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Cell biology of sarcomeric protein engineering: Disease modeling and therapeutic potential

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Abstract

The cardiac sarcomere is the functional unit for myocyte contraction. Ordered arrays of sarcomeric proteins held in stoichiometric balance with each other, respond to calcium to coordinate contraction and relaxation of the heart. Altered sarcomeric structure-function underlies the primary basis of disease in multiple acquired and inherited heart disease states. Hypertrophic and restrictive cardiomyopathies are caused by inherited mutations in sarcomeric genes and result in altered contractility. Ischemia mediated acidosis directly alters sarcomere function resulting in decreased contractility. In this review, we highlight the use of acute genetic engineering of adult cardiac myocytes through stoichiometric replacement of sarcomeric proteins in these disease states with particular focus on cardiac troponin I. Stoichiometric replacement of disease causing mutations has been instrumental in defining the molecular mechanisms of hypertrophic and restrictive cardiomyopathy in a cellular context. In addition, taking advantage of stoichiometric replacement through gene therapy is discussed, highlighting the ischemia-resistant histidine-button, A164H cTnI. Stoichiometric replacement of sarcomeric proteins offers a potential gene therapy avenue to replace mutant proteins, alter sarcomeric responses to pathophysiologic insults or neutralize altered sarcomeric function in disease.

Keywords

acute genetic engineering; myofilament; troponin; calcium sensitivity; sarcomere; molecular dynamics; adult cardiac myocytes

Introduction

The sarcomere is the subcellular functional unit of cardiac muscle. A near-liquid crystalline array of overlapping thin and thick filaments forms the sarcomere. Cardiac contractility is driven by the highly regulated, cyclical interaction of thin and thick filament proteins. By mass, the primary unit of the thin filament is actin. Actin monomers polymerize into an elongated filament of double helical strands. Tropomyosin (Tm), an elongated protein that spans seven actin monomers, polymerizes head to tail along the actin filament and resides in the groove between actin strands. Associated with every Tm subunit is the troponin complex

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(Tn) in a 1:1 molar ratio. Cardiac troponin is composed of troponin C (cTnC), the Ca sensitive subunit, troponin I (cTnI), the inhibitory subunit, and troponin T (cTnT), the tropomyosin binding subunit. The thin filament has a 1:1:7 stoichiometry of troponin:tropomyosin:actin that is strictly maintained (Figure 1). The molecular motor, myosin is the predominate protein of the thick filament. Myosin heavy chain dimers with the associated regulatory light chains and essential light chains align with the giant protein, titin, and the phospho-protein myosin binding protein C to make up the thick filament (Gordon et al., 2000). Cardiac contraction initiates with an action potential, activating the voltage gated L-type calcium channel, allowing a small influx of calcium across the sarcolemmal membrane (Bers, 2002). This small amount of calcium initiates calcium-induced calcium release from the sarcoplasmic reticulum through the ryanodine receptor resulting in a rapid and marked rise in intracellular calcium. TnC binds calcium which causes alterations in thin filament protein interactions allowing myosin to form strong cross-bridges with actin (Farah and Reinach, 1995; Filatov et al., 1999; Gordon et al., 2000; Vinogradova et al., 2005; Galińska-Rakoczy et al., 2008; Lehman et al., 2009). Hydrolysis of ATP by myosin produces force, and, if load permits, the relative sliding of myofilaments to cause contraction. The subsequent release of calcium from TnC and re-sequestration of calcium into the SR allows for relaxation to occur (Gordon et al., 2000; Bers, 2002). This highly regulated process of excitation-contraction coupling occurs more than a billion times over an average human lifespan.

