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Review

Ploidy in cardiovascular development and regeneration

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ABSTRACT

Somatic polyploidy, a non-inheritable form of genome multiplication, plays cell-type specific and context-dependent roles in organ development and regeneration. In the mammalian heart, embryonic cardiomyocytes are primarily diploid, which lose their ability to complete cell division and become polyploid as they mature. Unlike lower vertebrates like zebrafish, polyploid cardiomyocytes are commonly found across mammals, including humans. Intriguingly, the degree, timing, and modes of cardiomyocyte polyploidization vary greatly between species. In addition to the association with cardiomyocyte development and maturation, recent studies have established polyploidy as a barrier against cardiomyocyte proliferation and heart regeneration following cardiac injury. Hence, a thorough understanding of how and why cardiomyocyte become polyploid will provide insights into heart development and may help develop therapeutic strategies for heart regeneration. Here, we review the dynamics of cardiomyocyte polyploidization across species and how cardiomyocyte-intrinsic, -extrinsic, and environmental factors regulate this process as well as the impact of cardiomyocyte polyploidization on heart development and regeneration.

1. Introduction

Ploidy refers to the total number of chromosome sets in a cell. Polyploidy can occur as a result of genome duplication within a single nucleus or multinucleation, in which each nucleus within a cell contains two or more complete sets of chromosomes. Polyploid cells are observed in various tissues and organs across species and fulfill complex and multifaceted roles under both physiological and pathological conditions. During tissue development and homeostasis, polyploidy has been implicated in the maintenance of the integrity of blood-brain barrier in *Drosophila* [1], milk production in human and mouse mammary gland [2], platelet production by megakaryocytes [3], and maintenance of the urothelial barrier by superficial cells [4]. Under pathological conditions, polyploidization can have both protective and detrimental effects. In the kidney, polyploidization occurs in tubular epithelial cells following acute kidney injury as a compensatory response. While this process helps maintain short-term kidney function through hypertrophy, prolonged polyploidy leads to fibrosis and contributes to the development of chronic kidney disease [5,6]. In the liver, polyploid hepatocytes display increased genetic variability, allowing them to efficiently adapt to xenobiotic influences or nutritional changes [7], and create distinct cellular states that help mitigate age-related liver dysfunction [8]. Although dispensable for homeostatic liver function, hepatocyte polyploidization may have a tumor-suppressive role as livers composed of primarily diploid hepatocytes are more prone to hepatocarcinogenesis [9,10]. These findings suggest that polyploidy is required for specialized functions of differentiated cells and its physiological significance is highly cell type-specific and context-dependent.

In the heart, cardiomyocyte is the main cell type that undergoes developmentally-programmed polyploidization, which was first reported in rodents four decades ago [11]. Unlike cardiomyocyte proliferation that has been extensively studied and reviewed, the regulatory mechanisms of cardiomyocyte polyploidization and their role in heart development, homeostasis, and disease have just started to emerge. In this review, we discuss the cellular and molecular mechanisms of cardiomyocyte polyploidization and recent insights into the role of cardiomyocyte polyploidy under physiological and pathological conditions.

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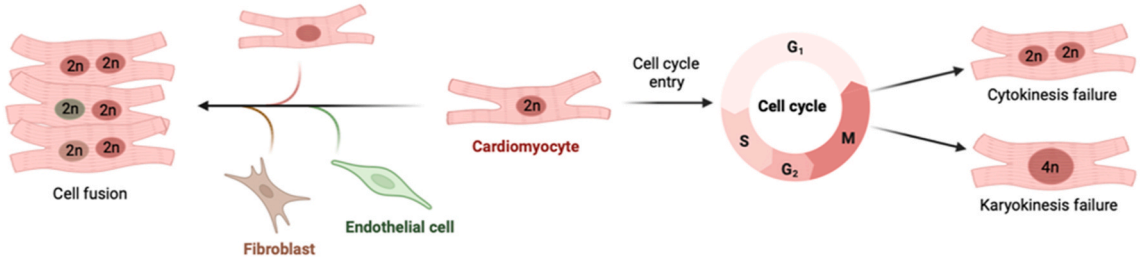
1.1. Modes and timing of cardiomyocyte polyploidization

Cardiomyocyte polyploidization is an example of somatic polyploidization, which occurs during the life cycle of a cell and is therefore non-inheritable. The degree of cardiomyocyte polyploidy varies across species [12], ranging from > 95 % diploid in zebrafish [13] to predominantly polyploid in humans [14], pigs [15], and mice [16]. Intriguingly, when and how mammalian cardiomyocytes become polyploid differs between species.

