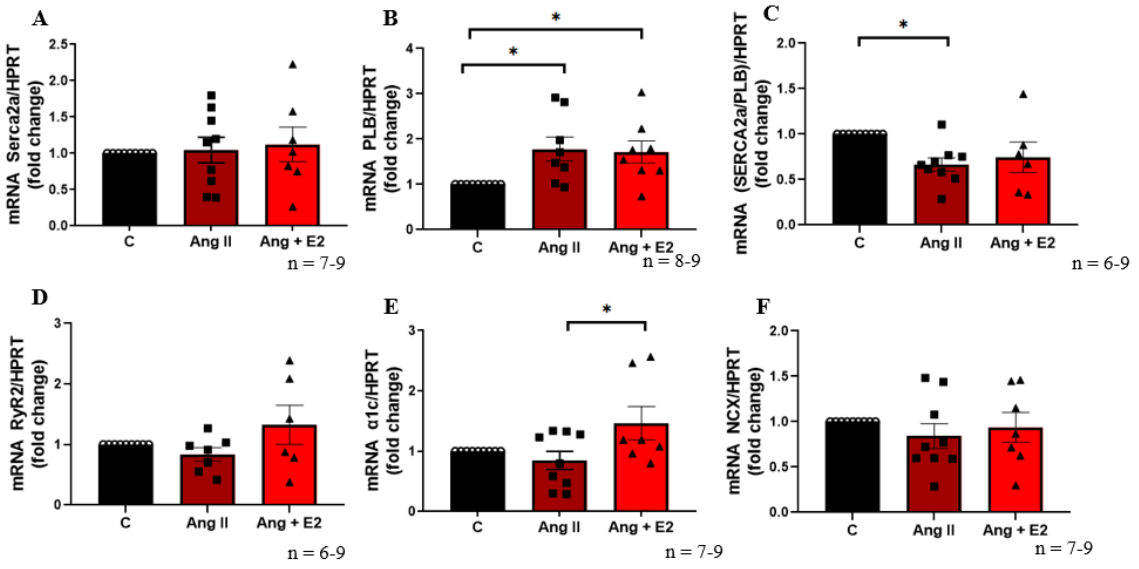

Figure 22. *Ca^{2+} handling proteins gene expression.*

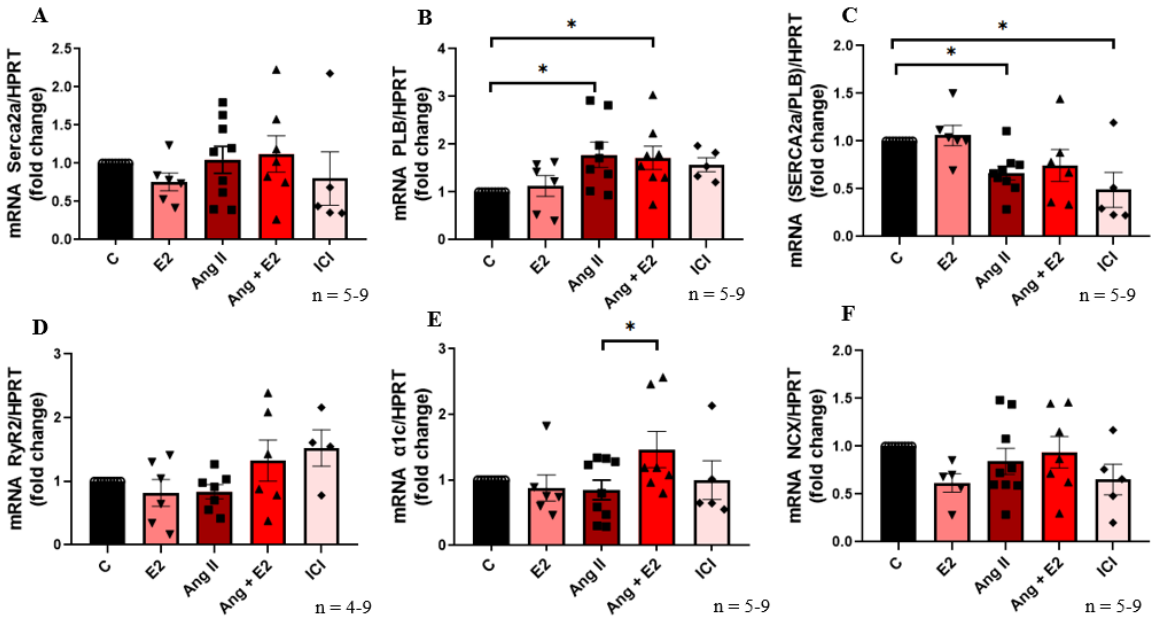

Figure 23. *Ca^{2+} handling proteins gene expression groups one to five.*

In figure 24 it is observed that Ang II treatment significatively increased pathological remodeling markers gene expression. It is evident a 2.1-fold change increase in figure 24 A, with reduced levels with E2 pre-treatment. In figure 24 C it is observed a 1.4-fold change increase in Il6 levels, and a tendency to increase levels with ICI-182,780 treatment is observed in figure 25 C. There is no significant