SYMPOSIUM REVIEW

Regulation of cardiomyocyte maturation during critical perinatal window

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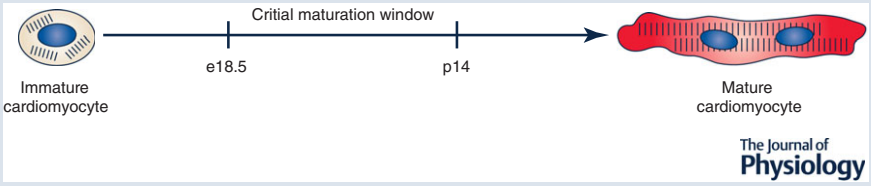

Abstract A primary limitation in the use of pluripotent stem cell-derived cardiomyocytes (PSC-CMs) for both patient health and scientific investigation is the failure of these cells to achieve full functional maturity. *In vivo,* cardiomyocytes undergo numerous adaptive structural, functional and metabolic changes during maturation. By contrast, PSC-CMs fail to fully undergo these developmental processes, instead remaining arrested at an embryonic stage of maturation. There is thus a significant need to understand the biological processes underlying proper CM maturation *in vivo*. Here, we discuss what is known regarding the initiation and coordination of CM maturation. We postulate that there is a critical perinatal window, ranging from embryonic day 18.5 to postnatal day 14 in mice, in which the maturation process is exquisitely sensitive to perturbation. While the initiation mechanisms of this process are unknown, it is increasingly clear that maturation proceeds through interconnected regulatory circuits that feed into one another to coordinate concomitant structural, functional and metabolic CM maturation. We highlight PGC1α, SRF and the MEF2 family as transcription factors that may potentially mediate this cross-talk. We lastly discuss several emerging technologies that will facilitate future studies into the mechanisms of CM maturation. Further study will not only produce a better understanding of its key processes, but provide practical insights into developing a robust strategy to produce mature PSC-CMs.

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Abstract figure legend Here, we postulate that there is a critical window, ranging from embryonic day 18.5 to postnatal day 14 in mice, in which interconnected regulatory circuits enable coordinated, concomitant structural, functional and metabolic cardiomyocyte maturation.

There is tremendous enthusiasm in the application of pluripotent stem cell (PSC) technology to cardiovascular medicine. PSCs (including embryonic stem cells (ESCs) and induced PSCs (iPSCs)) can be expanded and subsequently differentiated into cardiomyocytes (CMs) *in vitro*. Because adult CMs are non-proliferative and difficult to obtain from human patients, PSC-derived CMs (PSC-CMs) serve as the most viable approach to generating large quantities of CMs *ex vivo* (Batalov & Feinberg, 2015). Among many potential uses, PSC-CMs have been proposed for application in regenerative medicine, drug and toxicity screening, and disease modelling (Passier *et al.* 2006; du Pré *et al.* 2013; Wang *et al.* 2013; Chen *et al.* 2016; Youssef *et al.* 2016). Given the burden of cardiovascular disease (Benjamin *et al.* 2017) and the need for improved cardiac cellular models (Gwathmey *et al.* 2009), PSC-CMs offer potential solutions to significant unmet health needs in the United States.

Extensive analysis has led to an improved understanding of regulatory pathways in early cardiogenesis. This in turn has allowed for the development of improved and optimized protocols, inspired by *in vivo* development, to differentiate PSCs to CMs *in vitro* (Kattman *et al.* 2011; Burridge *et al.* 2012; Mummery *et al.* 2012; Uosaki *et al.* 2012; Cao *et al.* 2013; Lian *et al.* 2013). Despite these successes, however, use of PSC-CMs has been limited due to the failure of these cells to mature to a fully adult phenotype *in vitro*. *In vivo*, mature CMs demonstrate increased size, polyploidization, development of well-formed sarcomeres, improved calcium handling, t-tubules, large numbers of mitochondria, primarily oxidative metabolism, and other characteristic features (Robertson *et al.* 2013; Yang *et al.* 2014). However, PSC-CMs fail to show these same characteristics, and instead resemble mid-to-late embryonic CMs, even following extended culture (Beqqali *et al.* 2006; Cao *et al.* 2008; Snir *et al.* 2009; Davis *et al.* 2011; Robertson *et al.* 2013; Keung *et al.* 2014; Yang *et al.* 2014; Uosaki *et al.* 2015; Veerman *et al.* 2015). These differences significantly impede further research and clinical use of PSC-CMs.

As with advances in PSC-CM differentiation, improved maturation of PSC-CMs will likely require deeper understanding of CM maturation as it occurs during development *in vivo*. To date, the regulatory mechanisms underlying CM maturation remain an open area of investigation. The developing heart is a highly dynamic environment, characterized by changes in mechanical forces, electrical coupling, extracellular matrix composition, oxygen and cytokine gradients, and many others (Robertson *et al.* 2013; Yang *et al.* 2014); it is unclear which, if any, of these properties serves as upstream regulators or downstream effects of others. As many facets of maturation are conserved between species, animal models (including rodent as well as large animal, such as sheep, models) have been a primary tool for understanding regulation of CM maturation *in vivo*. Knowledge from these animal studies has been further supplemented by *in vitro* studies seeking to recapitulate aspects of the native milieu (Scuderi & Butcher, 2017). Though no single *ex vivo* method has captured the full complexity of *in vivo* maturation nor generated fully mature CMs, each has furthered our knowledge of the biological underpinnings of CM maturation.