1.1.1. Modes

Cardiomyocyte polyploidization is typically the result of

A



B

Species \ Mode	Developmental stage		Mononuclear (2n)	Mononuclear (≥4n)	Binuclear (2x2n)	>2 nuclei
Human	Embryonic		~99%	-	-	-
	Postnatal	First decade	~55%	~25%	~20%	
		Second decade	~40%	~40%	~20%	
Mouse	Embryonic		~99%	-	-	-
	Postnatal	P3	~80%	-	~20%	-
		P7	~20%	-	~80%	-
	Adult		-	-	~99%	-
Opossum	Embryonic		~99%	-	-	-
	Postnatal	P14	~80%	-	~20%	-
		P29	~20%	-	~80%	-
	Adult	P56	~10%	-	~90%	-
Pig	Embryonic		~99%	-	-	-
	Postnatal	P15	~50%	-	~50%	-
		P30	~10%	-	~60%	~30%
	Adult	6 month	-	-	~10%	~90%
Sheep	Embryonic	E95	~99%	-	-	-
		E135	~50%	-	~50%	-
	Postnatal	P0	~30%	-	~70%	-
		P60	~10%	-	>80%	~5%
Zebrafish	Whole lifespan		~99%	-	-	-

Fig. 1. Modes and timing of cardiomyocyte polyploidization (A) In a cell cycle-dependent manner, polyploid cardiomyocytes arise from defective karyokinesis (1x4n) or cytokinesis (2x2n). Alternatively, a small proportion of polyploid cardiomyocytes is generated by cell fusion with cardiomyocytes, endothelial cells or fibroblasts independent of cell cycle entry. (B) Summary of modes and timing of cardiomyocyte polyploidization across species. Rare cardiomyocyte population (e.g., 1x8n or 4x2n) are not depicted here.

endomitosis. In an archetypical cell cycle, a cell replicates its DNA during S-phase, followed by chromosomal segregation (i.e., karyokinesis) and cell division (i.e., cytokinesis) to generate two daughter cells. In contrast, endomitosis leads to the generation of different polyploid cells depending on the type of perturbations; defective karyokinesis results in a single polyploid nucleus while defective cytokinesis results in multinucleated cells with diploid or polyploid nuclei (Fig. 1A).

In mice [11,16] and rats [17], embryonic cardiomyocytes are primarily mononuclear diploid while the majority of their postnatal and adult counterparts are binucleated, correlating with their differential ability to complete cytokinesis. While embryonic cardiomyocytes undergo cytokinesis efficiently [18,19], cytokinesis defects characterized by mis-localization of ANLN (Anillin) [20], RHOA (Ras Homolog Family Member A) and IQGAP3 (IQ Motif Containing GTPase Activating Protein 3) [19], and defective midbody positioning [21] are common in postnatal rodent cardiomyocytes. Intriguingly, most pig cardiomyocytes are mononucleated at birth and harbor up to 16 nuclei per cell at 6 months of age [15], suggesting that they undergo multiple rounds of cytokinesis failure post birth. Cell fusion, a process critical for skeletal muscle development and polyploidization [22], has been reported in mouse cardiomyocytes [23] and contribute to their polyploidization in the postnatal heart, albeit at a low frequency [24].

Different cellular processes are thought to contribute to karyokinesis and/or cytokinesis defects in cardiomyocytes. Myofibril disassembly during metaphase and anaphase is critical for successful cytokinesis [25], and its absence leads to binucleation [20,25]. Binuclear cardiomyocytes often exhibit chromosomal abnormalities, such as nuclear bridging and micronuclei formation, caused by telomere dysfunction [26]. In postnatal mouse hearts, binuclear cardiomyocytes exhibited a 227 % increase in telomere fusions compared with their mononuclear counterparts [27]. Despite this correlation, whether and how telomere fusion directly contributes to postnatal mouse cardiomyocyte polyploidization is unclear.

Human cardiomyocytes are also primarily polyploid, however, the proportion between multinucleated and mononuclear polyploid cells differs significantly from the above-mentioned species. Instead of multinucleation, the majority of human cardiomyocytes contains only one nucleus (75 %), of which around half are polyploid (up to 16 n) [28,29]. These observations suggest that, unlike mice and pigs, human cardiomyocytes become polyploid mainly due to karyokinesis defects. Interestingly, single-nuclei sequencing and lineage analysis of healthy human hearts revealed that approximately 10 % of tetraploid cardiomyocytes contain somatic single nucleotide variants from distinct clades [30], indicating fusion of genetically distinct cells contributes to human cardiomyocyte polyploidization. The physiological and functional significance of different modes of cardiomyocyte polyploidization in mammals, however, is currently unknown and requires further investigation.

