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Relevance of the plasma membrane calcium-ATPase in the homeostasis of calcium in the fetal liver

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During the early stages of development, the embryo depends on the placenta as provider of oxygen and calcium, among other essential compounds. Although fetal liver accomplishes a well-known haematopoietic function, its contribution to calcium homeostasis upon development is poorly understood. The homeostasis of cell calcium contributes to diverse signaling pathways across developmental stages of most tissues and the calcium-ATPase located at the plasma membrane (PMCA) helps pumping excess calcium into the extracellular space. To date, the understanding of the equilibrium shift between PMCA isoforms during liver development is still missing. This review focuses on the characterization of the hepatic PMCA along the early stages of development, followed by a description of modern approaches to study calcium homeostasis involving several types of pluripotent cells. The application of interdisciplinary techniques to improve our understanding of liver development and the role calcium homeostasis plays in the definition of pathogenesis is also discussed.

Introduction

Our current knowledge of the mechanisms that regulate calcium homeostasis of fetal liver during the embryonic development remains incomplete. This review establishes the most important differences between fetal and adult liver physiology in order to highlight how calcium contributes to the specific physiological demands of each developmental stage.

Approximately 80% of liver tissue contains parenchymal cells (i.e., hepatocytes), while the remaining 20% includes nonparenchymal cells such as cholangiocytes, sinusoidal endothelial cells, Kupffer cells, lymphocytes, biliary cells, and hepatic stellate cells (or Ito cells). A fetal liver also contains stromal cells including macrophage, endothelial cells, epithelial cells, and fibroblasts that produce cytokines and synthesize extracellular matrix proteins.[1] Although nonparenchymal cells represent a small proportion of the total hepatic tissue, they are key contributors for proper hepatocyte function that is mediated by paracrine mechanisms under normal and pathological conditions. Furthermore, during the fetal stages, nonparenchymal cells are needed to support the differentiation of hepatoblasts into hepatocytes.[2,3] *In vitro,* nonparenchymal cells also importantly contribute to obtain hepatocyte-like cells from embryonic stem cells (ESCs).[4]

In contrast to the active proliferation observed in fetal hepatoblasts and hepatic progenitor cells during liver organogenesis, the adult liver has been considered as a quiescent tissue since it has been reported that one mitosis is detected for every 10,000 to 20,000 cells.[5] However, following exposure to certain chemicals or physical injury, mature hepatocytes reenter the cell cycle in a process known as liver regeneration (or compensatory hyperplasia) showing an extraordinary replicative capacity. In mice, this process involves only hypertrophy, or hypertrophy and hyperplasia after 30% and 70% partial hepatectomy, respectively.[6] Also, the nuclei number does not change and ploidy slightly increases in regenerated liver after 30% hepatectomy; nevertheless, nuclei number decreases and ploidy increases after 70% hepatectomy. Interestingly, in fetal and adult stages, even while regenerating, the liver performs complex functions involving the intermediary metabolism of amino acids, lipids, and carbohydrates, as well as the detoxification of xenobiotics and bile secretion. Moreover, functions such as vesicular trafficking, cell growth regulation, glycogen metabolism, and importantly, the synthesis of bile salts among others are related to the homeostasis of calcium in the liver. The canalicular membranes show a specialized composition to perform the secretion of bile including a 2-fold higher content of sphingomyelin and cholesterol than those present in basolateral and sinusoidal domains.[7]

Although the liver is not an excitable tissue, when several receptors are activated in the plasma membrane of hepatocytes, phosphoinositide signaling cascades are triggered and Ca^{2+} oscillations originating in the endoplasmic reticulum constitute part of the calcium signaling process.[8] In the canalicular zone of hepatocytes there is also a "trigger zone" that is rich in type II receptors (InsP3R2) and shown to be important for the initiation of Ca^{2+} waves.[9]

The fetal liver provides temporary functions related to hematopoiesis with the release of factors, primarily cytokines from