Throughout the continuous, rhythmical beating of the heart the sarcomere maintains a lattice like architecture with strict stoichiometry. The sarcomere is often simplified to a static crystalline structure whereby its machine-like properties gives rise to force production and motion. This could not be further from the truth. The sarcomere is a highly dynamic multi-protein complex with constant alterations in protein-protein interactions, post-translational modifications and protein production/ turnover (Kleerekoper et al., 1995; Miki et al., 1998; Filatov et al., 1999; Dong et al., 2003; Takeda et al., 2003; Vinogradova et al., 2005; Lehman et al., 2009; Sanger et al., 2009). The ability of the myocyte to maintain contractility while replacing “old” or “damaged” sarcomeric proteins in a stoichiometric manner is amazing biology. While the mechanistic details by which this occurs are lacking new evidence suggests that the balance between chaperons and degradation factors, such as E3 ubiquitin ligases and calpains play a key role (Willis et al., 2009). It is likely that each of the sarcomeric proteins have unique signals and partners which determine its turnover and replacement.

Despite the absence of a clear mechanism, the regular turnover of sarcomeric proteins is well documented. The turnover rate for sarcomeric proteins in adult heart has been investigated using whole animal H^3 leucine labeling experiments (Everett et al., 1981; Martin, 1981). These studies showed half-lives for troponin I and T ~3 days, troponin C, myosin and tropomyosin ~5.5 days, actin and myosin light chains ~7–10 days (Figure1). These experiments brought up many interesting questions which are still unresolved today. In the case of troponin, with troponin I and T having different half-lives compared to troponin C this suggests that TnC must recycle from “old” TnI-TnT to “new”. The same principle holds for myosin as the light chains which apparently must recycle from “old” to “new” in some regulated way. This also suggests that TnC and the myosin light chains have

the ability to dissociate from their respective partners and could be in a more dynamic equilibrium with them than a static interaction. In vitro biochemistry experiments support the ability of TnC and the myosin light chains to dissociate from the sarcomere under certain conditions, albeit non-physiological (Ling et al., 1996; Preston et al., 2006).

The use of adenovirus-mediated acute genetic engineering in adult cardiac myocytes has shed new light into the turnover and stoichiometry of the sarcomere. The principle of acute genetic engineering through adenoviral transduction of adult cardiac myocytes is depicted in figure 2. Treatment of adult cardiac myocytes with an adenovirus expressing an epitope tagged sarcomeric protein (i.e. TnI) results in a time dependent increase in expression and incorporation of the vector derived protein with a simultaneous stoichiometric decrease in the endogenous protein. This can be visualized through indirect immunofluorescence for proper incorporation and through Western blots which show shifted bands for the vector derived-epitope tagged protein for replacement. Many studies have confirmed stoichiometric replacement and proper incorporation into the sarcomere for myosin, TnI, TnT, TnC and Tm (Gulick et al., 1997; Michele et al., 1999; Rust et al., 1999; Tardiff et al., 1999; Robbins, 2000; Tardiff et al., 2000; Michele et al., 2002; Hernandez et al., 2005; Herron et al., 2007; Davis et al., 2008; Lim et al., 2008; Sadayappan et al., 2008).

Cell biology studies on incorporation

Adenoviral transduction of adult cardiac myocytes has been used to study the fundamental aspects of incorporation of thin filament proteins into the sarcomere. Early studies first showed stoichiometric replacement with ssTnI, the fetal TnI isoform in the heart (Westfall et al., 1997a). These studies showed replacement was time dependent and increased to nearly 100% replacement of cTnI with ssTnI, seven days after viral transduction. Permeabilization of the cells followed by Western blots showed the viral expressed protein is incorporated into the sarcomere and that there is no accumulation of expressed protein in the cytosol. This result can be interpreted as the endogenous cTnI turning over while the new vector derived protein competes for incorporation. The timing of replacement fits well with the documented half-life of cTnI of ~3 days. Transgenic (Tg) expression of sarcomeric proteins results in a similar replacement as is seen in the acute genetic engineering of adult cardiac myocytes (Robbins, 2000). Studies show that replacement can be from 10–90% (Ling et al., 1996; Gulick et al., 1997; Miki et al., 1998; Fentzke et al., 1999; Day et al., 2006; Sadayappan et al., 2008; Davis et al., 2012). Feng et al. probed the regulation of TnI incorporation through transgenesis (Feng et al., 2009). Homozygous knockout of cTnI is post-natal lethal at day ~19 (Liu et al., 2007). Transgenic rescue with an N-terminal truncated cTnI results is complete restoration of TnI. Through the analysis of multiple Tg lines with different mRNA expression of the transgene the authors were able to conclude that total TnI protein was unchanged from wild type mice. They also showed that heterozygous cTnI-KO mice have normal levels of cTnI protein in the face of 50% the mRNA level. These first studies help inform a stoichiometric system with defined kinetics that correlate well with the endogenous turnover of sarcomeric proteins. They also reveal that in the face of increased mRNA expression of TnI that the overall amount of TnI protein in the myocyte is constant. This suggests a limited amount of “parking spots” for TnI and competition for those spots is what