difference in Col1 observed in figure 24 C. However, Col1 mRNA levels tend to increase with Ang II (Figure 24 C).

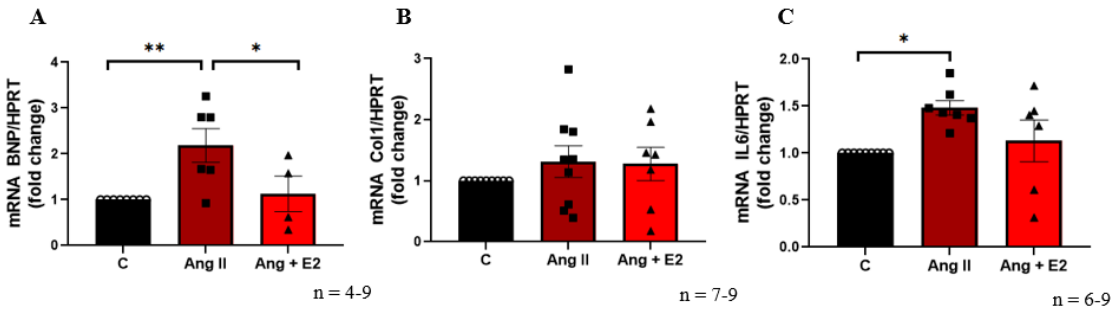

Figure 24. *Pathological remodeling markers gene expression.*

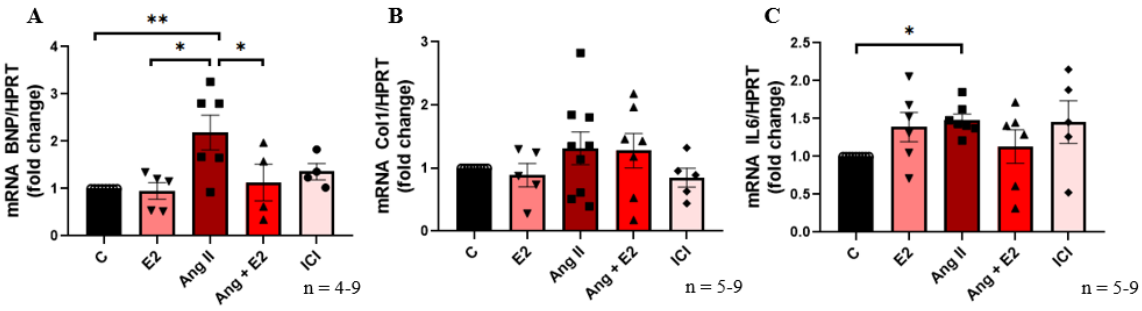

Figure 25. *Pathological remodeling markers gene expression groups one to five.*

Gene expression of proteins that regulate mitochondrial Ca^{2+} homeostasis, Micu 1 and MCU, had not significant change in Ang II group compared to control, as observed in

figure 26. When determining Micu1/MCU ratio, there is no changes observed in figure 26 and figure 27.