In this review, we aim to summarize potential regulatory mechanisms linking together the multiple facets of CM maturation. As our focus is on *in vivo* maturation, we primarily consider studies utilizing animal models. While *in vivo* human data regarding maturation is limited, where appropriate, we also comment on informative results from *in vitro* studies utilizing human PSC-CMs. We postulate a postnatal 'critical window' for maturation, in which interconnected positive feedback circuits regulate concomitant structural, functional, metabolic and transcriptomic CM maturation. We discuss the implications of this critical window in the generation of mature PSC-CMs. We lastly look forward to new technologies that may enable further studies of regulatory mechanisms in CM maturation.

Dynamics of CM maturation

The temporal dynamics of CM maturation have been comprehensively reviewed elsewhere (Robertson *et al.* 2013; Galdos *et al.* 2017). Nevertheless, we briefly summarize some of the most important aspects here, focusing particularly on the rodent model. In the perinatal period, CMs undergo significant structural changes as they transition from small, round cells with disorganized features to large, cylindrical cells with highly organized components. Beginning at embryonic day 18.5 (e18.5) and continuing through the first weeks of birth, CMs increase in cell length and length-to-width ratio while displaying longer and more aligned myofibrils (Hirschy *et al.* 2006).

From e19.5 to postnatal day 7 (p7), several sarcomeric proteins (including myosin heavy chain, cardiac troponin T, and cardiac troponin I) undergo isoform switching to enable more efficient contraction (Siedner *et al.* 2003; Yin *et al.* 2015). CMs fully cease proliferation around p3–p7 (Porrello *et al.* 2011; Notari *et al.* 2018) and subsequently undergo hypertrophic growth with polyploidization and multinucleation (Leu *et al.* 2001; Liu *et al.* 2010). From an electrophysiological perspective, ion channel activity and localization changes significantly postnatally (Liu *et al.* 2002; Harrell *et al.* 2007). Maturation of cell-cell and cell-ECM junctions, particularly at the intercalated disc, occur during the first week of birth (Wu *et al.* 2002; Hirschy *et al.* 2006). T-tubulation, which enables efficient excitation-contraction coupling, initiates at approximately p6 but continues through 2—3 weeks of birth (Sedarat *et al.* 2000). Lastly, CMs undergo several adaptive metabolic changes. While mitochondrial remodelling begins as early as e13.5 (Hom *et al.* 2011), significant improvements in mitochondrial number, size, distribution and internal organization occur in the peri- and postnatal periods (Hallman, 1971; Smolich *et al.* 1989; Marin-Garcia *et al.* 2000; Vega *et al.* 2015). Likewise, from p0 to p7, CMs undergo a transition from primarily glycolytic metabolism to one reliant on oxidative phosphorylation, and the primary energy source transitions from glucose to fatty acids (Marsh & Marsh, 1991; Itoi & Lopaschuk, 1993; Lopaschuk & Jaswal, 2010). Taken as a whole, these adaptive processes enable mature myocytes to meet their highly energetic demands. While many key changes occur up through the first week of birth, CM maturation progressively continues, with CMs reaching their maximal volume at approximately 3 months of age (Leu *et al.* 2001).

Species-specific differences in CM maturation

The hearts of different species have notably different force generation demands; correspondingly adult myocytes show phenotypic differences across species, particularly in terms of contractile and electrophysiological properties (Milani-Nejad & Janssen, 2015). While variations in maturation dynamics between species remain relatively unstudied, some prominent differences have been observed. For example, in mice, heart rate increases from approximately 150—190 beats per minute (bpm) at e12.5 to 245 bpm at e19.5 and eventually reaches a mature heart rate of 310–840 bpm (Yu *et al.* 2008). In contrast, the human fetal heart rate is 120–160 bpm, and progressively slows during maturation to achieve an adult heart rate of 60–100 bpm (Pildner von Steinburg *et al.* 2013). As a result, the shape of the rodent action potential is different from that of the human action potential, with a notably shorter duration and lack of a prominent plateau phrase. The differences are further reflected in expression dynamics of various isoforms of contractile proteins. While isoform switching is observed across species in CM maturation, the specific switches depend on each particular species' cardiac contraction/relaxation demands. One particularly well-known example of this phenomenon is myosin heavy chain (MHC) isoform switching. In rodent ventricles, MHC transitions from the β-isoform to the α-isoform over the course of maturation, enabling improved contractile velocity. In human ventricles, the β-isoform increases over maturation, enabling improved contractile economy and lower tension cost (Milani-Nejad & Janssen, 2015). T-tubulation dynamics also show species differences. Unlike in smaller mammals, primitive t-tubules may be observed in fetal development in large mammals such as sheep, cows, rhesus monkeys and humans (Kim *et al.* 1992). Lastly, nucleation dynamics also demonstrate significant species variations in maturation. For example, while terminal differentiation of CMs to multinucleated, quiescent cells occurs largely postnatally in rodents, this process may be up to 75% complete prior to birth in sheep (Burrell *et al.* 2003; Jonker *et al.* 2007). While binucleation occurs prenatally in humans, as in sheep, adult human hearts present with a notably smaller number of binucleated myocytes (25–60%) compared to both rodents and sheep (~90% binucleated) (Botting *et al.* 2012). It is estimated that human CMs reach an adult-like state by 10 years of age (Takamatsu *et al.* 1983; Peters *et al.* 1994).