dictates replacement. It also suggests that there are cellular mechanisms which limit the amount of unincorporated TnI in the cytosol.

Further studies helped to elucidate the spatial aspect to incorporation. Michele et al showed cTnI-Flag incorporates in a time dependent manner and through indirect immunofluorescences that incorporation is stochastic across the sarcomere (Michele et al., 1999). Through dual labeling experiments, using α-actinin as a Z-line marker, cTnI-Flag incorporated with even distribution between Z-lines. In time this staining became brighter and filled the area between Z-lines. In contrast, Flag-tropomyosin staining showed a restricted initiate pattern of incorporation, with staining at early time points being at the M-line and progressing towards the Z-lines with time. Thus tropomyosin has an ordered incorporation at the pointed-end of actin progressing towards the barbed end and Z-line. This study also documented that cTnI-flag replacement occurs faster that Flag-Tm consistent with the increased half-life of Tm protein of ~5days. The difference in incorporation suggests that thin filament proteins turnover with different temporal and spatial patterns. This points to specific partners and mechanisms of turnover for cTnI and Tm. Further studies are necessary to determine the mechanism of this difference and to reveal if all troponins incorporate in stochastic manner or if there is some ordered incorporation. In addition to revealing the spatial and temporal dependence of sarcomere turnover this study confirmed that function was unaltered by replacement of "self" proteins. The ability to replace sarcomeric proteins while maintaining contractile function suggests that the endogenous mechanisms of sarcomere maintenance are unaltered by adenoviral transduction of sarcomeric proteins.

Disease modeling through stoichiometric replacement

In addition to using acute genetic engineering to understand the turnover and stoichiometric replacement of sarcomeric proteins, this technique has been instrumental in understanding structure-function of sarcomeric proteins in disease. Hypertrophic and restrictive cardiomyopathies (HCM and RCM) are common inherited diseases of the sarcomere. Single amino acid mutations in thin and thick filament proteins are associated with HCM and RCM in an autosomal dominant manner (Seidman and Seidman, 2001; Teekakirikul et al., 2012). HCM is characterized by left ventricle hypertrophy, arrhythmias, and sudden cardiac death (Maron and Maron, 2013). More than 1000 mutations in 11 sarcomeric genes have been linked to HCM, with the highest prevalence being in β-myosin heavy chain, myosin binding protein C and cardiac troponin T (Lopes et al., 2013). Troponin was studied extensively early on due to its ability to be reconstituted and exchanged in permeabilized myofibers and its high turnover rate in intact cells which allows for high replacement in the time frame of viable adult cardiac myocytes in culture. Early myofibrillar ATPase assays along with calcium-force measurements in reconstituted myofibers indicated that cTnI HCM and RCM mutations result, in general, to increased calcium sensitivity of the sarcomere (Parvatiyar et al., 2010; Willott et al., 2010; Liu et al., 2012). RCM mutations tended to be hypersensitive compared to HCM mutations and were predicted to give rise to a more deleterious disease state. To fully elucidate the cellular implications of these mutations acute genetic engineering of adult cardiac myocytes with membrane intact functional measures of sarcomere shortening and calcium transient analysis were necessary. Davis et al. compared