1.1.2. Timing

In addition to cellular mechanisms, the timing of cardiomyocyte polyploidization also differs across species (Fig. 1B). In humans, cardiomyocyte nuclei remain primarily diploid throughout the first decade of life, and the average ploidy levels per nucleus increases by approximately 1.7-fold in the second decade and remains stable thereafter [14]. Another study reported that total polyploid cardiomyocytes, including both mononuclear polyploid and multinucleated, increase from ~40 % at 1 year after birth to ~58 % at 10 – 20 years of age and remain stable afterwards [28]. Unlike humans, cardiomyocyte polyploidization happens more rapidly post birth in several other species. In mice, over 80 % of cardiomyocytes become binucleated within the first week of life, starting approximately two days post birth [16]. Additionally, mouse cardiomyocytes with three (such as 2x2n; 1x4n) or four nuclei, which potentially result from a combination of karyokinesis and cytokinesis defects, have also been reported [31,32]. The binuclear proportion of bird cardiomyocytes also increases rapidly to approximately 30 % within two weeks after birth and then maintains a constant proportion [33]. In opossums, similar to mice, adult cardiomyocytes are predominantly binuclear, though this transition occurs later. Initially mononuclear during the first two weeks of age, these cardiomyocytes gradually binucleate, reaching about 90 % by postnatal day 56 (P56) [34]. Swine cardiomyocytes, on the other hand, display distinct timing of polyploidization. After birth, cardiomyocytes quickly binucleate, reaching about 50 % binucleation by P15 [15], which is followed by a significant rise in tetranucleated cells, reaching approximately 30 % within two weeks. By two months of age, cardiomyocytes with 6–8 nuclei can be observed and become the predominant ploidy class by six months. These sequential stages of polyploidization imply a regulated, stage-specific mechanism guiding myocardial development, with binucleated cells potentially serving as a "reservoir" for further polyploidization [15]. In sheep, however, the onset of cardiomyocyte polyploidization occurs before birth [35,36], suggesting that birth may act as a trigger but not the sole cause of polyploidization.

Importantly, correct timing of cardiomyocyte polyploidization is required for proper heart development. Premature cardiomyocyte polyploidization has been observed in humans, mice and *Drosophila* with cardiac developmental defects such as dilated cardiomyopathy, tetralogy of Fallot, and hypertrophy [37–39]. Consistently, experimentally-induced premature cardiomyocyte polyploidization in embryonic mice by *Rbpms* [40] and *Ect2* [41] loss-of-function was accompanied by heart malformation and lethality. However, despite these correlations, the casual relationship between induced cardiomyocyte polyploidy and heart defects remains to be formally established. In *Drosophila*, cardiomyocyte polyploidization is crucial for proper development and function of the heart, and inhibition of cardiomyocyte polyploidization resulted in smaller hearts with lower stroke volume and cardiac output [42]. In contrast, congenital cardiac malformations in humans have been associated with altered extent and timing of cardiomyocyte multinucleation. While human hearts only contain approximately 20 % multinucleated cardiomyocytes throughout life, patients with tetralogy of Fallot experience a steep increase in multinucleation up to 60 % mainly occurring in the first 2 months post birth [38,43].

The data described here suggest that the modes and timing of cardiomyocyte polyploidization is highly species-dependent. What underlies these differences and how it correlates with the physiology of the heart across species is currently unclear.

2. Molecular mechanism of cardiomyocyte polyploidization

In recent years, several cardiomyocyte-intrinsic (i.e., cell-autonomous) and -extrinsic factors have been identified to modulate different stages of cardiomyocyte cell cycle. While factors that enable adult cardiomyocytes to re-enter cell cycle have been discovered, they are not necessarily sufficient to enable cell division and result in further polyploidization. In this part, we will focus on factors and signaling pathways that were shown to affect cardiomyocyte karyokinesis and/or cytokinesis but not solely cell cycle entry (Fig. 2A).

2.1. Cardiomyocyte-intrinsic mechanisms

During postnatal heart development, cardiomyocytes undergo extensive epigenetic and transcriptional changes that contribute to the switch from cell division to polyploidization. Several known cell cycle regulators, such as cyclins and cyclin-dependent kinases (Cdks) have been shown to affect both cardiomyocyte proliferation and polyploidization [16,44]. *Ccng1* is upregulated postnatally and increases cardiomyocyte multinucleation, likely via cell cycle checkpoint activation [44]. On the other hand, *Cdk1*, *Cdk4*, *Ccnb1*, and *Ccnd1*, critical regulators of cell cycle progression, are progressively downregulated in mice from fetal to adult stages. Overexpression of *Ccnd1* induced cardiomyocyte cell cycle-entry and multinucleation [45], while loss of *Cdk1*

