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The Role of Reactive Oxygen Species in *In Vitro* Cardiac Maturation

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Abstract

Recent advances in developmental biology and biomedical engineering have significantly improved the efficiency and purity of cardiomyocytes (CMs) generated from pluripotent stem cells (PSCs). Regardless of the protocol used to derive CMs, these cells exhibit hallmarks of functional immaturity. In this Opinion, we focus on reactive oxygen species (ROS), signaling molecules that can potentially modulate cardiac maturation. We outline how ROS impacts nearly every aspect associated with cardiac maturation, including contractility, calcium handling, metabolism, and hypertrophy. Though the precise role of ROS in cardiac maturation has yet to be elucidated, ROS may provide a valuable perspective for understanding the molecular mechanisms for cardiac maturation under various conditions.

Unveiling the Molecular Mechanisms of Cardiac Maturation

Pluripotent stem cell-derived cardiomyocytes (PSC-CMs) are structurally and functionally immature, resembling fetal CMs [1]. Therefore, PSC-CMs cannot adequately recapitulate adult heart physiology, stalling their application in regenerative medicine and disease modeling. Numerous methods have been developed to promote cardiac maturation with varying degrees of success. These approaches range widely from biochemical strategies [2–4], such as triiodothyronine treatment (culturing PSC-CMs in a media containing thyroid hormone), miRNA overexpression, and metabolic modulation, to physical strategies [5–8], including mechanical stress, substrate patterning, and electrical pacing. Though these studies have resulted in substantial improvements in PSC-CM maturity, few studies have been dedicated to a thorough investigation of the underlying maturation mechanisms. Knowledge of these underlying maturation mechanisms is crucial for predicting potential synergy or antagonization between methods developed for PSC-CM maturation.

ROS 2013 byproducts of the metabolism of oxygen such as O_2^- , H_2O_2 , and radical $OH^\bullet$ – are extensively involved in cellular signaling, often by directly augmenting or suppressing protein activity [9]. More specifically to cardiac maturation, ROS can be generated in response to extracellular signals, such as biochemical signals (e.g., growth factor stimulation) or physical signals (e.g., mechanical stretching); [5]. ROS can also facilitate the

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transduction of these signals [10]. In other words, ROS may be a critical modulator of cardiac maturation, irrespective of the maturation approach. Indeed, recent studies suggest that ROS may promote or repress cardiac maturation, depending on the stage of development [11,12]. Furthermore, redox-sensitive signaling pathways, such as NF- κ B, have also been shown to regulate the maturation of CMs [13]. Though the precise role of ROS in cardiac maturation has yet to be further elucidated, we hypothesize that ROS may underpin the molecular mechanisms that govern cardiac maturation.

ROS in Cell Signaling and Homeostasis

ROS is a molecular byproduct of cellular metabolism. Examples of ROS include: superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^\bullet$). Cellular ROS mostly originate from O_2^- , which is produced by **NADPH oxidases** (see Glossary) or from the electron leak that occurs in the electron transport chain in the mitochondria [14]; (Figure 1). O_2^- is rapidly converted by superoxide dismutase, either in the cytosol or in the mitochondria, into H_2O_2 . Historically, excessive production of ROS, or oxidative stress, has been associated with disease pathogenesis, including the onset and progression of cardiovascular disease [15], neurodegenerative disease [16], and cancer [17]. For example, in the presence of ferrous ions, H_2O_2 can undergo a Fenton reaction and generate highly reactive and toxic hydroxyl radicals, leading to nonspecific oxidation of lipids, proteins, and nucleic acids. However, when ROS concentrations remain at physiological levels, these highly reactive small molecules are indispensable in maintaining cell signaling and homeostasis [9].

H_2O_2 can regulate cell signaling pathways by oxidizing cysteine residues near their functional motifs [9]. At physiological pH, the thiol groups in cysteine residues are presented as highly susceptible thiolate anions; oxidation of these anions by H_2O_2 into sulfenic forms can modify the activity and function of the proteins [18]. For example, upon stimulation with platelet-derived and epidermal growth factors, NADPH oxidase 4 (NOX4) promotes the generation of O_2^- , which in turn is converted into H_2O_2 . The increased production of H_2O_2 leads to the oxidation and inactivation of tyrosine phosphatase, a negative regulator of the cytokine receptor signaling pathways [9].