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haematopoietic cells. These factors are needed for the proper growth and differentiation of the liver.[10] In mice, the hematopoiesis process is initially observed around 12.5 d post-coitum (dpc). Between 11 dpc and 16 dpc, active erythropoiesis is observed, with the presence of precursors for myeloid and B-cells from 13 dpc up to birth. Lymphoid precursors of p-T type cells also increase 12 dpc, while precursors of p-B type cells are scarce and increase up to 18–19 d of gestation. In mice, at the start of hematopoiesis, the association between macrophages and erythroblasts during erythropoiesis has been clearly observed,[1] with macrophages at the center of erythroblastic islands contributing to maturation and enucleation of surrounding erythroblasts.[1]

While in the developing fetus calcium is transported through the placenta against a blood calcium gradient mediated by parathyroid hormone (PTH) and PTH-related protein (PTHrP), in adult tissue, PTH is the main regulator of blood calcium. In studies of mice deficient for *Hoxa3*, a transcription factor involved in parathyroid organogenesis, an absence of parathyroid glands and therefore PTH, were observed.[11] As a result, low calcium levels have been detected in both blood and amniotic fluid samples, while serum levels of PTHrP and the placental transport of calcium remained unaffected. In combination with studies of *Pthrp*$^{-/-}$ mutants, where serum PTHrP levels were found to increase concomitant with its overexpression in the liver and placenta, these findings suggest that these organs play active roles in fetal calcium homeostasis mediated by PTHrP instead of PTH.[11]

On the other hand, several Ca^{2+} mobilizing systems are located in the plasma membrane and cytosolic organelles of hepatic cells, also working to maintain very low levels of calcium. In the plasma membrane of hepatocytes, the plasma membrane calcium ATPase (PMCA) is primarily responsible for maintaining low levels of calcium, since under normal conditions, the Na^{+}/Ca^{2+} exchanger appears to be inactive.[12,13] This review focuses on the contributions of PMCA to the overall system that controls calcium homeostasis during the embryonic development of the liver.

Plasma Membrane Calcium ATPase (PMCA)

In eukaryotic cells, PMCA is part of a specialized battery of proteins that maintain cytosolic calcium levels by extruding calcium against a concentration gradient. These proteins belong to a family of P-type ATPases, which undergo phosphorylation to form high and low affinity conformations (E1-E2) for Ca^{2+} in order to mediate a calcium transport cycle. The function of several calcium ATPases has been elucidated from the first studies with erythrocytes conducted over 40 y ago to characterize their canonical features.[14] In particular, it has been identified that PMCAs are generally regulated by calmodulin (CaM), require as cofactors, Ca^{2+} and Mg^{2+} and exhibit inhibitory sensitivity to vanadate and lanthanum.

Although PMCAs exhibit ubiquitous distribution, tissue-specific combinations of PMCA isoforms have been observed. For example, in excitable cells where signal transduction is strictly regulated, a wide variety of PMCA variants have been described according to their kinetic properties. For our laboratory, the role of PMCA in liver function under normal and pathological conditions, as well as during the normal process of liver regeneration, has been of particular interest,[15-17] The PMCA present in the liver tissue seems not to be modulated by CaM since the purification of the enzyme employing CaM-coupled columns has been unsuccessful.[18] Therefore, it is hypothesized that alternative mechanisms of regulation are involved, since liver Ca^{2+}-ATPases can be modulated by several hormones, such as PTH via G protein mediated signaling[19] or by lipids such as cholesterol as recently studied by us (unpublished results).

To date, the PMCA crystal structure has not been reported, therefore the accepted topological model is based on the high resolution crystal structure from other member of the P-ATPases family, the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCAa1) solved by Toyoshima et al.[20] The crystal structure of SERCAa1 expressed in bovine fast-twitch skeletal muscle has also been solved showing slight differences in comparison to that from rabbit.[21] At this stage, it is important to note that the topological model of PMCA accepted for many years based only on predicted DNA sequences of SERCA, is still accurate.[22] The model includes a single polypeptide (125–140 kDa) corresponding to 10 transmembrane (TM) domains with the N- and C-ends located on the cytosolic side (**Fig. 1**). In addition, 2 main intracellular loops have been described composed of the A domain (located between TM domains 2 and 3), a nucleotide-binding/catalytic phosphorylation (N/P) domain (between TM domains 4 and 5), and a CaM-binding site in the C-terminus segment that extended from TM domain 10.[23]