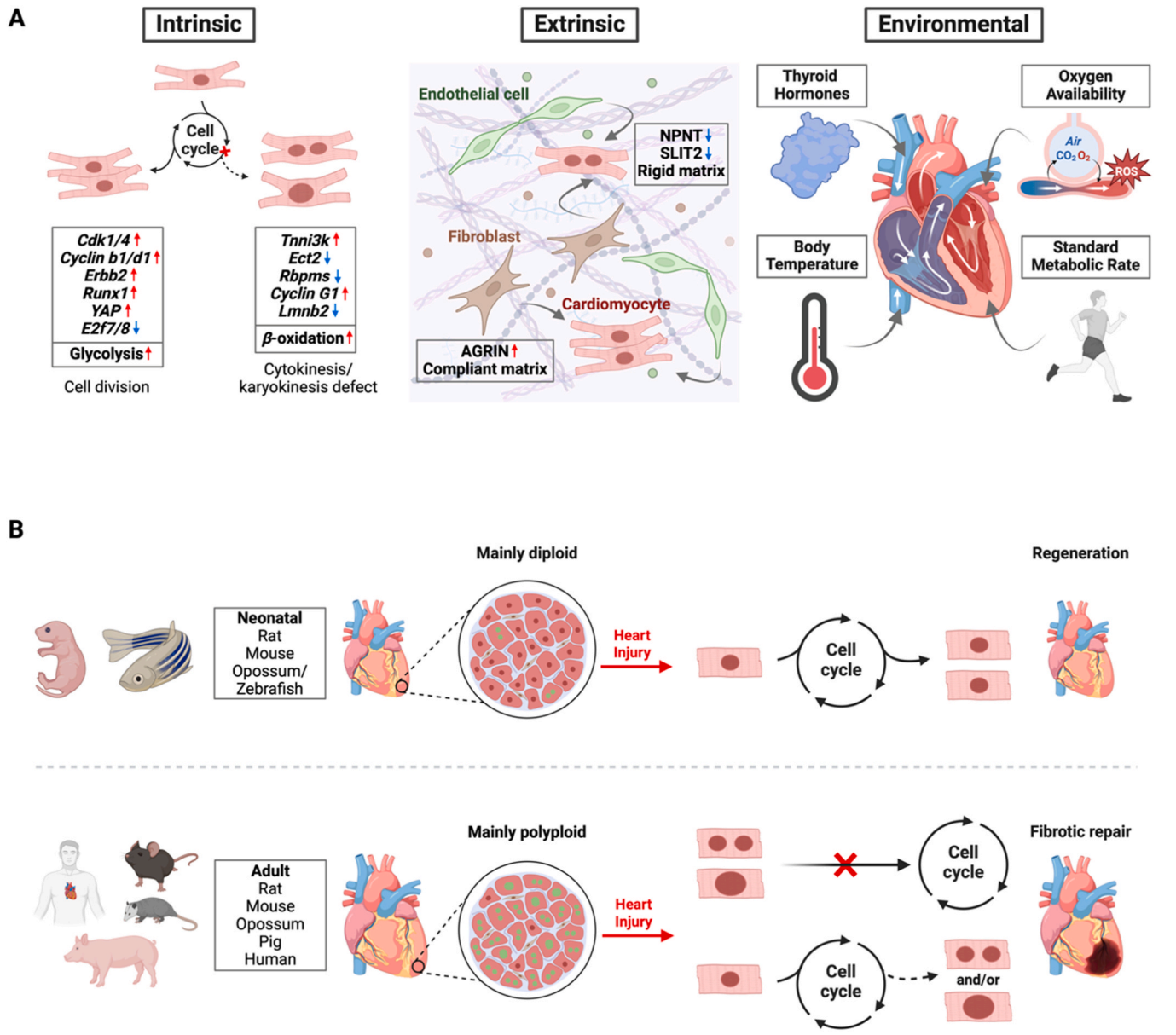


Fig. 2. Regulators of cardiomyocyte polyploidization and its role in heart regeneration (A) Cardiomyocyte polyploidization is regulated by factors intrinsic to cardiomyocytes (left), non-myocytes and the ECM (middle), as well as environmental factors (right). Red arrows indicate upregulation/higher abundance of genes or proteins, and blue arrows indicate downregulation/lower abundance of genes or proteins. (B) Organisms with predominantly diploid cardiomyocytes (dark red nuclei), such as neonatal mice and zebrafish, are able to effectively complete cell cycle and regenerate the heart following injury. In contrast, hearts with high levels of polyploid cardiomyocytes (green nuclei), such as those of adult mice and humans, undergo fibrotic repair following cardiac damage.

