An explosion of interest in new therapies for heart repair has occurred recently. Most notably, clinical trials of cell-based cardiovascular therapy have garnered much attention, and preliminary results are encouraging.1,2 However, the mechanism of action of cell-based therapies remains unclear, and many details with regard to characterization, quality control, and delivery of cells remain to be worked out before widespread therapeutic application. Nonetheless, the reported efficacy of cardiac cell–based therapies in human trials has generated interest in additional therapeutic development pathways. The identification of the release of paracrine-acting proteins as one mechanism by which cell-based therapies in the heart may improve cardiac function3 has spurred renewed interest in protein-based therapeutic approaches for cardiac repair.

Although protein therapeutics are increasingly becoming mainstream in a number of fields, including cancer and inflammatory diseases, (9 of the 20 top-selling drugs in 2012 were proteins4) proteins have relatively low penetration in the cardiovascular market. This is not to suggest a lack of progress in the cardiovascular therapeutic development, because small-molecule drugs such as statins5,6 and anticoagulants7 have been huge successes. Rather, as overall new drug approvals shift predominantly to proteins, protein therapeutic development represents an area of opportunity for cardiovascular medicine. Protein therapies allow for...
more targeted interventions, with well-defined regulatory pathways, improved scalability, and potentially reduced overall cost, compared with cell-based approaches. Furthermore, proteins have much larger surface areas than the small molecules of typical orally bioavailable drugs; thus, many of the molecular targets that are probably not druggable with small molecules can be targeted with proteins. A classic example of this concept is that no clinically successful small molecule activates the insulin receptor, and therefore patients with type I diabetes mellitus must inject insulin.

With regard to proteins for cardiovascular therapy, much effort has been put into enhancing cardiac microvasculature formation. More recently, the potential of proteins to induce cardiomyocyte proliferation has been identified, increasing optimism that protein-based approaches can be effective for cardiac regeneration. However, protein therapeutics often have limitations, including poor bioavailability, undesirable pharmacokinetics and biodistribution profiles, and off-target effects. One approach to overcome these deficiencies is protein engineering, a strategy that may not only render a therapeutic concept feasible but also generate the intellectual property necessary for development in patients.

The term protein engineering is a general one that covers many techniques for modifying proteins, including the use of molecular display technologies to enable targeted delivery, structural modification of proteins to impart enhanced properties such as resistance to enzymatic degradation via rational design or directed evolution, fusion of proteins to polymers or other protein domains to promote immune evasion, and incorporation of desired properties or functional groups via noncanonical amino acids (ncAAs). Despite this promise and widespread application in fields such as oncology, engineered protein therapeutics have to date failed to become a major part of the toolkit of cardiologists. In this article, we review the use of protein therapeutic approaches in the heart to date, with emphasis on therapies that have reached the clinical trial stage. We then highlight emerging and promising techniques in protein engineering and discuss how, when combined with new discoveries in molecular cardiology, these approaches might lead to a new generation of therapeutics for repairing the heart.

### Protein Engineering for Cardiovascular Therapeutics to Date

Although none has yet achieved widespread clinical use, a number of engineered proteins have been applied to cardiovascular diseases in clinical trials. Many other native protein therapeutic approaches have also been applied to cardiac repair, and several of these may benefit from protein engineering. Some of the earliest and most high-profile examples of protein engineering technology for cardiovascular therapy have been clinical failures. However, other examples have proven more successful, and ongoing clinical trials offer exciting possibilities.

### Tumor Necrosis Factor Antagonists

One of the most prominent, and ultimately unsuccessful, examples of translation of protein engineering technology for cardiovascular therapy involved the tumor necrosis factor (TNF) antagonist etanercept (Enbrel). Developed based on research by Beutler et al., etanercept is a dimeric fusion protein comprising the Fc region of human immunoglobulin G1 and the soluble human TNF receptor 2 domain that is approved for treatment of various forms of arthritis. After the description of overexpression of TNF in heart failure, it was hypothesized that TNF inhibition could be an effective treatment modality. However, despite initial promising results in carefully performed studies, both preclinically and in patients, 2 large-scale clinical trials revealed that etanercept did not show clinical benefit in chronic heart failure patients and it might have, in fact, increased the risk of chronic heart failure–associated morbidity and mortality.