Conversely, H_2O_2 is also capable of modulating cell signaling by directly activating protein tyrosine kinases, such as cellular SRC kinase [19]. It should be noted that the previously mentioned oxidations, from thiolate anion to its sulfenic form, are often reversible by thioredoxin reductase and glutaredoxin [20]. However, further oxidation of the thiolate into sulfinic or sulfonic forms is generally irreversible *in vivo*, leading to permanent changes in protein function and activity [18]. To counter this tendency towards oxidation, cells are equipped with specific antioxidants such as catalase, peroxiredoxins, and glutathione peroxidases to prevent potentially damaging overoxidation by H_2O_2 . Downregulation of catalase gene expression via the Hippo pathway activation leads to oxidative stress and induces cardiac cell death [21]. Thus, ROS regulate myocyte differentiation by modulating growth factor signaling pathways.

The role of O_2^- in cell signaling has also been extensively studied. However, since O_2^- is highly reactive and has a short half-life (~5 s), direct quantitative measurement of this free radical can present additional challenges [22].

Another important consideration is the compartmentalization and temporal profiles of physiological ROS. Though the accurate half-life for each ROS under its physiological condition is difficult to determine, the consensus is that these free radicals are generally very short-lived, with lifetimes ranging from several microseconds to several minutes [23–26]. These short half-lives limit ROS's diffusion distance and thus makes it difficult for these small molecules to act at a distance. Also, the negative charge of O_2^- prevents their crossing through cellular membranes (e.g., mitochondrial membrane), limiting the mitochondrial O_2^- influence on signaling events in the cytosol. Although membranes are not permeable to superoxide free radicals, H_2O_2 can diffuse through the mitochondrial and cell membranes. Additionally, during myocyte differentiation, the inner **mitochondrial permeability transition pore (mPTP)** is open, which allows for O_2^- diffusion into the cytoplasm [27]. Consequently, the subcellular location of ROS must be regulated to realize specific targeting. For example, cells achieve directed migration by concentrating NOX4, the primary source of ROS, into the leading edge microdomains (e.g., lipid rafts), allowing for localized ROS signaling [28]. Indeed, NOX4 is often found near relevant receptors and signaling molecules rather than randomly dispersed in the cytoplasm [28]. Similarly, the spatial mapping of O_2^- may determine its effect on cell fate. For example, mitochondrial O_2^- mediates glutamate-induced oxidative stress and apoptosis in cardiac cells [29], whereas cytosolic O_2^- has minimal impact on this process [30].

ROS also acts as a mediator for stem cell differentiation through redox signaling pathways [31, 32]. Scavenging ROS by using antioxidants during stem cell culture or by knocking down NOX4 expression inhibits cardiac differentiation in embryonic stem cells (ESCs). Adding free radicals such as H_2O_2 to the PSCs culture medium or forcing the expression of NOX4 leads to cardiac differentiation [33–36]. It has been shown that removal of antioxidants from mouse ESCs (mESCs) differentiation medium elevated ROS levels and reduced cell growth while increasing cardiac differentiation efficiency from 15% to 40%. Furthermore, increased ROS resulted in the significantly increased expression of cardiac progenitor and sarcomeric genes (*NKX2.5* by 100%; *ISL1* by 100%; *MLC2A* by 300%; *MLC2V* by 600%; *TNNI3* by 500%; *MYH6* by 100%; *MYH7* by 150%) and also altered the expression ratios of sarcomeric isoforms [37]. In summary, ROS-mediated cell signaling is vital in regulating basic cellular functions and is a significant determinant in specifying cell fate.

Current Challenges in PSC-CM Generation, Purification, and Maturation

Differentiation and Purification

Cardiac differentiation from PSCs has significantly improved in the past two decades. Early reports utilized high serum concentrations (20% fetal calf serum, [38]) coupled with the hanging drop method, which generates spherical aggregates termed **embryoid bodies (EBs)**. This methodology typically leads to low-efficiency CM generation (<10%, [39]) and

inconsistency due to the batch-to-batch variations in the serum and the resulting geometry of the EBs. Later protocols have employed a monolayer differentiation method that relies on the temporal modulation of the **WNT pathway** with small molecules; this protocol typically generates very pure populations of CMs, up to 98% [40].