There are 4 genes (Atp2b1-Atp2b4) that encode the isoforms PMCA1–4. These genes undergo complex alternative splicing at sites A and C to produce various combinations of PMCAs that have specific kinetic properties and tissue-specific expression profiles (**Fig. 2**). Site A is located in the first cytosolic loop close to a phospholipid-binding domain, while Site C is located close to the C-terminus and comprises a CaM-binding domain and internal splicing sites that serve to increase the number of variants that are produced.[24-26] The C-end domain also modulates enzyme activity based on an autoinhibitory sequence which mediates interactions with both intracellular loops to potentially decrease PMCA activity at low intracellular calcium concentrations.

In general, it is well-accepted that PMCA1 and PMCA4 have a lower affinity for Ca^{2+} with respect to PMCA2 and PMCA3, and represent housekeeping isoforms that are ubiquitously expressed. In contrast, isoforms PMCA2 and PMCA3 are expressed in excitable tissues,[16,27] that during embryonic development exhibit spatiotemporal expression profiles. Although PMCA has also been shown to play a role in the homeostasis of calcium in the placenta,[28] the role of this enzyme in fetal liver remains to be investigated.

PMCA Expression in the Early Stages of Liver Development

In order to study hepatic cells during fetal and adult stages of development, several methodological issues related to the