extended the postnatal proliferative phase and led to more mononuclear cardiomyocytes [46]. Intriguingly, overexpression of a combination of these four factors was sufficient to promote adult cardiomyocyte cell cycle entry and cytokinesis both *in vitro* and *in vivo* [47,48]. ECT2, a small GTPase involved in the formation of the cytokinetic furrow, is also developmentally downregulated in cycling mouse cardiomyocytes [38]. Inactivation of *Ect2* in mouse cardiomyocytes led to increased cardiomyocyte binucleation due to impaired cytokinesis, causing perinatal lethality without affecting cell cycle entry [38]. Consistent with these observations, transient overexpression of a dominant-negative form of *ect2* in zebrafish cardiomyocytes (primarily diploid) resulted in highly polyploid hearts that are unable to regenerate [13]. In addition to cell cycle machinery, various signaling pathways have been implicated in cardiomyocyte cell cycle activity and cytokinesis. Hippo signaling activity increases sharply in mouse hearts from P2 to P10, correlating with the onset of cardiomyocyte polyploidization [49]. Overexpression of a constitutively-active form of Yap was sufficient to induce expression of cell cycle genes and a fetal gene program in adult mouse cardiomyocytes, leading to cell cycle re-entry, cytokinesis, and a significant increase in cardiomyocyte number [50,51]. Similar findings have been reported for *Erbb2,* a co-receptor for NEUREGULIN 1. *Erbb2* is downregulated in the postnatal mouse heart and overexpression of a constitutively-active form of *Erbb2* in cardiomyocytes was sufficient to promote cardiomyocyte dedifferentiation, cell cycle entry, and cytokinesis [52,53]. Additionally, the E2F family transcription factors, *E2f7* and *E2f8*, have been identified as positive regulators of mouse postnatal cardiomyocyte polyploidy; cardiomyocyte-specific *E2f7/8* double knockout resulted in a 10-fold increase in mononuclear diploid cardiomyocytes in adult mice [54]. Incorrect post-transcriptional modification can also affect cardiomyocyte binucleation. *Rbpms*, a RNA-binding factor, is essential for proper heart development. *Rbpms* knockout resulted in the accumulation of the short isoforms of the

heart-enriched LIM domain protein *Pdlim5*, which promoted cardiomyocyte binucleation in both mouse and human cardiomyocytes [40].

The degree of cardiomyocyte ploidy is also determined by genetic background. A comparison of 120 inbred mouse strains revealed a significant variability in the degree of mononuclear diploid cardiomyocyte in the adult heart, ranging from 2 % to 15 % across strains. Subsequent genome-wide association analysis revealed that this variability is associated with *Tnni3k*, a cardiomyocyte-specific kinase [55]. However, how TNNI3K modulates cardiomyocyte cell cycle activity, progression, and cytokinesis is not entirely clear. Mouse strains with a hypomorphic allele of *Tnni3k* (e.g., A/J) showed a significantly increased proportion of mononuclear diploid cardiomyocytes compared with strains harboring a functional allele such as C57BL/6 J [56]. Genome-wide association study between these strains further identified the transcription factor RUNX1 as a positive regulator of cardiomyocyte polyploidy; A/J mice (15 % mononuclear diploid cardiomyocytes) have almost 3-fold more cardiomyocytes that were RUNX1-positive compared to C57BL/6 J (5 % mononuclear diploid cardiomyocytes) at P21. Consistently, overexpression of *Runx1* during the first postnatal week in C57BL/6 J mice increased cardiomyocyte proliferation and produced a higher proportion of diploid cardiomyocytes, mimicking the A/J phenotype [32].

One hallmark of cardiomyocyte maturation is the metabolic switch from glycolysis to fatty acid oxidation after birth [57]. While fatty acid oxidation is more energy-efficient, it produces reactive oxygen species (ROS) and triggers DNA damage responses, which has been shown to drive cardiomyocyte cell cycle exit in postnatal mice [58]. In line with these observations, promoting glycolysis in mice by fat-free diet or cardiomyocyte-specific *Pdk4* knockout increased cardiomyocyte cell cycle activity as well as cytokinesis completion, suggesting that metabolism plays a direct role in regulating cardiomyocyte polyploidy [59].

While the majority of mouse cardiomyocytes contain two diploid nuclei (i.e., 2x2n), additionally, a small population of mononuclear or multinuclear polyploid cardiomyocytes (i.e., 1x4n, 2x2n or higher) has been reported [32,54], suggestive of karyokinesis defects. A recent study has reported the nuclear lamina protein LAMIN B2 (Lmnb2) as a regulator of mouse cardiomyocyte karyokinesis. LMNB2 facilitates the breakdown of the nuclear envelope, a necessary step for chromosome alignment and M-phase progression. Consequently, cardiomyocytes lacking *Lmnb2* showed impaired nuclear envelope breakdown, leading to defective spindle microtubule attachment and increased nuclear ploidy levels but not the number of nuclei postnatally [60].

2.2. Cardiomyocyte-extrinsic mechanisms

In addition to cell-intrinsic signals, cardiomyocyte function and behavior is affected by a range of extrinsic factors such as paracrine signals from non-myocytes and environmental factors like thyroid hormone levels, oxygen availability, and the stiffness and composition of the extracellular matrix (ECM) they are anchored to. In this part, we will review how these environmental factors contribute to cardiomyocyte polyploidization.