These differentiation protocols generate contracting CMs in less than two weeks [40,41]. In contrast, human heart development, from the formation of the primitive heart tube in the embryo to postnatal maturation, occurs over significantly longer timeframes (months to years). Indeed, when compared with their *in vivo* counterparts, human PSC-derived CMs more closely resemble early embryonic CMs [42]. This immaturity limits the possible applications of PSC-CM for cardiovascular disease modeling and drug screening; these technologies, when mature, are expected to mimic or predict responses in the adult myocardium [43]. Ventricular CMs are the most dominant subtype (compared with atrial CMs or pacemaker cells), and also the primary cell type that is lost during myocardial infarction; the CMs discussed in this and the next sections, therefore, refer exclusively to ventricular CMs.

Maturation

Generally, the phenotype changes associated with maturation allow CMs to efficiently generate a stronger contraction force in a rhythmic and synchronized fashion. Morphologically, mature CMs are significantly larger, more elongated, and display well-organized sarcomere structures [44], providing the physical foundation to more powerful contractions. As the heart develops after birth, CMs in both rodents and humans undergo a 30-fold to 40-fold increase in size. Also, it has been reported that adult CMs in collagen constructs have 550 times higher contraction power compared with human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs); [1]. The well-developed **sarcoplasmic reticulum (SR)** and transverse tubules (T-tubules) present in mature CMs facilitate **calcium-induced calcium release (CICR)** from the SR, a process necessary for rapid and synchronized beating, whereas in immature CMs lacking T-tubules, calcium ions rely on passive diffusion through the cell membrane, a much slower process [42,44]. To support the large energetic demands associated with increasing workload, mature CMs are equipped with extensive mitochondria network, occupying 25%–33% of their cytosol [45], whereas immature CMs contain a fewer number of mitochondria, occupying less than 3% [46,47]. The detailed functional and structural differences between mature and immature CMs have been well summarized in previous reviews [1,42,48].

Irrespective of the differentiation protocol, generated hPSC-CMs share the characteristics described earlier and are thereby classified as immature. Numerous efforts have been made to increase the maturation of hPSC-CMs to recapitulate adult heart physiology. The employed methods included: (i) mechanical stimulation, such as passive stretching of the cells [49] and dynamic culture on shakers [7], and (ii) electrical stimulation with biowires [4] or, more recently, the application of a high-frequency electrical field [8]. Additionally, (iii) biochemical stimulation, such as **insulin-like growth factor-1 (IGF1)**, neuregulin-1 β [50], and Tri-iodo-L-thyronine 3 (T3); [51], and (iv) metabolic modulation, such as depletion of glucose [52] or use of galactose and **fatty acids (FAs)**-containing medium [2,6] have been

added to cardiac maintenance medium to encourage further maturation. Despite these notable advances, improvements in hPSC-CM maturity have remained mostly relative and partial. Currently, there is still no method that is capable of maturing hPSC-CMs to a state indistinguishable from adult CMs. More importantly, the detailed underlying molecular mechanism of maturation is mostly unclear. We propose that ROS may serve as a critical mediator of hPSC-CM maturation. ROS is already implicated in a number of cardiac pathways, and early studies have shown that ROS is present and an active signaling component in hPSC-CMs. In the following sections, we review how ROS impacts hPSC-CM contractility, calcium handling, metabolism, and hypertrophy, all of which are associated with cardiac maturation (Figure 2, Key Figure).

ROS as a Mediator of PSC-Cardiac Maturation

Contractility

The sarcomere is the main component responsible for contraction in CMs. Adult CMs have an average sarcomere length of 2.2 μm in their relaxed state, while this number is 1.65 μm in immature CMs differentiated from human induced PSCs (hiPSC-CMs); [1]. The expression of sarcomeric proteins, such as α -actinin, β -myosin heavy chain, cardiac troponin I, and cardiac troponin T, leads to sarcomere development and eventually encourages the maturation of CMs [1,51]. As CMs continue to develop, their sarcomere organization and length increase, enabling increased force generation, which is a critical function of CMs [53–55]. Immature CMs have been reported to have a lower peak twitch tension of about 550-fold compared with adult human cardiac tissues in collagen constructs [1].