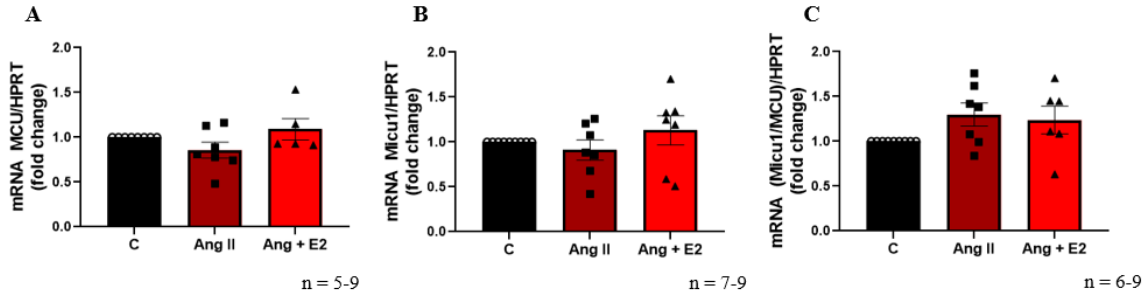

Figure 26. *Mitochondrial Ca^{2+} homeostasis proteins gene expression.*

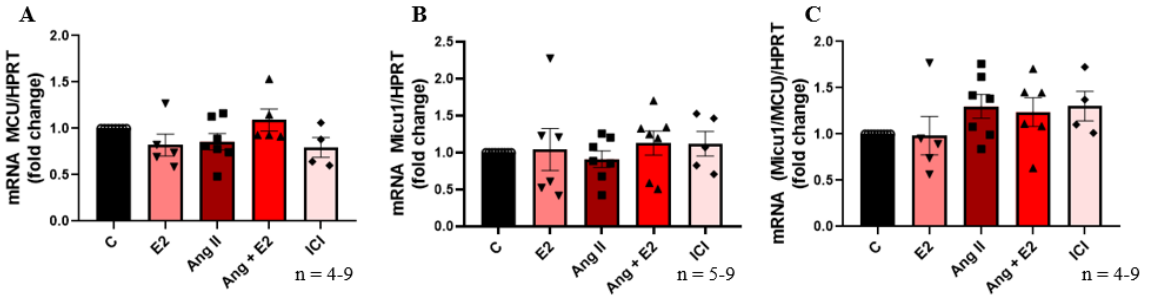

Figure 27. *Mitochondrial Ca^{2+} homeostasis proteins gene expression groups one to five.*

Chapter 5- Analysis and discussion

In the past decades, it has been explored the H9c2 myotubes differentiation process to obtain an *in vitro* model which more closely resembles the cardiac phenotype. In this study, we started assessing the differentiation protocol of 14 days, as followed in ITESM Cardiovascular Research Group for larger containers (T-25 flasks). However, best results were obtained when the differentiation process was reduced to 7 days, when plated in 6-well plates. This time frame coincides with previous reports (seeding 35,000 cells/ml), who followed the differentiation process of H9c2 cells from 5 to 7 days (Menard et al. 1999; Branco et al. 2015; Pereira et al. 2011; Szalai et al. 2000).

H9c2 cells were able to differentiate in T-25 flasks, but not in 6-well plates. This might be related to the fact that cell-to-cell interaction contributes to the differentiation process. On a study using mesenchymal stem cells (MSCs) it was demonstrated that MSCs co-cultured with cardiomyocytes the contact and soluble factors promote the differentiation to a cardiac type (Wang et al. 2006). The microenvironment of the cells determines the differentiation, being stimulated in cell clusters compared to isolated cells (Mao, Shin, and Mooney 2016). In H9c2 cells it has been observed a moderate increase in ROS signaling in 6-day differentiated cells, which can be mediator of growth arrest by activating PKC- δ signaling pathway (di Giacomo et al. 2010). Furthermore, it has been observed a linear relationship between cells differentiation and cell contact, that allows them to communicate. Exist evidence that supports gap junctions formation, given that AGA (18α-glycyrrhetinic acid, gap junctions formation inhibitor) reduce extent of differentiation in bone marrow stromal cells from neonatal rats (Tang, Peng, and Ding 2010).

With the initially reported conditions, cell differentiation process was more favorable in T-25 flasks compared to 6-well plates. This might be related to the different conditions in gas exchange between both conditions. It has been demonstrated differences in dissolved oxygen concentrations in T-25 flasks, 24-well plate, and 35 mm dish, being in T-25 flasks at 5.8 % O_2 more variations in oxygen concentrations (Zhang et al. 2016). Other reports also conclude differences among types of culture vessels used. It was found an increase in oxygen transfer rates in microliter plates with increased well size (Running and Bansal 2016). Despite that the evidence is not sufficient to clearly conclude why differences between T-25 flasks and 6-well plates was observed, it is evident that the differences in cell culture vessels might contribute.

H9c2 derived myotubes were found to have an elongated morphology and polynucleated, with 2-4 nuclei >20% in all cell groups. This coincides with previous reports, describing small (2-5 nuclei) and large (>5 nuclei) myotubes population (Menard et al. 1999).