cTnI HCM and RCM mutations for replacement and cellular function (Davis et al., 2008). R145G, an HCM mutation, resulted in a limited contractile phenotype with slowed relaxation. This effect was replacement dependent, with increased phenotype with increased replacement. Two RCM mutations in cTnI, A172T and R193H showed decreased diastolic sarcomere length and very slow relaxation. Although the phenotypes of the HCM and RCM mutations mirror each other qualitatively, the RCM mutations quantitatively have hyper-phenotypes. This study also suggested that different mutations have altered ability to compete for replacement with wild type cTnI. Using unlabeled cTnI adenovirus with increasing dosage to compete with the Flag labeled mutant adenovirus showed that R145G competes less well with wild type cTnI while R193H has increased competition (Davis et al., 2007, 2008). These findings suggest that the disease phenotype is dependent on the replacement percentage of the mutant protein. In addition they suggest that the intrinsic alteration of calcium sensitivity does not fully determine the disease phenotype as replacement percentage can influence the cellular function. Further experiments with R193H cTnI transgenic mice showed similar dose-dependent functional alterations. R193H cTnI mice have ~10–15% R193H in two independent lines suggesting that the heart can't tolerate higher levels of replacement (Du et al., 2008; Davis et al., 2012; Li et al., 2013). Indeed recent studies confirmed this through crossing R193H transgenics to the cTnI knockout. These mice have ~80% R193H cTnI incorporated in the sarcomere and die at post-natal day 30 (Li et al., 2013). This marked phenotype again suggests that the extent of replacement in addition to the intrinsic calcium sensitivity determines the disease phenotype. The ability to study cTnI mutations in the context of living cells has advanced our understanding of the genotype-phenotype relationship. In addition to the contractile phenotypes these studies also advanced the idea of calcium alterations in HCM. Calcium transient analysis for the HCM and RCM mutations showed increased diastolic calcium, decreased calcium transient amplitude and slow reuptake of calcium into the SR (Davis et al., 2007, 2008). All together this suggests that the heightened calcium sensitivity allows troponin to "buffer" calcium in the sarcomere and stay in a partially active state. This has been shown with cTnT HCM mutations as well and may be a common feature of HCM and RCM mutations (Knollmann et al., 2003; Haim et al., 2007; Guinto et al., 2009).

The ability to stochiometrically replace sarcomeric proteins has the potential as a therapy for HCM and RCM. As discussed above, the extent of replacement of the mutant polypeptide determines the phenotype. It follows that decreasing mutant peptide content in the sarcomere would be therapeutic. This could be through specifically targeting the transcript of the mutant allele for degradation through shRNA as was shown recently for a MHC mutation which causes HCM (Jiang et al., 2013). Another avenue is to out-compete the mutant protein through viral-mediated transduction and expression of the wild type protein (Davis and Metzger, 2010). Both avenues hold promise because the myocyte has the intrinsic ability to dictate the level of incorporated protein in the face of decreased or increased levels of transcripts by preservation of overall sarcomeric protein stoichiometry.

Therapeutic use of stoichiometric replacement

Altered sarcomeric function is a hallmark of many disease states, including ischemic cardiomyopathy and myocardial infarction. Ischemia causes an alteration of the intracellular

milieu of the myocyte resulting in acidosis. This acidosis results in decreased contractility of the cardiac myocyte which a significant portion is due to calcium desensitization of the sarcomere (Orchard and Kentish, 1990). Early biochemical studies showed that the isoform of TnI is a critical determinate of acidosis-induced desensitization of the sarcomere (Metzger and Westfall, 2004). The fetal cardiac TnI, slow skeletal TnI (ssTnI), shows resiliency to acidosis in comparison to the adult isoform cTnI. Acute gene transfer experiments in adult cardiac myocytes with ssTnI results in stoichiometric replacement of cTnI with ssTnI (Westfall et al., 1997b). Cellular tension-calcium measurements showed that ssTnI confers increased calcium sensitivity at pH 7 and maintains calcium sensitivity at pH 6.2 in comparison to cTnI. Independently, an ssTnI transgenic mouse showed a similar gain in calcium sensitivity and pH resiliency (Fentzke et al., 1999; Wolska et al., 2001).