Several recent studies have demonstrated that ROS are associated with increased CM contractility. In rat CMs, increased contractility leads to an increase in oxygen consumption and production of ROS [56,57]. Through modulation of endothelin-1 reception, ROS can increase the contractile force in CMs [58–60]. Kubin *et al.* showed that ROS from NOX plays an important role in the regulation of contractility in adult rat hearts. Specifically, endothelin-1 was shown to promote contractility through the ROS mediated ERK1/2–p90 ribosomal S6 kinase– Na^+ - H^+ exchanger-1 pathway [12]. These studies elucidate the relationship between ROS and contractility, suggesting that modulating intercellular ROS might increase contractility and lead to CM maturation.

Calcium Handling

We have discussed how the application of varied electrical stimuli has emerged as a viable technique to encourage neonatal-like hiPSC-CMs to exhibit a more mature phenotype. To ensure a physiological response to these electrical stimuli, hiPSC-CMs should express mature isoforms of specific ion channels and contain calcium-sensitive organelles that are necessary to shuttle calcium and other ions from the intracellular space to the cytoplasm. Cardiac contractility is wholly dependent on this series of calcium transfers (commonly denoted ‘calcium handling’). Briefly, an initial action potential triggers a release of calcium from the SR through **ryanodine receptor 2 (RyR2)**; this is the fundamental building block of CICR, which is largely responsible for activating the components of the contractile

machinery [61]. This burst of ions from the SR is commonly denoted as a ‘cardiac spark’ and was first described by Cheng *et al.* in 1993 [62].

Reports so far demonstrate the PSC-CMs have partial mature calcium handling machinery and functionality. Li *et al.* observed that human ESC (hESC)-derived ventricular CMs displayed physiologically relevant calcium handling; however, they also noted that hESC-CMs had a compromised SR [63]. Zhang *et al.* were the first to demonstrate that hiPSC-CMs displayed cardiac sparks with similar spatiotemporal profiles attained in mature, adult CMs; furthermore, these cells contained two proteins vital for effective CICR: L-type Ca^{2+} channels and RyR2 [64]. However, as noted by Zhang *et al.* and in a separate review [65], these sparks were stochastic and tended to localize to the same location in the cell, indicating possible immaturity. Differentiated hiPSC-CMs lack T-tubules, which are a vital compartment for calcium storage and transfer [65]. Without well-developed T-tubules, it is possible that hiPSC-CMs could remain trapped in a neonatal-like developmental stage. Due to the lack of T-tubules and an organized SR, the immature CMs rely mainly on extracellular calcium for excitation–contraction coupling.

It has been reported that physiologic stretch of CMs produces ROS through NADPH oxidase 2 (NOX2) rapid activation. Generated ROS increases RyR2 sensitivity, triggering a cardiac spark [66]. The amplitude and number of cardiac sparks can be influenced by mechanical cues present in the surrounding CM microenvironment; for example, rat ventricular CMs stretched by carbon fibers exhibited an increased rate of cardiac sparking of up to 30% [67]. Furthermore, passive stretch [66] and shear stress [11] in cardiac tissue leads to the NOX2-mediated generation of mitochondrial ROS, which sensitizes the RyR2 channels to calcium activation. These findings indicate that ROS interacts with the proteins of the SR, thereby fine-tuning the production and frequency of cardiac sparks. ROS signaling via mechanotransduction plays an integral role in regulating the calcium handling pathway and is, therefore, a viable target in hiPSC-CM maturation.