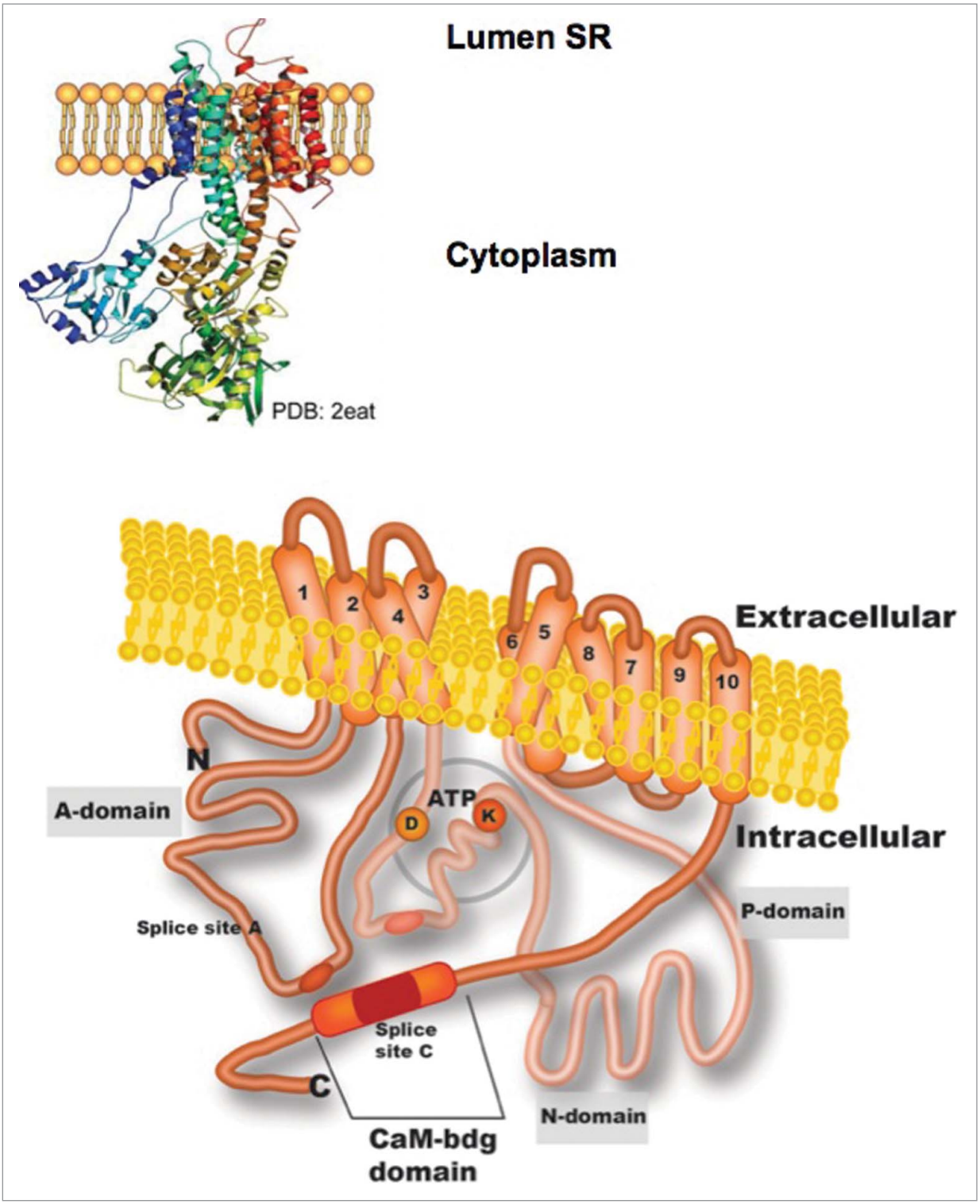

Figure 1. Illustrated topology of a plasma membrane Ca^{2+}-ATPase (PMCA). The three-dimensional representation of reticulum sarcoplasmic Ca^{2+}-ATPase (SERCA; PDB:2eat) that is shown at the top of the figure was used to construct the PMCA model below. The structure predicted for PMCA includes most of the domains being oriented toward the cytoplasmic face and 10 transmembrane domains (TM). Domain A, which is between TM domains 2 and 3, contains splice site A and a site for phospholipid binding. The intracellular loop located between TM domains 4 and 5 comprises the P- and N-domains where phosphorylation of an aspartate residue (D) and ATP-binding of a lysine residue (K) occur. The C-terminus also contains a calmodulin-binding site in splice site C.

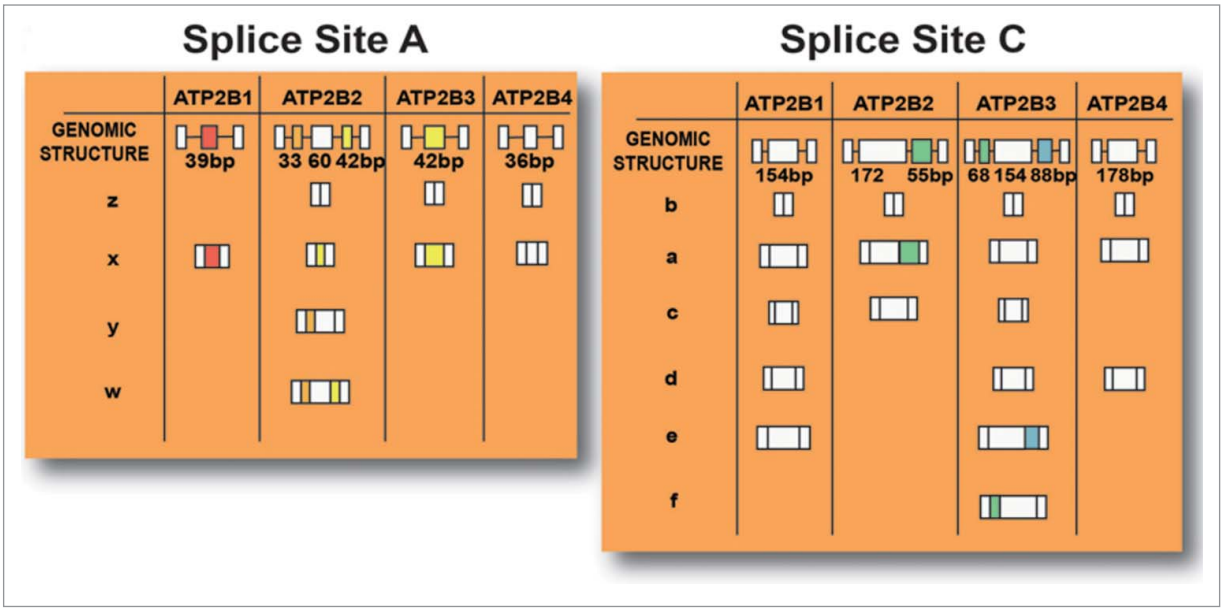

Figure 2. Possible isoforms of PMCA which are produced based on alternative splicing sites that are present at site A (resulting in w-z variants) and site C (resulting in a-f variants) of the 4 genes that encode PMCAs.