Further investigation is required to understand the mechanisms underlying these species-specific differences in CM maturation dynamics and adult CM properties. Intriguingly, comparative gene expression analysis indicates that transcriptomic changes across maturation are broadly conserved in mouse and humans (Uosaki & Taguchi, 2016). On the other hand, xenogenic transplantations of PSC-CMs typically yield only partially mature myocytes, suggesting significant environmental or regulatory differences between species with regards to maturation (Dai *et al.* 2007; Laflamme *et al.* 2007; Shiba *et al.* 2012; Chong *et al.* 2014; Liu *et al.* 2018). It is possible that while gene trends remain similar across species, species-specific gene dosages or relative gene expression levels contribute to species differences in maturation. Moreover, these species-specific effects may be regulated through post-translational processes. Identifying a mechanism underlying species-specific differences in CM maturation remains a significant unanswered question in cardiac developmental biology.

Initiation of CM maturation

To date, the factor(s) responsible for initiating CM maturation remain unknown. Birth, which is accompanied by significant changes in haemodynamics, oxygenation and biochemical milieu (Rudolph, 1970;

Dawes *et al.* 1980; Teitel *et al.* 1987), has frequently been thought of as a trigger for maturation. During birth, the closure of shunts in fetal circulation results in significant changes in cardiac load and output (Agata *et al.* 1991; Schubert *et al.* 2013), which in turn promotes structural, contractile and force generation improvements in CMs (Barbera *et al.* 2000; Ruan *et al.* 2015). Oxygenation of arterial blood doubles (Teitel *et al.* 1987), leading to metabolic and mitochondrial maturation through reduction in HIF1a signalling (Breckenridge *et al.* 2013; Neary *et al.* 2014). The timely regulation of these changes is critical; for example, in pediatric patients, when the fetal-to-neonatal transition in circulation does not occur (often called 'persistent fetal circulation'), myocardial maturation may become delayed (Hines, 2013). In several studies conducted in both humans and other animals, premature birth was found to be associated with abnormal CM hypertrophy and nucleation and premature cell cycle cessation (Bensley *et al.* 2010, 2018; Aye *et al.* 2017). As a caveat, however, patients in these studies were often treated antenatally and/or postnatally with corticosteroids, which may in turn affect CM maturation (Agnew *et al.* 2017).

Despite these observations, the idea of birth as a triggering event for CM maturation has been contested (Jonker *et al.* 2015). Indeed, many facets of maturation appear to be initiated *in utero,* including increase in myocyte length and volume, myofibril length and number, sarcomeric isoform switching, cessation of cell cycle, aerobic metabolism, and other changes (Kim *et al.* 1992; Burrell *et al.* 2003; Siedner *et al.* 2003; Hirschy *et al.* 2006; Jonker *et al.* 2007, 2015; Porter *et al.* 2011; Baker & Ebert, 2013; Yin *et al.* 2015). Moreover, disruption of the intrauterine environment, particularly in the period prior to birth, can lead to retardation of CM maturation (Bubb *et al.* 2007). Thus, it appears that the initiation of CM maturation occurs in the period prior to birth (e.g. e16.5–e18.5 in mice, gestational days 110–130 of 147 in sheep, pregnancy weeks 28–32 in human), perhaps in anticipation of significant changes in cardiac function in the fetal-to-neonatal transition.

The evolving hormonal and neuroendocrine environment of the fetus may influence these maturation phenomena. For example, in mice, endogenous glucocorticoid production increases from e15.5 and peaks at e17.5; correspondingly, maternal plasma glucocorticoid levels peak at approximately e16–e17 (Rog-Zielinska *et al.* 2015). This spike in glucocorticoids has been shown to mediate CM myofibrillar structure, oxygen consumption, ion channel expression and calcium handling, with many of these processes mediated in a PGC1α-dependent manner (Rog-Zielinska *et al.* 2013, 2015). Similarly, in rodents, thyroid hormone secretion initiates at approximately e17.5, and mediates transcriptional effects on CM contractile function, among other effects (Li *et al.* 2014). Other hormones that have been implicated in CM maturation include IGF1 and NRG1 (Rupert & Coulombe, 2017).

Evidence for a critical perinatal window in maturation

While the initiation of CM maturation is still unknown, recent data strongly demonstrate the critical importance of the perinatal cardiac environment in regulating CM maturation. For example, we recently demonstrated that transplantation of ESC-CMs into the neonatal rat heart at p0–p3 led to generation of CMs that were structurally, functionally and transcriptomically indistinguishable from adult CMs (Cho *et al.* 2017). Similarly, Kadota *et al.* (2017) have shown that full maturation of neonatal rat ventricular CMs (NRVCMs) is possible following transplantation in the neonatal heart. On the other hand, results of same-species transplantation experiments in adult hearts have been more equivocal. For example, some studies have observed significant and near-complete maturation of transplanted cells by structural and electrophysiological analysis, including primary cells (Klug *et al.* 1996; Gojo *et al.* 1997; Roell *et al.* 2002; Rubart *et al.* 2003) and PSC-CMs (Didié *et al.* 2013). On the other hand, other studies have shown only partial and limited maturation even following extended transplantation, with failure to achieve full adult size and structure (Leor *et al.* 1996; Watanabe *et al.* 1998; Reinecke *et al.* 1999; Müller-Ehmsen *et al.* 2002; Christoforou *et al.* 2010; Shiba *et al.* 2016). Likewise, we have observed that transplantation of ESC-CMs at p14 resulted in limited maturation with incomplete sarcomere alignment (Cho *et al.* 2017). In addition to these transplantation experiments, other studies have demonstrated that the proliferation-to-hypertrophy transition (Anatskaya *et al.* 2010) and mitochondrial/metabolic maturation (Gong *et al.* 2015) processes are exquisitely sensitive to perturbation up to approximately 2—3 weeks after birth in rodents. Based on these data, we believe that the perinatal time period (which we define as e18.5–p14 in rodents) may represent a critical window for CM maturation, analogous to the perinatal regenerative window. Perturbations to normal developmental phenomena during this time period may lead to an immature CM phenotype.