Metabolism

PSC differentiation into somatic cells is characterized by significant transitions in metabolism. PSCs contain relatively few mitochondria, which are globular in shape with poor cristae structure, and therefore mostly rely on glycolysis to fulfill metabolic demand. In contrast, differentiated adult CMs have elongated, cristae-rich mitochondria, allowing for the derivation of ATP through **oxidative phosphorylation (OXPHOS)**; [68,69]. As PSCs differentiate, oxidative metabolism gradually takes over as the primary energy source. Specifically, pyruvate generated from glycolysis is transported into the mitochondria and further utilized in the tricarboxylic acid (TCA) cycle for energy metabolism [70]. The glycolysis–OXPHOS transition during stem cell differentiation dramatically changes the intracellular metabolite profiles: PSCs are rich in unsaturated/reduced metabolites (e.g., arachidonic and eicosapentaenoic acids), whereas somatic cells such as neuronal cells and CMs contain increased levels of oxidized metabolites (e.g., saturated free FAs and their derivatives); [71]. Notably, CMs further undergo radical changes in their metabolic profile as they continue to mature. Fetal and immediate newborn CMs produce almost half of their ATP via glycolysis (44%) and half by oxidative metabolism of lactate (25%), glucose (18%),

and FA (13%); [72]. After 7 days of CM maturation, ATP production is mainly derived from lactate (49%) and FA (41%) oxidation and the anaerobic glycolysis contribution drops down to 5%. As the CMs further mature (21-days-old), ATP production is derived mainly from FA β -oxidation (~80%) and glucose oxidation (~12%); [72]. Most importantly, the metabolic change during stem cell differentiation or adult cell maturation is not merely a byproduct of cell phenotype change, but an active regulator of cell fate decision [73]. Inhibition of the mitochondria respiratory chain with antimycin or rotenone significantly disrupted cardiac differentiation, suggesting the critical role of oxidative metabolism in cardiogenesis [74]. Mimicking the postnatal nutritional environment by supplementing culture media with FAs and galactose improved mitochondrial bioenergetics of human PSC-derived CMs, providing a new path for CMs maturation [75].

Similarly, 3D microphysiological cardiac tissue functionally matured in FA supplemented media [2]. Alternative induction of OXPHOS metabolic switching in hPSC-CMs, via overexpression of Let-7 miRNA [76] or small molecule-based inhibition of hypoxia-inducible factor 1 α [77], led to cardiac physiological and functional maturation. Collectively, these results highlight the promise of improving both cardiac differentiation and CM maturity by directly modulating cell metabolite levels. A potential route to achieve such modulation can be presented by changing the level of ROS.

ROS and the energy metabolism in the mitochondria are tightly linked. ROS is the byproduct of these processes produced during the electron transport chain. Additional sites for ROS production occur in FA oxidation, which has been shown to increase ROS production [78,79] at the very long Acyl-CoA dehydrogenase [80] and the electron transfer flavoprotein [81] sites. In turn, ROS-generating enzymes, such as NOX, may play a crucial role in the metabolic switching from glycolysis to OXPHOS. NOX4 for example, one of two NOXs that is expressed in the heart, has been shown to reduce glucose oxidation and glycolysis while concomitantly increasing FA oxidation during heart remodeling [82].

It has been shown that ROS can activate cardiac differentiation [33,83,84]. Chung *et al.* reported that hESC-CM switch to mitochondrial oxidative metabolism and consume 40 times more oxygen than hESCs and prevention of NADPH oxidation reduced the amplitude of beating CMs by 90% [74]. Hom *et al.* showed that treating E9.5 mouse embryos with cyclosporin A (an immunosuppressant that blocks the calcium ion-induced permeability transition) promoted the closure of mPTPs, thereby enhancing mitochondrial maturation and cardiac differentiation. Conversely, the authors reported that oxidant and antioxidant treatments inhibited and enhanced CM differentiation, respectively, suggesting that the redox signaling myocyte differentiation takes place after the closure of mPTP [27]. Based on the ongoing discussion, it is evident that ROS play an integral role in the metabolic switch required for PSC-CMs maturation.

Cardiac Growth

During mammalian embryonic development, heart growth is mainly accomplished through the proliferation of CMs, or hyperplasia, whereas once in the postnatal period, the proliferation capability of CMs gradually diminishes with most cells withdrawing from the cell cycle. Heart growth is then mainly achieved via the growth in cell size, or hypertrophy

[85]. From a physiological point of view, cardiac tissue hypertrophy is required to reduce ventricular wall stress and to keep pace with the increased cardiovascular demand of the growing organism. During this transition, neonatal CMs are exposed to the oxygen-rich environment and their metabolism shifts from glycolysis to FA oxidations (as described previously); [84]. This metabolic shift in turn significantly increases mitochondrial ROS production in mouse hearts during the first postnatal week and coincides with cardiac cell cycle arrest (i.e., transition to hypertrophy); [84]. It has also been shown that exposure to chronic hypoxia (2 weeks, 7% O₂) decreases ROS generation in adult mouse CMs and induces proliferation. Mice with myocardial infarction exposed to the chronic hypoxia experienced significant recovery (~25%) of LV systolic function 1 week after recovery compared with the control group [87]. Therefore, modulating ROS levels in mice impacts both hyperplasia and hypertrophy.