Chimeras of cTnI and ssTnI were used to decipher the critical domains which confer these attributes. cTnI has a unique N-terminal extension of 32 amino acids that has a critical PKA phosphorylation site necessary for β-adrenergic stimulated calcium desensitization and relaxation that is not present in ssTnI (Guo et al., 1994; Metzger and Westfall, 2004; Yasuda et al., 2007; Feng et al., 2009). Other than the N-terminal extension ssTnI and cTnI differ only in amino acid composition while having preserved domains (Palpant et al., 2010). Through the analysis of an N-cardiac/slow-C and an N-slow/cardiac-C in tension-pCa measurements it was revealed that the C-terminal portion of ssTnI confers protection from pH-mediated calcium desensitization (Westfall et al., 2000). Single amino acid changes between cTnI and ssTnI were analyzed through substitution of the ssTnI for the cTnI residue. These studies showed that a single residue, H132 in ssTnI confers protection from acidosis (Westfall and Metzger, 2007). cTnI has an alanine at this same position. In an effort to confer the pH resiliency to cTnI, Day et al made the opposite substitution, creating A164H cTnI (Day et al., 2006). A164H cTnI transgenic mice conferred protection from ischemia/reperfusion injury, hypoxia, and age-related cardiac dysfunction (Day et al., 2006; Palpant et al., 2008; Palpant et al., 2009). Importantly they also showed no deleterious effects under physiologic conditions. Cellular studies showed a resiliency to pH dependent decrease in tension-pCa measures as well as maintenance of contractility at acidic pH in membrane intact myocytes (Day et al., 2006; Palpant et al., 2012). The cellular studies also showed that unlike ssTnI, A164H cTnI did not increase tension-pCa measurements at physiologic pH. This was confirmed in contractility measures in membrane intact cells (Palpant et al., 2012). This finding suggests that A164H is a titratable molecular inotrope which behaves like cTnI under normal physiologic conditions but reacts to pathophysiologic conditions such as acidosis to maintain contractile function. This is an important distinction between ssTnI and A164H cTnI. As discussed above in HCM most of the mutations result in increased calcium sensitivity of the sarcomere much like ssTnI. In accordance, ssTnI transgenic hearts show significant diastolic dysfunction at baseline similar to HCM mutant mice (Fentzke et al., 1999). Additionally, A164H cTnI maintains the N-terminal extension and responds like wild type cTnI to β-adrenergic stimulation. Thus A164H cTnI, by its titratable function has the attributes to be a viable therapy for ischemia induced disease states such as ischemic cardiomyopathy.

Mechanistic detail as to how an engineered histidine button in cTnI can confer protection under acidic conditions comes from a combination of cellular functional studies with

molecular dynamic (MD) simulations. Histidine is a unique amino acid in that its pKa for protonation is in the physiologic range. At normal physiologic pH most histidines are deprotonated while a slight shift to acidic conditions will result in increased protonation of a histidine. Codon A164 is located next to the switch peptide of cTnI. The switch region of TnI interacts with cTnC N-terminal domain in a calcium dependent manner (Li et al., 1999). cTnC binds calcium which partially opens a hydrophobic patch, the switch region of TnI interacts with the hydrophobic patch inducing the full opening of the hydrophobic patch. This interaction is modeled to pull the inhibitory region of TnI away from tropomyosin allowing myosin strong binding sites on actin to be revealed (Gordon et al., 2000). Thus cTnI switch region binding to cTnC allows contraction to occur. Protonation of the histidine in close proximity to cTnC lead to the hypothesis that the histidine could interact with an acidic residue on cTnC.