Critical window for maturation in PSC-CMs

Intriguingly, a similar critical window for maturation may be observed in PSC-CM differentiation. *In vitro*, PSC-CMs mature through the first 20 days of culture before undergoing maturation arrest (Uosaki *et al.* 2015). At this time, they structurally, functionally and transcriptomically resemble fetal CMs (Robertson *et al.* 2013; Galdos *et al.* 2017), though they display numerous aberrant regulatory networks (Uosaki *et al.* 2015). While it is thought that long term culture improves maturation

of PSC-CMs (Kamakura *et al.* 2013; Kuppusamy *et al.* 2015; Dias *et al.* 2018), recent analyses have suggested that even at 1 year of culture, PSC-CMs continue to resemble late embryonic/early fetal CMs (DeLaughter *et al.* 2016). We hypothesize that, analogous to *in vivo* development, PSC-CMs are receptive to signalling cues prompting them to undergo maturation during an early critical window. Perturbation during this window (e.g. due to stresses induced by cell culture) leads to failure of complete maturation.

This hypothesis is supported by transplantation studies. For example, Kadota *et al.* (2017). observed that transplantation of early human PSC-CMs at day 5 of differentiation into neonatal rat heart led to significantly improved maturation over PSC-CMs transplanted at days 18–20 of differentiation. Similarly, we have observed complete maturation of mouse ESC-CMs transplanted into neonatal heart at days 5–7 of differentiation (Cho *et al.* 2017), but incomplete maturation of the same cells when transplanted at day 14+ of differentiation (authors' unpublished data). We summarize these results in Fig. 1. It is possible that the results of these transplantation studies are confounded by difficulties in handling/dissociating late stage PSC-CMs from culture or their poor retention *in vivo*. Interestingly, however, Ronaldson-Bouchard *et al.* (2018) recently described a similar phenomenon in an *ex vivo* bioreactor system in which iPSC-CMs were subjected to various electrical simulation regimes. They found that an intensity training-based simulation protocol resulted in significant force generation, calcium handling and ultrastructural maturation in early stage (day 12) iPSC-CMs but only limited maturation in late stage (day 28) iPSC-CMs.

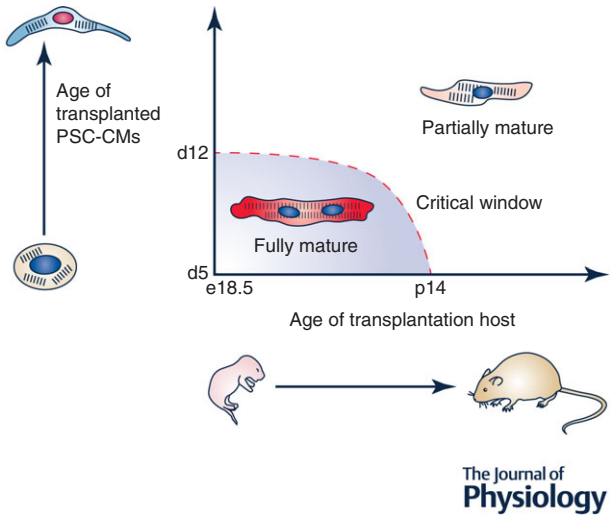

Figure 1. Summary of transplantation experiments for PSC-CM maturation
When early PSC-CMs are transplanted *in vivo* during the perinatal period, they achieve full structural, functional and transcriptomic maturity. However, when either late PSC-CMs are transplanted or an older host is used, only partial maturation occurs. These results support the existence of a critical window for CM maturation both *in vitro* and *in vivo*.

These results have significant research implications for the use of PSC-CMs. In particular, interventions designed to improve the maturation of PSC-CMs may be limited if they use cells that have already passed the critical window and have undergone subsequent maturation arrest. Likewise, early stage PSC-CMs may be most optimal for regenerative medicine therapies to ensure that transplanted cells achieve full maturity in their transplanted niche.

Regulation of coordinated maturation processes

While a number of studies have aimed to identify factors regulating various individual aspects of CM maturation (Galdos *et al.* 2017)., it is still unclear how maturation is coordinated. A number of individual maturation-related processes have been identified, as discussed in previous sections (e.g. structural, functional, metabolic, cell cycle maturation). However, a major open question is whether these processes are independently regulated or, if they are co-regulated, whether they are organized hierarchically or interdependently. To date, it has been difficult to individually manipulate each maturation-related process *in vivo* to observe the effect on other processes. *In vitro* studies, typically using PSC-CM models, have facilitated this type of perturbation study. Intriguingly, several studies have hinted at significant co-regulation of maturation processes. As an example, fatty acid treatment of PSC-CMs not only results in expected improvements in PSC-CM metabolic maturation, but also leads to improvements in sarcomeric gene expression and structure, calcium handling and cell cycle inhibition (Correia *et al.* 2017; Mills *et al.* 2017). This in turn suggests potential regulation of structural, functional and cell cycle maturation through a metabolic mechanism. In Table 1, we summarize studies in which perturbation of one facet of CM maturation results in novel observations of improvements in other maturation-related processes. What emerges from these data supports the notion of maturation as composed of intertwined regulatory circuits that feed into one another to allow concomitant structural and functional maturation (Fig. 2).