Treatment with antioxidants delayed cell cycle arrest, while treatment with ROS generators increased cardiac cell size in the postnatal heart, implying that ROS is an activator of transitioning from hyperplasia to hypertrophy [84]. ROS is also modulated via the Hippo signaling pathway, which plays a crucial role in regulating cardiac cell proliferation and heart size [88]. A major downstream target of the Hippo pathway is the transcriptional coactivator **Yes-associated protein (YAP)**. YAP can promote cell growth and proliferation through interaction with the WNT pathway and IGF pathway [89]. The Hippo pathway phosphorylates YAP and prevents its nuclear entry and thus inhibits cell proliferation [89]. Hippo inhibition/Yap activation can reprogram adult cardiac cells back into the hyperplasia state and lead to cardiac proliferation and cardiac regeneration in adult mice hearts [86]. Xin *et al.* showed that loss of Yap led to lethal cardiomyopathy in mice, whereas activated Yap increased heart size and improved survival post-myocardial infarction [90]. Conversely, Shao *et al.* reported that YAP and **FoxO1** form a complex in the nucleus of mouse CMs and promote expression of antioxidant genes. They reported that inhibition of YAP decreased antioxidant gene expression, increased accumulation of ROS in the mice hearts, and induced cardiac death in response to oxidative stress, suggesting that YAP modulates cardiac redox state [21]. Hypertrophy can also be induced chemically in healthy PSC-CMs using ROS modulating adrenergic receptor agonist (e.g., norepinephrine and phenylephrine); [91], or via endothelin-1 stimulation [12,92–94]. ROS signaling is therefore at the root of the hyperplasia to hypertrophy transition and can be modulated to induce PSC-CM maturation.

Concluding Remarks

In addition to the traditional methods for CM maturation, ROS-based techniques have tremendous potential to aid in the creation of adult-like phenotype CMs. Understanding the detailed role of ROS in the differentiation and maturation process will allow us to fine-tune existing protocols or devise novel strategies. ROS have long been implicated as critical small-molecule messengers in cell signaling and homeostasis. In this Opinion, we demonstrate that these signaling pathways are largely conserved in CMs derived from PSCs. Table 1 shows a summary of results from selected studies conducted on the effects of ROS on CM maturation and differentiation.

Modulation of redox signaling may offer us a powerful new tool to enhance both the efficiency and consistency of differentiation as well as maturation. Though existing methods (e.g., monolayer protocol) are capable of generating high-purity CMs, there is still room for improvement. For instance, subtle changes in drug concentrations or initial cell seeding density can significantly reduce efficiency [46]. Zhang, J. *et al.* and Zhang, D. *et al.* studied the effects of 3D culture in hydrogels containing extracellular matrix proteins (Matrigel) and reported improvements in function and maturity of differentiated hPSC-CMs [95,96]. Although they did not examine ROS generation and migration, it is possible that ROS is a participant in the paracrine signaling that regulates this CM maturation.

Nevertheless, an all-encompassing understanding of the effects of ROS on the maturation of PSC-CMs remains elusive. It does appear that ROS regulates contractility, calcium handling, metabolism, and hypertrophy in primary and PSC-derived CMs; all four of these functional parameters are typically used to define the maturity of a given CM (see Outstanding Questions).