Moreover, the chimeric monoclonal antibody infliximab (Remicade), a TNF inhibitor that works through a mechanism different from that of etanercept, also did not improve the clinical outcome of patients with heart failure, with high doses showing profound adverse effects. It should be noted that both etanercept and infliximab are efficacious against various forms of arthritis, and infliximab is highly effective against Crohn’s disease. Thus, protein engineering–based antagonism of TNF has proven to be a case of a successful product development pathway that has been unsuccessfully applied to cardiovascular therapy despite compelling preclinical investigation.

### Natriuretic Peptides

The natriuretic peptides offer an example of a promising application of protein engineering to cardiovascular therapy. Atrial natriuretic peptide and B-type natriuretic peptide (BNP), also known as brain natriuretic peptide and basic natriuretic peptide, have similar activities and are well-established as useful biomarkers in the guidance of cardiovascular therapy. In addition, exogenous administration of BNP has been shown to improve multiple parameters in patients with systolic heart failure. However, BNP as a therapeutic (nesiritide) may be limited because of its hypotensive effects.

C-type natriuretic protein causes a lower hypotensive response compared with atrial natriuretic peptide and BNP because of its lack of activity on arteries attributable to differential receptor expression. Additionally, a related peptide isolated from snake venom, Dendroaspis natriuretic peptide, acts similarly to atrial natriuretic peptide and BNP but is highly...
potent and resistant to enzymatic degradation. To leverage the advantageous properties of C-type natriuretic protein and Dendroaspis natriuretic peptide, Burnett et al synthesized a chimeric natriuretic peptide that comprises domains of both (CD-NP). Initial results from clinical trials for this engineered protein as a heart failure therapy were promising, and a follow-up trial may shed more light on the potential of CD-NP for clinical use.

**Insulin-Like Growth Factor-1**

Insulin-like growth factor-1 (IGF-1) is a protein with a molecular structure similar that of insulin and has been shown to provide protection from the progression of heart failure in mice. In humans, low serum levels of IGF-1 are associated with an increased risk of ischemic heart disease. However, the undesirable side effects of IGF-1 systemic delivery are well-noted and include increased risk of diabetic retinopathy and cancer. Thus, as of early 2013, there were only 2 active clinical trials examining IGF-1 (Mecasermin) as a cardiovascular therapy. As an attempt to overcome these effects by promoting local delivery, Tokunou et al engineered an IGF-1 fusion with the heparin-binding domain of heparin-binding epidermal growth factor to make heparin-binding IGF, which proved effective in stimulating biosynthesis of chondrocytes. Additionally, Hubbell et al engineered a variant of IGF-1 with increased immobilization capacity within fibrin that improved smooth muscle cell proliferation, introducing the possibility of a cofactorial local delivery approach. Notably, an IGF-1 modified to enable interaction with self-assembling peptides for cardiac delivery has demonstrated efficacy in improved cardiac function after myocardial infarction (MI). Thus, although not yet applied in clinical trials, engineered variants of IGF-1 may yield therapeutics for cardiovascular therapy.