How the interplay between the various facets of CM maturation is regulated remains unknown. It is still unclear whether circuits are co-regulated through direct means, for example a common upstream transcriptional mechanism, or through indirect methods. Moreover, while *in vitro* methods have been highly informative in terms of demonstrating potential co-regulation, the numerous differences between experimental methodologies, as well

Table 1. *In vitro* studies demonstrate co-regulated circuits in maturation

Perturbation	Primary response	Secondary effect	Reference
Structural			
Anisotropic ECM micropatterning	• Myofibril alignment • Increased contractile force	• Improved anisotropic calcium propagation • Improved action potential amplitude and maximum upstroke velocity • Primitive t-tubulation • Improved basal and maximal respiration, and spare respiratory capacity	(Ribeiro *et al.* 2015; Lyra-Leite *et al.* 2017)
3D CM aggregate formation	• Improved sarcomeric gene expression and structure	• Increased oxidative phosphorylation gene expression • Increased TCA cycle flux	(Correia *et al.* 2018)
Functional			
Auxotonic contraction of engineered heart tissue (EHT)	• Increased cellular alignment and sarcomeric structure	• Improved mitochondrial structure • Increased mitochondrial protein content and mass • Increased oxidative metabolism	(Ulmer *et al.* 2018)
Electrical stimulation with increasing intensity	• Increased structural, calcium handling, and mature ion channel gene expression • Improved calcium handling and contraction force	• Improved ultrastructural organization • Improved mitochondrial density • Increased oxidative phosphorylation gene expression and activity	(Ronaldson-Bouchard *et al.* 2018)
Metabolic			
Differentiation with fatty acids and galactose	• Increased oxidative metabolism	• Improved transcription of contractile and sarcomeric genes • Improved sarcomeric structure and alignment • Improved calcium transient velocity • Improved fractional shortening and force generation	(Correia *et al.* 2017)
Palmitate treatment	• Increased fatty acid oxidation and oxidative metabolism	• Cell cycle inhibition • Increased sarcomeric isoform switching	(Mills *et al.* 2017)
Glucose deprivation following differentiation	• Increased mitochondrial structure and oxidative capacity	• Increased sarcomere and contractile gene expression • Cell cycle inhibition • Improved calcium handling dynamics • Increased maximal upstroke velocity	(Nakano *et al.* 2017)

(*Continued*)

Table 1. Continued

Perturbation	Primary response	Secondary effect	Reference
Cell cycle			
Mitomycin C treatment	• Abrogated Ki67 expression and cell cycle cessation	• Increased sarcomere assembly • Improved beat rate	(Zhou *et al.* 2017)

Here, we describe several *in vitro* studies of myocyte maturation in which one pathway of maturation (e.g. structural, functional, metabolic, cell cycle) was perturbed experimentally. We describe putative primary and secondary effects of the intervention. This table is not an exhaustive list of tissue engineered approaches to improved CM maturation; for more comprehensive reviews on that topic, please see Zhu *et al.* (2014) and Scuderi & Butcher (2017). Instead, we compile studies in which perturbation of one maturation pathway led to previously undescribed changes to other maturation-related processes, suggesting potential co-regulation between pathways.

as potential divergence from *in vivo* biology, have made it difficult to identify common mechanisms responsible for coordination of maturation. We anticipate that new studies will shine further light on this question.

Nevertheless, given the tight coordination of the maturation regulatory network, we predict the existence of factors that must be upstream of and simultaneously directly regulate multiple facets of maturation. Here we highlight three factors that may fit this description: PGC1α, SRF and MEF2. We do not intend this to be an exhaustive list, but rather highlight these factors because they have previously been implicated in multiple maturation circuits *in vivo*. These factors are known to interact with one another and have been implicated as key nodes in the CM maturation regulatory network (Xu *et al.* 2009; Schlesinger *et al.* 2011). Moreover, PGC1α and SRF in particular are dysregulated in PSC-CM maturation and may contribute to maturation arrest (Uosaki *et al.* 2015). While it is possible that these factors may exhibit species-specific regulatory patterns, the largely conserved