Our 7-day myotube model was able to generate hypertrophy at 48 hr after Ang II exposure, increasing cell area by ~63% compared to control. This result is in accordance with other reports in H9c2 cells, that showed increased cell area upon 48 hr Ang II treatment (Watkins, Borthwick, and Arthur 2011; An et al. 2020; A et al. 2018; Lu and Yang 2009). The Ang II dose ranged between 100 nM to 1 μM in previous reports, however, all experimental protocols reported similar cell area increase, approximately duplicating the initial cell area. Furthermore, it has been demonstrated that cell viability is maintained with 1 μM Ang II treatment during 24 and 48 hours(Hernández et al. 2014).

17β-E2 effect in hypertrophy was observed in a dose dependent manner. Three doses were tested, 1 nM, 10 nM, and 100 nM of 17β-E2, being 10 and 100 nM able to attenuate cell area increase. Physiological 17β-E2 circulating levels in females is about 1.5 nM, this concentration demonstrated cardio protection in animal models and isolated cardiomyocytes (Turdi et al. 2015; Paigel et al. 2011; Kravtsov et al. 2007; Machuki et al. 2019). However, in cell lines 10 nM and 100 nM of 17β-E2 have been used. In H9c2 cells, 100 nM 17β-E2 were reported as the best condition to attenuate cell increase area upon β-adrenergic stimulation (H.Y. Fang et al. 2018). This might be related to differences in the microenvironment of immortalized cell lines compared to animal model. Cell culture flasks does not mimic the complexity of living beings microenvironment, such as cell interaction with different cell types, membrane receptor expression and cytoskeleton interaction with extracellular matrix (Rijal and Li 2018).

It is interesting to observe that an ER inhibitor abolishes the anti-hypertrophic effect of E2. This has been previously reported in H9c2 cells β-adrenergic agonist-induced hypertrophy (H.Y. Fang et al. 2018). ICI- 182,780 is an ERα and ERβ antagonist, so it can be said that 17β-E2 has an antihypertrophic effect in the genomic signaling pathway. However, since it also interacts with GPER, further information is needed to determine the relative antihypertrophic effect contribution of a specific E2 signaling pathway (Muler et al. 2020).

About the gene expression of Ca^{2+} handling proteins, it was observed a decrease in the SERCA2a/PLB ratio with Ang II treatment as expected. Although no significant difference was found in SERCA2a, the increase in PLB mRNA levels increase with Ang II indicated a possible reduced SERCA2a activity. It has been reported a SERCA2a

down-regulation in failing cardiomyocytes (Lipskaia et al. 2014; H. Chen, Liu, et al. 2018). However, SERCA unchanged expression is not necessarily an indicator of unchanged activity. Even with SERCA2a unchanged mRNA and protein levels, a decrease activity was reported in failing human hearts and cardiomyopathy animal model (Schwinger et al. 1995; Linck et al. 1996; Willis et al. 2015). A possible explanation for this result is the post-translational modifications of SERCA2a (Lancel et al. 2010). Nevertheless, it is needed to measure SERCA2a activity and protein levels to strengthen a conclusion.

For RyR2 gene expression, there is not a solid evidence about its changes during cardiac hypertrophy. What is most reported is RyR2 oxidation and increased Ca^{2+} sparks in failing cardiomyocytes (Lou, Janardhan, and Efimov 2012). A reduction in RyR2 with E2 treatment have been previously reported, however this result is not consistent among different experimental protocols. Decreased levels of RyR2 have been reported, which might contribute to attenuate Ca^{2+} overload damage (Machuki et al. 2019; Chu et al. 2006).

NCX expression did not change between groups. It is reported a NCX upregulation in animal models with failing hearts which impairs Ca^{2+} cycle and contractility in cardiac cells (Hasenfuss and Pieske 2002; Münch et al. 2006). In failing hearts, it has been reported a decrease in LTCC activity, which contributes to CIRC process alteration and impaired contractility (Goonasekera et al. 2012; Machuki et al. 2019). Interestingly, it is observed an increase in LTCC subunit α1c in cells pre-treated with E2 compared to Ang II group. Reports about α1c expression are not homogeneous.

However, in cellular models, similar results have been observed in synergy with PI3K/AKT pathway activation (Yang et al. 2018).

It is commonly reported an increase in SERCA2a/PLB ratio in E2 treated models (Paigel et al. 2011; Ren et al. 2003). However, although a tendency to decrease was observed, this was not statistically significant, as observed in figure 22 C. As mentioned before, a possible explanation for these differences between what is already reported, and our results is the possible protein modifications, heterogenicity in cell changes, or heterogenicity in aliquots of different treatments. Further investigation is needed to determine if E2 has cardioprotective effect through SERCA2a modulation.