availability of biological material and proper tracing techniques need to be addressed. Correspondingly, there have been diverse efforts to study PMCA expression in different species during the fetal stages of liver differentiation (**Table 1**). Zacharias and Kappen have reported the ATPases identified throughout the stages of embryonic development in the mouse from 9.5 dpc to 18.5 dpc.[29] *In situ* hybridization images have shown that PMCA1 is expressed as a constitutive variant during all stages studied. In contrast, PMCA4 is abundantly expressed in fetal liver at 12.5 dpc (**Table 1**). The latter result is interesting since it corresponds with the initiation of hematopoiesis and the observation that PMCA4 is the predominant isoform in erythrocytes.[30] Zacharias and Kappen have also demonstrated that PMCA1–4 transcripts are expressed in ESCs that can potentially differentiate into hepatocytes.[29] In this study, PMCA1 and PMCA3 were found to be expressed at high levels, while PMCA2 and PMCA4 were expressed at low levels.[29] However, in another study only PMCA1 and PMCA4 expression were detected in mouse ESCs.[31] These differences may be due to the methodological approaches used in each study.

On the other hand, employing a cDNA library of human fetal liver at an unspecified gestational age, expression of *PMCA1b*, *PMCA4a*, and *PMCA2* was detected.[32] However, Heim et al. only detected expression of isoform 2x when using a cDNA library constructed from human fetal liver.[33] Furthermore, in a quantitative report of PMCA expression in human adult liver, splicing of PMCA1 at site A (1x) or at site C (1b) was found to produce 70% of the total PMCA expression detected for each splicing site. This was followed by PMCA4 isoforms (4x and 4b) representing 28% of total PMCA expression.[25] In addition, the expression of isoform 2w was detected in fetal human livers between 7.6 and 19.5 weeks of gestation.[34]

Using the liver from adult rats, Howard et al. identified PMCA1b and PMCA4b as the main isoforms in this tissue, with levels of PMCA1 being greater than those of PMCA4. In contrast, adult human liver samples exhibited higher levels of PMCA4 expression than PMCA1.[35] Previously, we have characterized the expression of the housekeeping isoforms of PMCA (i.e., PMCA1 and PMCA4) in both normal liver and liver tissue undergoing regeneration after 70% partial hepatectomy.[15] These isoforms have also been studied in several fetal development stages and in a rat hepatocarcinoma model.[15] Transcripts for isoforms 1x, 1b, and 4b have consistently been found to be the most abundant, while the pattern of expression for these isoforms during regeneration was found to be more similar to that of normal liver than that of fetal liver (**Table 1**). This phenomenon can be explained due to the significant role that mature hepatocytes play during liver regeneration.[36] In addition, 2w transcripts were detected in all samples analyzed.[15] Although a comprehensive analysis of PMCA expression during the fetal stages of rat liver development is still ongoing, 4 PMCA variants have been analyzed in our laboratory using single-cell quantitative reverse transcription PCR techniques. Various cell types derived from hepatic tissue, including hepatocytes, Kupffer cells, and hepatic stellate cells, have been analyzed, and for the first time facilitated the study of the PMCA3 isoform in adult rat liver tissue.[17]

Taken together, these observations show that available knowledge related to the expression of PMCAs in liver tissue during the embryonic stages of development, is scarce, largely due to challenges that remain to be solved related to the obtention of sufficient and good quality biological material to study.