Future studies must work towards identifying the detailed molecular mechanisms of these ‘maturation pathways’ and the role of ROS in their regulation. Advancements in the super-resolution live cell imaging systems will allow researchers to identify the key events and small-molecule messengers that encourage embryonic-like CMs to undergo a transition into a more mature phenotype. Furthermore, the addition/removal of antioxidants to/from commonly employed differentiation and maturation cell culture media may have a profound impact on the final phenotype of the differentiated cell. Additionally, optogenetic ROS-generating molecules can be used to spatially and temporally target bursts of ROS in cells. The development of antioxidant-conjugated probes that can specifically modulate ROS in different organelles may further provide means to tightly regulate the expression levels of these maturation pathways.

In summary, PSC-CMs have shown great promise in applications such as disease modeling, drug screening, and cell transplantation. Maturation of CMs is an essential step towards these applications. ROS play an important role in the regulation of signaling pathways for cellular processes and can alter features such as contractility, calcium handling, metabolism, and hypertrophy in CMs. Therefore, maturation techniques based on ROS modulation can be very effective in producing adult-like CMs.

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Glossary

Calcium-induced calcium release (CICR)

a process in which calcium activates calcium release from the intracellular Ca_2^+ stores

Embryoid bodies (EBs)

3D clusters of pluripotent stem cells (PSCs)

Fatty acid (FA)

a carboxylic acid with a long saturated or unsaturated aliphatic chain

FoxO1

a transcription factor that regulates cell cycle, apoptosis, atrophy, autophagy, and energy homeostasis

Insulin-like growth factor-1 (IGF1)

human gene that codes for a protein hormone that is important in childhood growth

Mitochondrial permeability transition pore (mPTP)

a protein pore that opens in the mitochondrial membrane under certain pathological conditions

NADPH oxidase (NOX)

an enzyme that catalyzes the reduction of molecular oxygen to ROS and acts as an oxygen sensor by using nicotinamide adenine dinucleotide phosphate as a cofactor and reducing agent

Oxidative phosphorylation (OXPHOS)

a metabolic pathway in which cells oxidize nutrients using enzymes to release energy for ATP production

Ryanodine receptor 2 (RyR2)

a protein that acts as the major mediator for calcium release in the CICR

Sarcoplasmic reticulum (SR)

a structure found within muscle cells

WNT pathway

signal transduction pathways which begin with proteins passing signals into a cell through cell surface receptors

Yes-associated protein (YAP)

a protein that can promote cell growth and proliferation through interaction with the WNT and IGF pathways

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Highlights

Pluripotent stem cell-derived cardiomyocytes (PSC-CM) must exhibit adult-like characteristics before they can be applied in the pharmaceutical and clinical setting.

Reactive oxygen species (ROS) can cause oxidative stress when produced in excess; however, they also play a crucial role in mediating cell signaling pathways, and it has been shown that they can activate cardiac differentiation of PSCs.

There is growing evidence that ROS are involved in the maturation process of CMs. Through modulation of endothelin-1 reception, ROS can extend the contractile force in CMs. ROS located closely to the T-tubules increases RyR2 sensitivity, tuning the cardiac sparks.

CM maturity can be modulated directly by cellular metabolism, and prevention of ROS production reduces the amplitude of beating CMs.

ROS is modulated via the Hippo signaling pathway, which regulates cardiac cell proliferation and hypertrophy.

Clinician's Corner

PSC-CMs have great potential for disease modeling, drug screening, and cell transplantation.

Current protocols for differentiating CMs from iPSCs result in immature fetal-like CMs, and methods to induce their maturation fail to generate CMs that are indistinguishable from mature CMs as the underlying maturation mechanisms remain unknown.

While at high levels ROS cause cellular damage and have been associated with degenerative diseases, at physiological levels, these highly reactive small molecules play an indispensable role in maintaining cell signaling and homeostasis.

Here, we propose that ROS modulation and compartmentalization may serve as a critical mediator of hPSC-CM maturation.

ROS regulates hPSC-CM contractility, calcium handling, metabolism, and hypertrophy, all of which are associated with cardiac maturation.

Though the precise role of ROS in cardiac maturation has yet to be further elucidated, ROS may underpin the molecular mechanisms that govern cardiac maturation.

Outstanding Questions

Can the fine-tuned modulation of intracellular ROS effectively induce a mature phenotype in stem cell-derived CMs?

Can CMs subjected to the ROS treatments exhibit increased maturity and thereby be more convincing drug screens and disease models?