As expected, pathological remodeling markers, BNP and IL6 increased its gene expression, and Col1 tends to increase as seen in figure 24 A-C. BNP mRNA and protein levels increase during cardiac hypertrophy upon Ang II treatment. In a study of Ang II induced hypertrophy in H9c2 cells it was reported a ~33% increase in BNP protein expression (A et al. 2018). Inflammation is also characteristic in failing and hypertrophic hearts. It has been observed increased IL6 expression and activity in hypertrophic H9c2 cells (Akhondzadeh et al. 2020). Our results did not report Col1 increase with Ang II treatment compared to control, contrary to an increase already reported. However, we only measured mRNA levels, therefore, it would necessary this study to measure protein levels to strengthen our conclusions (Ji et al. 2018).

It was explored changes in the gene expression of MCU and Micu1, which are essential for mitochondrial Ca^{2+} homeostasis. We found no change in the Micu1/MCU ratio in the E2 treated group compared to Ang II group (figure 26 and figure 27). Further investigation is needed to determine mitochondrial Ca^{2+} homeostasis contribution to

hypertrophy in this cell model. It is reported that an increase in Micu1/MCU ratio might contribute to cardiac damage tolerance (Chapoy-Villanueva et al. 2019). This change can contribute to improve mitochondrial Ca^{2+} homeostasis and energy metabolism, which is potentially beneficial for an adequate ATP supply for SERCA2a activity (Lipskaia et al. 2010).

Chapter 6- Conclusions and future perspectives

The aims of this thesis work were to implement an *in vitro* model of hypertrophy that resembles the phenotype of cardiac myocytes and to assess the 17β-E2 role in hypertrophy and explore the relation with the expression of the genes for Ca^{2+} handling proteins and of pathological remodeling markers. With the information presented in this document, we can conclude that H9c2 cells differentiated to myotubes is a useful model to study hypertrophy. This 7-day model results in small myotubes population, which is an important tool to obtain cell phenotype closer to that of cardiac cells in culture, without the challenges implied in the use of animal models. Furthermore, the adequate cell culture conditions were found, being 35,000 cells/ml in T-25 flasks for RNA or protein quantification, and 25,000 cells/coverslip during 7 days. These conditions will allow to explore other functional experiments such as Ca^{2+} dynamics experiments.

It can also be concluded that the model proposed is valid to assess pathological cardiac remodeling, as the increase in BNP and IL6 with Ang II treatment validates that the increase in cell area observed is linked to a pathological condition. Moreover, with the changes in SERCA2a/PLB ratio it can be concluded that Ca^{2+} homeostasis should importantly be taken into account during cardiac hypertrophy and E2 can directly modulate gene expression of Ca^{2+} handling proteins in this model.

17β-E2 pre-treatment to hypertrophied myotubes reduce cell area in a dose-dependent way. This evidence strengthens the idea of E2 potential cardioprotective role. With our results, it cannot be certainly concluded whether the cardioprotective effect of E2 is only mediated through gene expression changes in Ca^{2+} handling proteins and

exclude other mechanisms. However, it can be concluded that the genomic signaling pathway of E2 has an important role in E2 cardio protection.

To strengthen the conclusions presented in this work, it is necessary to measure Ca^{2+} handling protein content, as well as functional parameters of cytosolic Ca^{2+} dynamics, mitochondrial Ca^{2+} signaling and metabolism, and protein post translational modifications such as oxidation and phosphorylation of Ca^{2+} handling proteins.

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Graduate

- Master in Science (Biomedical Sciences)- Escuela de Medicina del Tecnológico de Monterrey, Nuevo León, México (2019- to date)

College

- Nutrition – Facultad de Salud Pública y Nutrición, UANL, Nuevo León, Mexico (2013- 2018)
- Accumulated grade throughout college- 96

High School

- Centro de Investigación y Desarrollo de Educación Bilingüe (CIDEB)– Nuevo León, México (2011-2013) Final grade- 94
- Bilingual High School Modality

Conference Presentations

- Poster Presentation in session “51 Congreso de Investigación y Desarrollo” with poster “Effect of estradiol on Ca^{2+} dynamics in hypertrophied H9c2-derived myotubes”
    - February 2021

Non-CONACYT awards

- Permanencia de 3 años en el "Programa Institucional Desarrollo de Talentos Universitarios, nivel Licenciatura”, 2018
- Premio Ceneval al Desempeño de Excelencia-EGEL, 2018

Languages

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