MD simulations of sTnI (a.a.115–141) from the chicken fast skeletal troponin crystal structure (PDB 1YTZ)(Vinogradova et al., 2005) or A164H cTnI (a.a.148–174) from the cardiac troponin crystal structure (PDB 1J1E)(Takeda et al., 2003) bound to cTnC (a.a.1–90) provided evidence for a histidine (cognate codon 164 in cTnI) to glutamic acid (codon 19 in cTnC) interaction in acidic conditions. The sTnI simulations showed H132 had an electrostatic interaction with cTnC E19 (Palpant et al., 2010). Substitution of sTnI with the cTnI residue H132A showed this electrostatic interaction is eliminated and sTnI moves away from cTnC. In agreement, cTnI shows no interaction around A164 with cTnC and adopts a significantly different conformation than sTnI. Simulations with A164H cTnI showed the potential for a similar electrostatic interaction between H164 and E19 of cTnC (Palpant et al., 2012). NMR studies using similar portions of sTnI and A164H bound to cTnC in acidic conditions support the potential for a histidine to E19 interaction (Pineda-Sanabria et al., 2012; Robertson et al., 2012). The NMR studies also indicate that there is increased affinity of sTnI and A164H for cTnC under acidic conditions. These results in combination with the cellular functional studies suggest that under acidic conditions H164 is protonated, increasing TnI to cTnC affinity through the electrostatic interaction of H164 with E19 which in turn increases contractile function. Further evidence supports this electrostatic interaction as a determinate of contractile function. An engineered cTnI A164R was used to mimic the charge on H164 during acidosis (Palpant et al., 2012). A164R MD simulations showed R164 to E19 cTnC interaction is present and overall its confirmation is similar to sTnI and A164H. In adult cardiac myocytes A164R results in increased tension-pCa calcium sensitivity and increased contractility in live cells under physiologic pH conditions. Under acidic conditions A164R has similar function as A164H. These studies support the idea that A164H titratable gain in function is due to an electrostatic interaction between H164 and E19 of cTnC, increasing TnI-TnC affinity and thereby altering contractility in acidotic conditions.

The titratable gain-in-function of A164H cTnI under pathophysiologic stimuli while maintaining normal function under physiologic conditions makes it an attractive potential therapeutic strategy. In addition, due to the cell intrinsic capacity of stoichiometric replacement, “molecular torture” due to extraneous protein production or accumulation is not a concern. This makes A164H cTnI a viable gene therapy for conditions which result in ischemia, whether transient or chronic.

Sarcomeric replacement offers a suitable system to exploit for therapeutic intervention in diseases of the sarcomere. HCM, RCM and ischemia result in alterations of sarcomere function as the prime mechanism of the disease phenotype. As such, therapeutic intervention at the level of the sarcomere is necessary to alleviate the disease phenotype. Whether this is through gene therapy, taking advantage of the stoichiometric replacement of sarcomeric proteins or through small molecules targeting the sarcomere, this therapeutic avenue needs to be explored. Acute genetic engineering of adult cardiac mocytes offers a viable system to test both therapeutic avenues as a disease model and test bed for beneficial engineered sarcomeric proteins.