Is differential ROS regulation linked to discrepancies in the generation of physiologically-relevant CMs *in vitro*?

What studies need to be conducted to demystify the role of ROS in cardiac maturation signaling pathways?

Is mitochondrial or NOX ROS a more significant contributor to cardiac function? Is one aberrantly elevated/depressed in stem-cell derived CMs?

Trends in Molecular Medicine

Figure 1. Pluripotent Stem Cell-Derived Cardiomyocytes Produce Superoxide As a Byproduct of Homeostatic Metabolism.

Primarily, this superoxide (O_2^-) is generated from: (i) the action of NADPH oxidases (NOXs), membrane-bound protein complexes associated with respiratory burst, and (ii) the activity of transmembrane proteins that constitute the electron transport chain. This O_2^- is converted into hydrogen peroxide (H_2O_2), which is a critical messenger molecule in redox regulation. Furthermore, H_2O_2 can be further converted into free hydroxide (OH^-), which is responsible for DNA damage, oxidative stress, and lipid oxidation. O_2^- and H_2O_2 , which are both highly reactive small molecules composed of oxygen, and hydrogen and oxygen, respectively, can be classified as reactive oxygen species (ROS).

Key Figure

The Production of ROS (Hydrogen Peroxide, Superoxide, and Hydroxide) by Pluripotent Stem Cell-Derived Cardiomyocytes Can Be Potentially Leveraged to Induce *In Vitro* Maturation

Figure 2.

(A) Reactive oxygen species (ROS) have been well-implicated as messenger molecules in cardiomyocyte (CM) contractility, and it has been demonstrated that CMs conditioned via mechanical stretching exhibit more mature characteristics. Furthermore, the limitations of the contractile mechanism of pluripotent stem cell-derived cardiomyocytes (PSC-CMs) has been well documented; adult CMs cultured in collagen constructs have 550 times higher contraction power than PSC-CMs in the same constructs [1]. (B) ROS is a molecular byproduct of cellular metabolism; by presenting immature PSC-CMs with carefully

formulated cell culture medium (e.g., containing galactose), PSC-CMs can be purified and subsequently undergo further maturation. (C) Current PSC-CMs lack transverse tubules (T-tubules) and an organized sarcoplasmic reticulum (SR); ROS have been demonstrated to be messenger molecules that guide calcium signaling in mature primary CMs and therefore, might be leveraged to induce the formation of more mature calcium handling machinery. (D) While hypertrophy is often associated with a pathological state in mature CMs, it is also a necessary process in the transition from fetal-like CMs to CMs which exhibit more mature protein expression and electrical functionality. ROS play a significant role in regulating hypertrophy and therefore, might be leveraged to induce a 30–40-fold increase in CM size, as has been observed in rodent and human CMs [1].

Table 1.

Summary of Selected Studies of Reactive Oxygen Species (ROS) Effects on Cardiomyocytes Maturation and Differentiation

Time points of study	Models	ROS measurement techniques	Effects on CMs maturation/differentiation	Refs
E9.5-E13.5 days	Mouse embryos (primary isolated cells)	2',7'-dichlorofluorescein diacetate (H ₂ DCFDA)	The closing of mPTP via cyclosporin A (CsA) promoted mitochondrial structural maturation and increased number of Z bands in the cells. ROS modulation with Trolox recapitulated this process.	[27]
14–28 days	hESC-CMs (H9 and UCLA4 cell lines)	H ₂ DCFDA	Glucose depletion promoted metabolic maturation and increased contractile function. ROS scavenging did not alter troponin T2 (TNNT2) expression.	[52]
10–20 days	Mouse mESC-CMs (D3 cell line)	CM-H ₂ DCFDA	Cyclic strain increased expression of <i>Nkx2.5</i> and <i>CX43</i> genes and beating EBs in mESC-CMs. Scavenging ROS with antioxidants neutralized the effect.	[5]
1–14 days	Neonatal rat CMs (primary isolated cells)	H ₂ DCFDA	The postnatal oxygen-rich environment promotes ROS, DNA damage, and cell cycle arrest. The effects could be amplified by H ₂ O ₂ treatment or inhibited by <i>N</i> -acetylcysteine (NAC) antioxidant treatment.	[84]