Energy metabolism during heart development is strongly linked to polyploidy. After birth, the basal metabolic rate increases significantly as newborns must regulate body temperature, digest food, maintain organ function, and support growth. Evaluation of ploidy levels across 41 vertebrate species indicated a strong correlation between metabolic rate and cardiomyocyte polyploidy [12]. Ectothermic species, such as fish and reptiles with lower metabolic rates, exhibited lower levels of polyploidy, while endothermic animals, like mammals with higher metabolic rates, displayed a greater degree of cardiomyocyte polyploidy [12]. Metabolic rate is largely regulated by body temperature and thyroid hormone signaling, linking these factors and cardiomyocyte ploidy. In mice, thyroid hormone levels increase rapidly in the neonatal period, coinciding with the onset of cardiomyocyte polyploidization. Pharmacological and genetic inhibition of thyroid hormone signaling increased the proportion of proliferating and diploid mouse cardiomyocytes, suggestive of cytokinesis completion [12]. These effects are further augmented by simultaneous inhibition of adrenergic receptors [61]. Mechanistically, thyroid hormone receptor alpha directly targets several mitochondrial genes such as *Cpt2*, which is involved in β-oxidation of fatty acids. Consistently, partial depletion of *Cpt2* in cardiomyocytes promoted their proliferation and the proportion of diploid cardiomyocytes [12]. These findings highlight the hormonal control of metabolism and cardiomyocyte polyploidization, which may explain, at least in part, the variance of the degree of cardiomyocyte polyploidy across different species.

In addition to thyroid hormone, oxygen levels also rise substantially in the early postnatal period as the pulmonary system transitions to supplying oxygen via the lungs [62]. The oxygen-rich postnatal environment increases the production of ROS, leading to oxidative damage, activation of the DNA damage response, and increase in mouse cardiomyocyte polyploidization [58]. These observations are supported by a hypoxia fate-mapping study in which hypoxic mouse cardiomyocytes were found to be smaller, more proliferative, and have fewer nuclei [63], potentially via HIF1α-mediated repression of the transcription factor *Atf4* and deactivation of p53 [64].

The ECM, which is mainly produced, secreted, and maintained by fibroblasts, is not only a structural scaffold but influences several cellular processes, including cell migration, differentiation, proliferation, and adhesion. Depletion of periostin-positive activated cardiac fibroblasts in postnatal mice significantly increased the proportion of mononuclear (and presumably diploid) cardiomyocytes [65], suggestive of an important role of the ECM in cardiomyocyte polyploidization. Specific ECM proteins have been identified as regulators of cardiomyocyte proliferation. For example, AGRIN, a proteoglycan secreted by endothelial cells, was present in the ECM of neonatal mouse hearts but reduced in abundance in the early postnatal period. Treatment with recombinant AGRIN was sufficient to promote cardiomyocyte proliferation both *in vitro* and *in vivo* following myocardial infarction, likely through the DAG1 receptor and activation of the YAP signaling pathway [66]. Furthermore, embryonically-enriched cardiac ECM proteins like SLIT2 and NPNT have been shown to promote postnatal mouse cardiomyocyte cytokinesis [67]. In addition to specific ECM components, mechanical properties of the ECM also influence cardiomyocyte cell cycle behavior. Neonatal mouse cardiomyocytes cultured on compliant matrices (5 kPa) exhibited increased cell cycle activity, whereas those on rigid matrices (2 MPa) showed aligned sarcomeres and increased cytokinesis defects [68]. Altogether, these observations highlight the complex regulatory mechanisms of cardiomyocyte polyploidization by environmental factors. In addition to fibroblasts, it would be interesting to investigate whether and how other non-myocytes in the heart, e.g., endothelial cells, immune cells, epicardial cells, contribute to cardiomyocyte polyploidization.

3. The role of polyploidy in cardiomyocyte renewal and heart regeneration

In contrast to lower vertebrates like zebrafish and newt, mammals possess very limited heart regenerative capacity, which can be attributed to the low proliferative capacity of adult mammalian cardiomyocytes. Contrary to other cell types like hepatocytes, adult human and mouse cardiomyocytes are considered largely post-mitotic under both physiological [69,70] and pathological conditions [71]. Understanding what regulates mammalian cardiomyocyte cell cycle exit and hence the lack of regenerative capacity has been a subject of intense interest. Recent studies on various model organisms have established a clear correlation between cardiomyocyte ploidy and the natural regenerative capacity of the heart (Fig. 2B).