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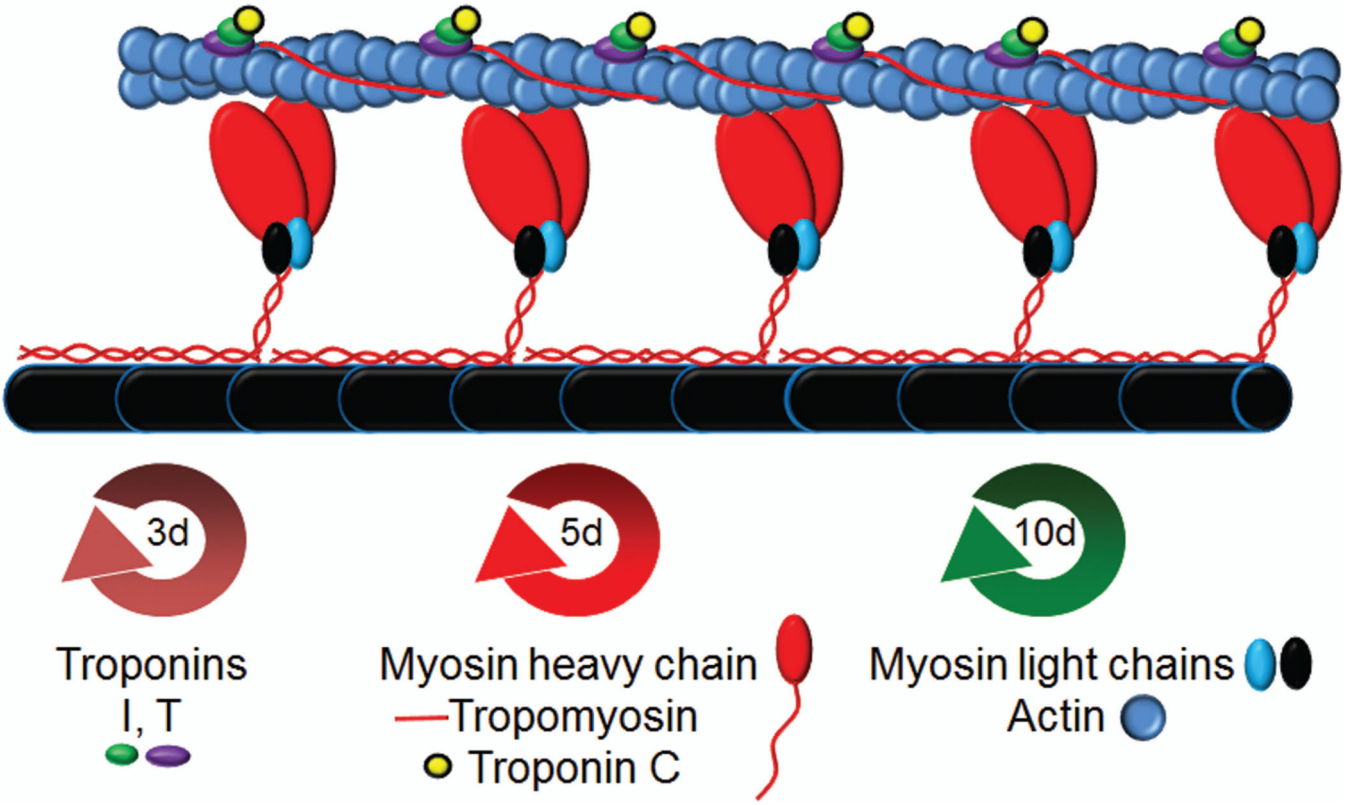

Figure 1.

Schematic representation of the structure of the cardiac sarcomere. The sarcomere is made up of the thin filament containing actin, troponin, and tropomyosin and the thick filament containing myosin, myosin light chains, and titin (black) along with myosin binding protein C (not depicted). Troponin interacts with tropomyosin and actin in 1:1:7 stochiometry. Myosin motor domains (red) interact with actin in a calcium dependent manner to produce force. Turnover half-lives of the depicted sarcomeric proteins are presented below (d=days).