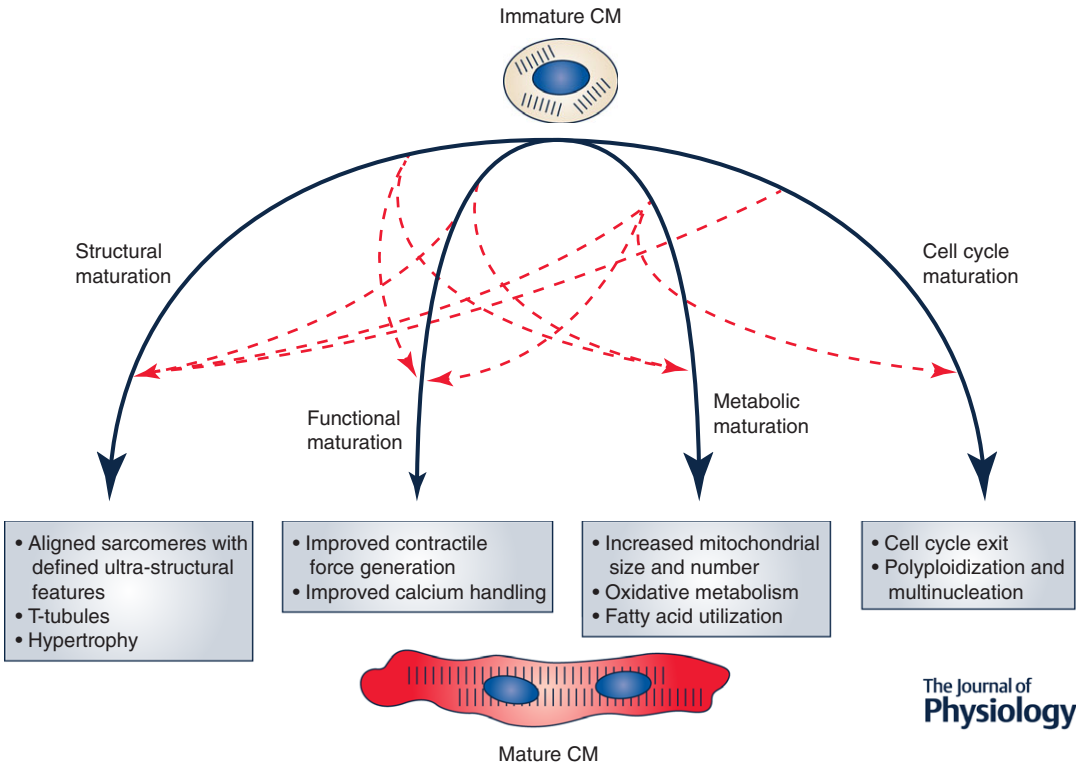

Figure 2. Cross-talk between processes involved in CM maturation
During maturation, CMs undergo significant changes in structure, function, metabolism and cell cycle, among other processes. Evidence from various *in vitro* studies suggests that these processes may function in an interdependent manner, allowing for coordinated CM maturation.

nature of these factors suggests they may play a significant role in CM maturation across species (Uosaki & Taguchi, 2016).

PGC1α

Peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC1α) is a critical regulator of mitochondrial biogenesis in a variety of tissues (Finck & Kelly, 2006) and plays a role in metabolic regulation in the heart in development and disease (Duncan & Finck, 2008). In addition to PPARg, PGC1α binds to a large number of nuclear receptor and non-nuclear receptor transcription factors to mediate chomatin remodelling and gene transcription (Finck & Kelly, 2006). In the mouse fetal heart, PGC1α is a target of glucocorticoid activity (Rog-Zielinska *et al.* 2015) and is expressed beginning at e15.5 (Lai *et al.* 2008). Critically, PGC1α has been implicated in mediating the metabolic switch away from glycolysis to oxidative phosphorylation and fatty acid metabolism (Lehman & Kelly, 2002). Over-expression of PGC1α at birth leads to a dramatic increase in mitochondrial volume density and size (Russell *et al.* 2004). Developmentally, PGC1α has partial redundancy with PGC1b; indeed, while knockout of either alone leads to a minimal phenotype under physiological conditions, the double knockout is lethal shortly after birth (Lai *et al.* 2008). PGC1α/b$^{-/-}$ mice show severe defects in mitochondrial number and size, and demonstrate a failure to transition from anaerobic glycolysis to oxidative metabolism with fatty acid utilization. Interestingly, PGC1α may have effects on CM sarcomeric structure and function as well. For example, the same study showed that PGC1α/b$^{-/-}$ mice display CMs with significant disarray or even absence of sarcomeric structure. In an *in vitro* study of fetal myocytes, PGC1α siRNA-mediated knockdown eliminated myofibril maturation induced by glucocorticoid treatment (Rog-Zielinska *et al.* 2015). Likewise, PGC1α knockdown in PSC-CMs led to decreased CM beat rate, altered action potential and a failure of sarcomeric integrity (Birket *et al.* 2013). Currently, it is thought that PGC1α mediates its effects on sarcomeric organization and contractile function indirectly through energetic/metabolic regulation (Birket *et al.* 2013; Rog-Zielinska *et al.* 2015), though studying maturation-specific direct targets of PGC1α is an area of ongoing investigation.