Table 1. Comparative expression of PMCA transcripts in fetal and adult liver

Species	Age	PMCA1	PMCA2	PMCA3	PMCA4	Reference
Human	Fetal (20–22 w)	1b	2	ND	4a	32
Human	Fetal	U	2x	U	U	33
Human	Fetal	U	2w	U	U	35
Human	Adult	1b	2w	ND	4b	35
Human	Adult 75 y	1x (70%)	2x, 2w (≤1%)	ND	4x (28%)	25
		1b (70%)	2b (≤1%)		4b (28%)	
Rat	Adult	1b	2w	ND	ND	35
Rat	13 d	1x > 1b	2w	U	4b > 4x	15
Rat	17 d	1x > 1b	2w	U	4b > 4x > 4z	15
Rat	Neonatal	1x > 1b	2w	U	4b > 4x > 4z	15
Rat	Adult	1x > 1b	2w	U	4b > 4a, 4d, 4x	15
Rat	Adult	H 1x, 1c	H 2A*	H 3A*	H 4a, 4d	17
		KC 1x, 1c	KC 2A, 2a	KC 3A, 3e	KC 4a, 4d	
		HSC 1x, 1c	HSC 2A, 2a	HSC 3A, 3e	HSC 4a, 4d	
Mouse	Fetal 12.5 d	Present	ND	ND	Maximal	29
Mouse	Fetal 18.5 d	Maximal	ND	ND	Minimal	29

Isoforms 2A and 3A correspond to variants detected with primers designed upstream of splicing site A. Isoform 3e: HSC> KC>H. ND; not detected; U; undetermined; HSC: Hepatic stellate cells, KC: Kupffer cells; H: Hepatocytes.

Stem Cells as a Tool for Studying Embryonic Development

In the early 1980s, the use of pluripotent stem cells such as ESCs, emerged as an alternative to the low availability of hepatocytes for transplantation. However, important ethical issues emerged since the method for obtaining ESCs implied the destruction of an embryo. In 2006, Takahashi and Yamanaka described the use of induced pluripotent stem cells (iPSCs) produced from mouse embryonic and adult fibroblasts that could overcome the immunological rejection associated to ESCs.[37]

To date, iPSCs have been found to be produced by several species including humans, and shown to differentiate to form distinct cell types.[38] Also, iPSCs can be obtained from diverse cell lineages that are "forced" through reprogramming mediated by retroviral or lentiviral induction of 4 essential pluripotency transcription factors, to differentiate into a hepatocyte phenotype.[37] Among these factors, apparently c-myc plays a role as a major component promoting the expression of features associated to ESCs and repressing fibroblast-specific genes.[39] Subjacent mechanisms involved in the reprogramming are under not only genetic, but under complex epigenetic control addressing the strict transcription of genes.[37,40,41] In other studies where reprogramming is performed in the absence of transgenes, no transcriptomic differences were identified in cells that were reprogrammed with or without c-myc.[42] Of particular interest is the finding that among more than 2,000 genes that exhibited significant differential expression when the transgene is absent (most of them related to epigenetic regulation and chromatin assembly), the housekeeping PMCA isoforms were expressed. Furthermore, PMCA4 appeared to be overexpressed, while PMCA1 was underexpressed.[42] Although the meaning of these results is not readily apparent, they may be understood in terms of the unique features of each isoform associated to the specific demands that the microenvironment might impose.

In this sense, an advantage of reprogramming is that the experiments can be performed under conditions that mimic the natural development of the liver. As a result, hepatocytes can be obtained for transplantation or for studies of developmental biology.[43-47] For example, there is a 25-day protocol for obtaining hepatocyte-like cells from ESCs or iPSCs that reproduces the main stages of embryonic development in mice.[44,45] Recently, it has been shown that human iPSCs spontaneously form 3-dimensional clusters 48 h after seeding, where after 6 d of culture they develop a bud-like structure which corresponds to 9.5–10.5 d of gestation in mice provided of a functional endothelial network and proliferating hepatoblasts.[46]

To date, few reports have explored the phenomena of calcium homeostasis in excitable tissues using human iPSC and ESC lines differentiated into cardiomyocytes. It has been demonstrated that iPSCs exhibit an apparent immaturity in the expression of important calcium mobilizing systems involving the ryanodine receptor and SERCA.[48] Employing iPSCs derived from patients suffering from hypertrophic cardiomyopathy or Parkinson disease, alterations in calcium handling and an increase in the levels of intracellular Ca^{2+} have been found in close relationship to the genesis of these pathologies.[49,50] Therefore, the role of PMCAs in regulating calcium perturbations in excitable and nonexcitable tissues represents an active area of ongoing research.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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