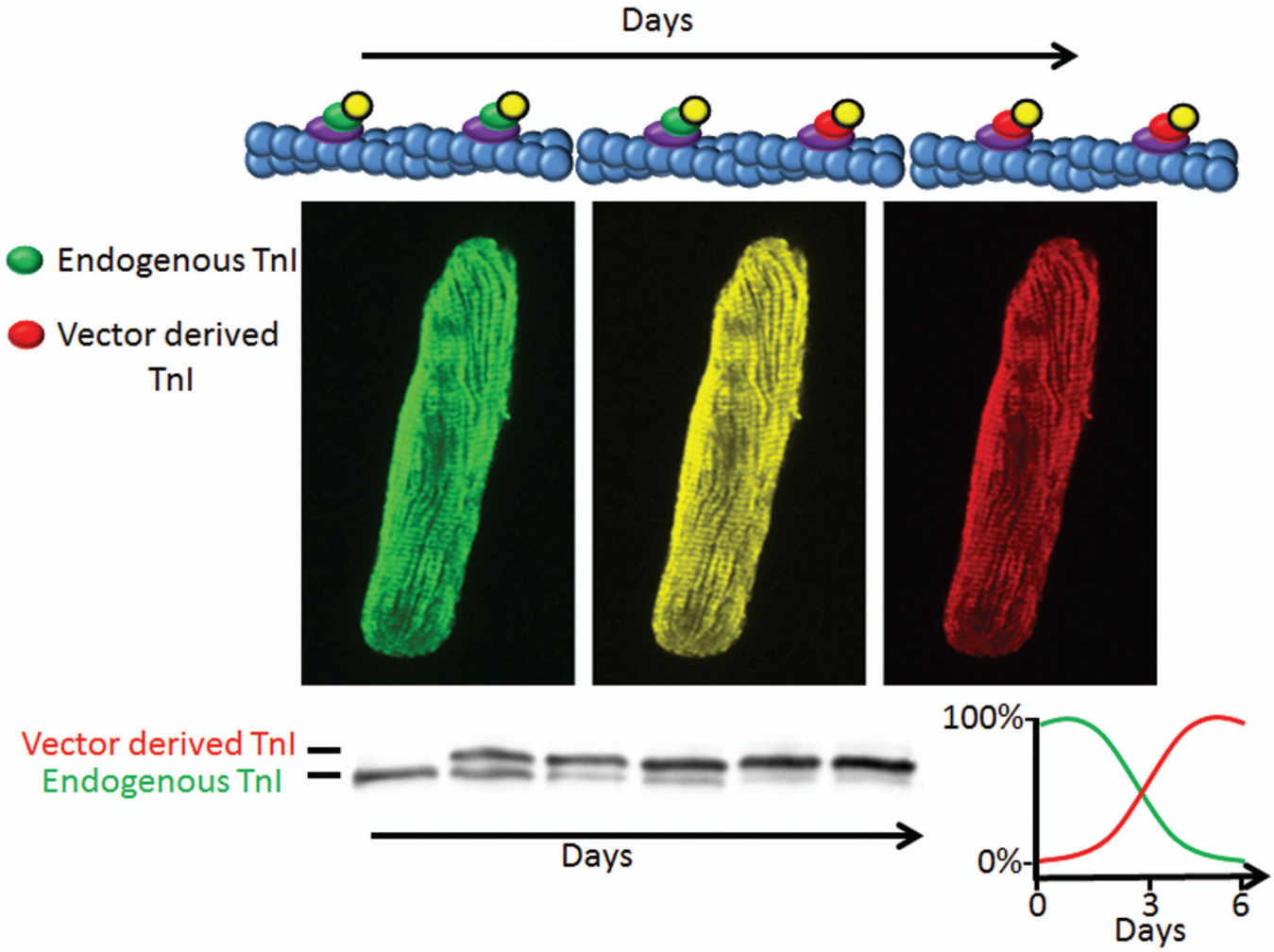

Figure 2.

Guiding principles of acute genetic engineering of adult cardiac myocytes. Adenoviral expression of vector-derived cTnI-Flag (Red) results in a time dependent increase in replacement of the endogenous cTnI (Green). Myocyte images are artistic renderings for discussion purposes only. The pseudo colored change from green (endogenous cTnI) to red (vector derived cTnI-Flag) of the sarcomere are used to visually demonstrate incorporation as cTnI-Flag replaces the endogenous cTnI over time. Generic Western blot of cTnI shows replacement percent with the endogenous band decreasing over time with the vector derived-epitope tagged band increasing over time as it replaces endogenous TnI while total TnI is unchanged. For TnI, replacement reaches ~100% 6 days after adenoviral transduction.