SRF

Serum response factor (SRF) has been implicated in key processes in mesoderm formation and muscle development (Arsenian *et al.* 1998), and is essential to cardiomyocyte differentiation and maturation (Parlakian *et al.* 2004; Dirkx *et al.* 2013). CM-specific deletion of SRF leads to embryonic lethality between e10.5 and e13.5 in mice, and is characterized by failure of chamber maturation and disruption of the CM contractile apparatus (Parlakian *et al.* 2004; Balza & Misra, 2006). SRF also mediates cardiac function postnatally. For example, Zhang *et al.* (2001) generated a CM-specific SRF mutant with impaired binding of SRF to target binding sites. These mice died within 12 days of birth and demonstrated significant dilated cardiomyopathy. SRF disruption in adult mice similarly leads to dilated cardiomyopathy and heart failure-induced death, with significant defects in CM structural integrity and contractile function (Parlakian *et al.* 2005). It is increasingly recognized that SRF forms a key node in the cardiac transcription network, and may regulate a range of CM processes including contraction, conduction, growth/apoptosis, miRNA regulation, and others (Schlesinger *et al.* 2011; Schueler *et al.* 2012). Intriguingly, SRF may mediate its effects in a stage-specific manner, and may play a particularly critical role in perinatal CM maturation. In a recent study, CRISPR/Cas9-driven knockdown of SRF at p1 led to CM defects in cell size, sarcomeric structure, and T-tubulation, as well as gene-expression changes related to mitochondrial biogenesis and oxidative metabolism (Guo *et al.* 2018*a*). SRF may regulate these latter processes in a multifactorial way – both directly through target gene binding and indirectly through disruption of overall CM cytoarchitecture (Schlesinger *et al.* 2011; Guo *et al.* 2018*a*).

MEF2 family

The myocyte enhancer factor 2 family (MEF2) consists of a family of transcription factors responsible for regulating a range of processes in cardiac development and differentiation. The full dynamics of MEF2 isoform expression is outside the scope of this review and may be found elsewhere (Desjardins & Naya, 2016). Nevertheless, we summarize by noting that expression of individual isoforms of MEF2 initiates between e7.5 and e8.5 in mice; postnatally, MEF2A, MEF2C and MEF2D are expressed (Iida *et al.* 1999). In the perinatal period, these MEF2 isoforms may have non-overlapping and even potentially antagonistic function (Desjardins & Naya, 2017). In particular, MEF2A and MEF2D appear to be required for cell-cycle inhibition and activation of sarcomeric gene expression, while MEF2C performs the opposite function. MEF2A knockout *in vivo* leads to death within the first week of life, with mice exhibiting significant myofibrillar disarray, mitochondrial disorganization, and failure to activate mature gene expression patterns (Naya *et al.* 2002). By contrast, the MEF2D knockout has no phenotype under physiological conditions, though these mice display attenuated hypertrophy and remodelling following application of cardiac stressors (Kim *et al.* 2008). Intriguingly, while MEF2C has primarily been

implicated in early cardiac development (Lin *et al.* 1997), it may regulate mitochondrial function and oxidative metabolism during the perinatal period (Desjardins & Naya, 2017).

Emerging technologies for studying CM maturation

Elucidating the CM maturation regulatory network remains a major area of investigation. It is being appreciated that this network is extraordinarily complex, comprising not only transcription factors (such as those discussed above) but other regulatory molecules such as microRNAs (Kuppusamy *et al.* 2015; Lee *et al.* 2015; White *et al.* 2016; Alfar *et al.* 2018) and long non-coding RNAs (Touma *et al.* 2016), as well as epigenetic regulation (Schlesinger *et al.* 2011). Here, we survey two major scientific tools that we believe will influence ongoing research in maturation: CASAAV and transcriptomics.

CASAAV

In vivo analysis of factors regulating maturation has been limited, owing not only to the challenge of generating mouse models for a large number of candidates, but also due to confounding results from secondary effects of heart failure in knockdown models. The recent development of the CRISPR/Cas9/AAV (CASAAV)-based somatic mutagenesis platform may facilitate future *in vivo* loss-of-function studies (VanDusen *et al.* 2017; Guo *et al.* 2018*b*). In this system, an AAV9 vector delivers guide RNAs (under a ubiquitous promoter) and Cre (under a CM-specific promoter) to Cre-dependent Cas9-P2A-GFP knock-in mice. Thus, knockdown of target genes is done in a CM-specific mosaic pattern, enabling the study of cell autonomous effects of knockdown without confounding secondary effects. Moreover, the use of the CRISPR/Cas9 system enables rapid testing of many target genes. Thus far, this system has been used to study the cell autonomous effects of a variety of genes in cardiac maturation, including junctophilin-2 (Guo *et al.* 2018*b*), GATA4/6 (Prendiville *et al.* 2015) and SRF (Guo *et al.* 2018*a*).

Transcriptomics

In addition to novel methods for performing *in vivo* studies such as the CASAAV system, improving technologies in the field of transcriptomics (particularly RNA-sequencing (RNA-seq)) will enable an improved understanding of regulatory networks in CM maturation. To date,