3.1. Increased cardiomyocyte ploidy levels are associated with loss of cardiac regeneration capacity

In mice, embryonic cardiomyocytes are primarily mononuclear diploid and can regenerate upon genetic ablation [72]. After birth, mouse cardiomyocytes rapidly become binucleated from less than 10 % at P3 to over 80 % by P7 [16]. This transition from cytokinesis to polyploidization coincides with the loss of proliferative and regenerative capacity at P7 [73]. Consistently, in adult mice, the degree of polyploid cardiomyocytes varies across inbred mouse strains (from 85 % to 98 %), which negatively correlate with the degree of cardiomyocyte proliferation and functional recovery following myocardial infarction [55]. Opossum cardiomyocytes, on the other hand, remain primarily mononucleated in the first two weeks of age. Correspondingly, cardiac injury at P14 (when cardiomyocytes are mostly mononucleated) resulted in augmented cardiomyocyte proliferation and functional recovery; these responses were significantly blunted following cardiac injury at P29 when 25 % of cardiomyocytes have become binucleated [34]. On the contrary, zebrafish, whose cardiomyocytes are primarily diploid throughout life [13], can efficiently replace lost cardiomyocytes following cardiac injury via proliferation of pre-existing cardiomyocytes [74,75]. Altogether, these findings highlight that cardiomyocyte ploidy levels negatively correlate with the natural regenerative capacity of the heart across species. However, one notable exception is pigs, in which heart regenerative capacity is lost at P2 [76] when the majority of cardiomyocytes are still mononucleated [15], suggesting that, in addition to cardiomyocyte ploidy, other cell types/environmental factors (e.g., the ECM) are also critical in determining the regenerative outcome following cardiac injury.

The correlation between polyploid cardiomyocytes and the lack of heart regenerative capacity is further supported by several lines of experimental evidence. Zebrafish cardiomyocytes are predominantly diploid, and their polyploidization can be induced by transient overexpression of a dominant-negative form of *ect2*, which causes karyokinesis and cytokinesis defects [13]. Intriguingly, although zebrafish with a high level of polyploid cardiomyocytes (combination of multinucleated and mononuclear polyploid) are viable and healthy, cardiomyocyte proliferation as well as heart regeneration were significantly inhibited compared with their diploid-enriched counterparts following cardiac injury [13]. Manipulation of *Lmnb2*, a karyokinesis regulator, also modulates the regenerative outcome following cryoinjury of P1 mice. Overexpression of *Lmnb2* increased the proportion of mononuclear diploid cardiomyocytes, and was sufficient to promote cardiomyocyte proliferation and functional recovery of the heart following cryoinjury at P1 [60], which, unlike apical resection and myocardial infarction models, has been reported to be non-regenerative [77]. Genome-wide association study across 120 inbred mouse strains have identified *Tnni3k*, a cardiomyocyte-specific kinase, as a regulator that influences the variation of the proportion of mononuclear diploid cardiomyocytes in adult mice. Correspondingly, cardiomyocyte-specific *Tnni3k* knockout in C57/Bl6 mice resulted in an increase in mononuclear diploid cardiomyocytes as well as cardiomyocyte proliferation following myocardial infarction, while *tnni3k* overexpression in zebrafish increased cardiomyocyte nuclear ploidy and compromised heart regeneration [55]. However, these findings are questioned by a recent study showing that (1) *Tnni3k* overexpression in D2J mouse strain (which normally lacks TNNI3K) resulted in a significantly increased cardiomyocyte cell cycle activity following cardiac injury, and that (2) the majority of cycling adult cardiomyocytes increase in nuclear ploidy irrespective of the abundance of TNNI3K (comparing DBA/2J and C57Bl6/NCR strains) [71]. Intriguingly, in contrast to the DBA/2J strain, A/J strain (another *Tnni3k* hypomorphs) and *Tnni3k* global knockout mice established and maintained on an isogenic C57Bl/6J background showed increased cardiomyocyte cell division following cardiac injury [56]. The mechanims underlying these strain-specific differences in TNNI3K function in cardiomyocyte proliferation requires further investigation. Inhibition of thyroid hormone and ß-adrenergic signaling alone [12,38] or in conjunction [61] in neonatal mice increased diploid cardiomyocyte content and promoted cardiomyocyte proliferation and heart regeneration following myocardial infarction and ischemic-reperfusion in non-regenerative P14 or adult mice. In line with these findings, thyroid hormone treatment increased the percentage of binucleated cardiomyocytes in adult zebrafish heart, which reduced cardiomyocyte proliferation and regeneration following apical resection [12]. Altogether, the data described here suggest that heart regeneration is tightly linked to cardiomyocyte ploidy status.