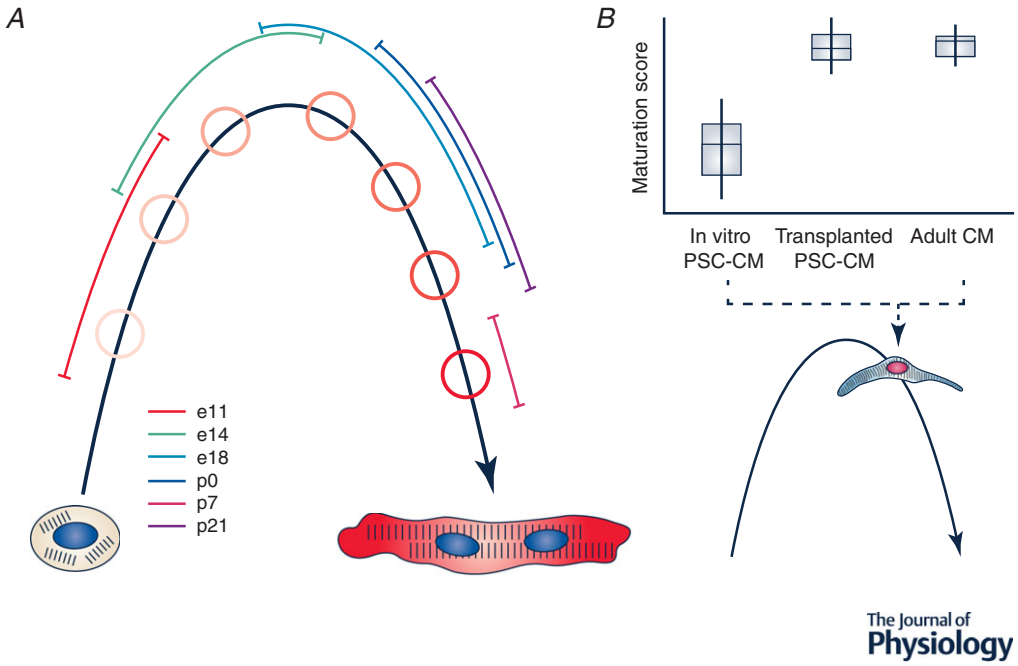

Figure 3. scRNA-seq enables improved understanding of CM maturation
A, scRNA-seq data of CMs at various stages of development reveals that while maturation proceeds in a stage-specific manner, individual CMs proceed heterogeneously across the maturation trajectory before converging on the final mature phenotype. *B*, scRNA-seq profiles may enable more precise benchmarking of PSC-CM maturation. By comparing individual PSC-CMs to *in vivo* CM data, their position along the maturation trajectory can be ascertained and used to quantify a maturation score for single cells. This approach has enabled us to identify, for example, that PSC-CMs transplanted *in vivo* achieve a maturation score greater than those cultured *in vitro* and comparable with adult CMs (authors' unpublished data).

transcriptomic analysis has been used to: elucidate stage-specific regulatory networks guiding cardiac development and maturation (Uosaki *et al.* 2015); identify nucleosome and histone-modifying genes in maturation (van den Berg *et al.* 2015); identify the role of Let-7 family of microRNAs in guiding CM maturation through metabolic switch (Kuppusamy *et al.* 2015); and identify miR-200c as a regulator of mature ion channel expression and calcium handling (Poon *et al.* 2018). In concert with chromatin immunoprecipitation-sequencing (ChIP-seq), transcriptomic analyses have also been used to develop a stronger understanding of epigenetic dynamics of maturation (Sim *et al.* 2015; Gilsbach *et al.* 2018). Lastly, transcriptomics has provided a powerful tool to benchmark the maturation status of *in vitro*-generated CM tissues (Kuppusamy *et al.* 2015; Uosaki *et al.* 2015; van den Berg *et al.* 2015; DeLaughter *et al.* 2016).

Single cell RNA-seq (scRNA-seq) represents a major opportunity for a further understanding of CM maturation. DeLaughter *et al.* (2016) performed a seminal study in which they generated scRNA-seq libraries for $>$ 1,200 cardiac cells from various developmental time points ranging from e9.5 to p21. They subsequently analyzed the developmental dynamics of CMs. As expected, they observed distinct stage-specific progression of maturation, with a notable transition from e14.5 to e18.5/p0 representing the initiation of perinatal maturation. Crucially, however, while transition states were observable on bulk, there was significant heterogeneity of maturation state at any given discrete time point. In particular, between e18.5 and p3, individual cells spanned an overlapping spectrum of maturation states before converging to a final mature phenotype at p21. These results indicate that maturation may be best viewed at the level of the single cell which, upon receiving the appropriate cues, proceeds through maturation at its own unique rate before reaching maturity (Fig. 3*A*).

The use of scRNA-seq may additionally provide a powerful method for benchmarking the precise maturation state of PSC-CMs (Fig. 3*B*). Indeed, to date, one of the primary challenges in PSC-CM research is the lack of a consensus metric or metrics to precisely quantify maturation state, particularly with reference to physiological maturation *in vivo*. scRNA-seq is a particularly useful tool as it integrates information from the range of phenomena perturbed in maturation (e.g. sarcomeric, electrophysiological, metabolic, cell cycle and other changes). By comparing scRNA-seq profiles of PSC-CMs to the inferred trajectory of *in vivo* CM maturation, the maturation state of PSC-CMs can be quantified in a biologically meaningful manner. This analysis may facilitate more comparable and reproducible studies of CM maturation, and may allow further biological insight to be gleaned from studies using PSC-CMs as model systems.

Conclusion

The biology of CM maturation remains a fast-moving and highly exciting area of research, with emerging technologies offering new opportunities for insight. Here, we aim to emphasize the perinatal period as a critical window for maturation, consisting of interconnected regulatory modules guiding concommitant structural and functional maturation of CMs. We believe that new breakthroughs in understanding CM maturation can be leveraged towards improving patient health.

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Additional information

Competing interests

The authors declare no competing interests with regard to this manuscript.

Author contributions

Suraj Kannan was responsible for conception and design of the work, acquisition, analysis and interpretation of data for the work, drafting the work and revising it critically for important intellectual content, approving the final version of the work, and agreeing to be accountable for all aspects of the work. Chulan Kwon was responsible for conception and design of the work, drafting the work and revising it critically for important intellectual content, approving the final version of the work, and agreeing to be accountable for all aspects of the work.

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