3.2. Induced cell cycle re-entry of adult cardiomyocytes leads to further polyploidization

Similar to their postnatal/juvenile counterparts, adult mammalian cardiomyocytes are also susceptible to polyploidization under pathological conditions. Following myocardial infarction, cell cycle activity can be detected in a small population of cardiomyocytes at the wound border zone. However, adult cardiomyocyte cell cycle activity usually results in increase in ploidy but not the generation of new cells. Using multi-isotope imaging mass spectrometry [70], genetic lineage-tracing [78] and base analogs pulse-chase approaches [31,32,71], it is estimated that, following myocardial infarction, only a minor population of cycling adult mouse cardiomyocytes undergo cytokinesis and divide, and that this cell division capacity varies between mouse strains. Intriguingly, similar observations have been made in experimental model where diploid cardiomyocytes are significantly enriched in adult mice. Cardiomyocyte-specific knockout of *E2f7* and *E2f8*, two E2F transcription factors known to promote hepatocyte polyploidization [79], resulted in a 10-fold increase in mononuclear diploid cardiomyocytes in adult mice. Following myocardial infarction, despite augmented cardiomyocyte cell cycle activity as expected, the majority of them became mononuclear polyploid (presumably via karyokinesis defect) and the heart failed to regenerate similar to control mice whose cardiomyocytes are primarily polyploid [54]. Analogous to observations in mice, a significant increase in nuclear ploidy (likely a result of defective karyokinesis) has been reported in human cardiomyocytes from heart failure patients [29,80]. Altogether, these results suggest that (1) cycling adult cardiomyocytes (likely diploid) are also prone to karyokinesis and cytokinesis defects, which may explain, at least in part, why adult mammalian hearts cannot regenerate efficiently via proliferation of pre-existing cardiomyocytes, and that (2) approaches that augment cardiomyocyte cell cycle re-entry alone are not necessarily sufficient to promote heart regeneration. Hence, a mechanistic understanding of how cardiomyocyte polyploidization is regulated may help develop new therapeutic approaches to promote adult cardiomyocyte cell cycle re-entry as well as successful cell division.

3.3. Role of polyploid cardiomyocytes in heart regeneration?

Polyploid cells are typically considered post-mitotic and their proliferation is associated with aberrant cell division [81]. These findings raise the question whether adult polyploid cardiomyocytes can enter the cell cycle and proliferate to generate new cells. Several studies in hepatocytes and cardiomyocytes have provided insights into this question. Hepatocytes undergo developmentally-programmed polyploidization (increase in both nuclear ploidy and nucleation) that begins at weaning and increases with age [82]. Intriguingly, both diploid and polyploid hepatocytes can enter the cell cycle, undergo centrosome clustering, and divide in a bipolar manner to generate new hepatocytes [9,83]. While the underlying molecular mechanisms require further investigation, these findings suggest that polyploid cells have the ability to enter the cell cycle and divide. Intriguingly, proliferation of polyploid mammalian cardiomyocytes has been reported as well. Live imaging analysis revealed that a small population of primary postnatal rat binuclear cardiomyocytes can enter the cell cycle and divide in a bipolar manner

similar to hepatocytes [19]. Proliferation of normally non-proliferative polyploid cardiomyocytes (P7 - adult) can be augmented upon different experimental stimulations, including co-culture with neonatal rat cardiomyocytes [84], induction of a constitutively-active form of *Erbb2* [52], and a combinatorial overexpression of *Oct4*, *Sox2*, *Klf4*, and *c-Myc* [85]. These *in vitro* findings indicate that, although polyploid cardiomyocytes are often considered terminally differentiated and post-mitotic, they may retain some proliferative potential and may contribute to cardiomyocyte regeneration under the right conditions.

4. Perspectives

Cardiomyocyte polyploidization was discovered more than four decades ago, and we are just now starting to gain insights into the regulation of cardiomyocyte polyploidy and its role in heart development and regeneration, with many questions remaining to be answered. For example: (1) Non-myocytes are critical in different aspects of heart development, homeostasis, and disease. While most studies to date focused on cardiomyocyte-intrinsic regulatory mechanisms, how cell-cell and cell-ECM interactions modulate cardiomyocyte polyploidization remain obscure. (2) The degree and modes of polyploid cardiomyocyte vary significantly across species, ranging from >95 % mononuclear diploid in zebrafish, >90 % multinucleated in rodents, and a mixture of multinucleated and mononuclear polyploid in humans. These observations argue that polyploidization is not an absolute necessity for cardiomyocyte maturation and function. Understanding the functional and physiological significance of different modes and degree of cardiomyocyte ploidy will provide insights into what underlies these species-specific differences. (3) Diploid mammalian cardiomyocytes have been viewed as a unique population that is smaller in size, less mature, and more proliferative compared to their polyploid counterparts, which may hold the key to successful heart regeneration. This notion is not supported by transcriptomic analysis, in which only minimal differences have been identified between diploid and polyploid adult cardiomyocytes in both mice and humans [29,86]. It would be interesting to investigate the post-transcriptional differences between diploid and polyploid cardiomyocytes, e.g., the proteome, and how they influence cardiomyocyte behavior under physiological and pathological conditions. With rapid technological advances in single-cell OMICs, human engineered heart tissue and organoids, and methodologies to more reliably assess cardiomyocyte ploidy [31,87–90], we anticipate significant progress in our understanding of the regulatory mechanisms and physiological importance of cardiomyocyte polyploidization in the near future.

Declaration of Competing Interest

All authors declare no competing interests.

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