**Stromal Cell–Derived Factor-1α**

One protein undergoing active clinical investigation for cardiac regeneration—although not currently as a protein therapy—is stromal cell–derived factor-1α (SDF-1). SDF-1 is a chemokine that plays important roles in angiogenesis and leukocyte trafficking. The discovery that SDF-1 induces stem cell homing to the heart after injury spurred interest in its therapeutic application. However, SDF-1 is proteolytically cleaved by both matrix metalloproteinase-2 and dipeptidyl peptidase IV; thus, the likelihood of retained bioactivity in the myocardium after injury—a highly inflammatory environment—is low. For this and other reasons, the only active clinical trial of SDF-1 for cardiac therapy uses plasmid delivery, which offers the potential for prolonged SDF-1 expression but is also limited by issues of safety and unpredictability common to gene therapy approaches. Protein engineering applied to SDF-1 offers an alternative; we developed a protease-resistant form of SDF-1 by mutating a single amino acid within the matrix metalloproteinase-2 cleavage site. This protease-resistant SDF-1 successfully induced endothelial progenitor cell recruitment to the heart after MI that resulted in improved cardiac function and led to increased angiogenesis and improved ventricular function after onset of myocardial ischemia. Protein engineering efforts to improve delivery and tissue retention of SDF-1 have also been reported, as has the creation of a polypeptide analog of SDF-1 that induced improved recovery after MI compared with the native protein. Future synergy of these and other protein engineering strategies may allow for a therapeutic approach that overcomes the limitations of the native SDF-1 protein.

**Granulocyte Colony–Stimulating Factor**

Granulocyte colony–stimulating factor (G-CSF) is a glycoprotein that selectively induces a reduction of SDF-1 and an increase in the SDF-1 receptor CXCR4 (C-X-C motif chemokine receptor) in the bone marrow. A majority of clinical trials involving G-CSF in the heart have focused on its use as an adjunct to cell therapy, but others have assessed its potential as a primary therapy as G-CSF has been shown to prevent deleterious remodeling after MI. A potential next step in these active lines of therapeutic investigation could involve the use of fusion proteins incorporating G-CSF moieties, because improved functionality relating to delivery and stability of G-CSF has already been reported.

**Interleukin Receptor Antagonists**

Interleukins (ILs) are proteins that induce pleiotropic effects in a wide variety of cell types. In the heart, IL-1 is a potent mediator of inflammation during ischemia/reperfusion injury and elevated plasma levels of IL-6 have been linked with increased risk of future MI; thus, their inhibition is of therapeutic interest. IL-1 has a naturally occurring competitive inhibitor for its receptor (IL-1 receptor), the IL-1–related protein IL-1 receptor antagonist (IL-1 receptor a), also known as anakinra. Both forced overexpression and injections of anakinra have been shown to reduce apoptosis after heart injury in rodents and this drug has been applied in clinical trials with promising results. In addition, IL-6 inhibition has been pursued via antagonism of its receptor (IL-6 receptor or CD126) by an engineered, humanized monoclonal antibody, tocilizumab. Encouragingly, a human case report of tocilizumab administration resulting in successful improvement of cardiac dysfunction associated with multicentric Castleman disease has been published, and a clinical trial investigating the efficacy of tocilizumab in MI is ongoing. Thus, IL receptor antagonists may represent a promising path forward for protein engineering applied to cardiovascular therapy.

**Erythropoietin**

A glycoprotein that regulates red blood cell production, erythropoietin (EPO), has well-documented cardioprotective properties and works via multiple mechanisms. Preclinical studies in both rat and rabbit acute MI models showed that EPO administration improves cardiac contractility and hemodynamic parameters, prompting human clinical trials. Initial findings indicated that EPO, although failing to improve left ventricular ejection fraction, may reduce heart failure in acute MI patients. However, a more recent trial has cast doubt on the potential for EPO as a therapy for heart failure, indicating that it may lead to increased infarct size. Because of these results, focus has shifted toward examining the efficacy of low doses of EPO, an approach that might be aided by applying...
protein engineering techniques to improve potency and stability of EPO, as has already been demonstrated.87

**Neuregulin-1β**

Neuregulin (NRG) is an essential regulator of cardiovascular development and plays an important role in cardiovascular disease.88 The cardioprotective potential of NRG has been well-established in preclinical models,89,90 and enthusiasm for future therapeutic application of NRG in heart failure increased on the report of its potential ability to induce cardiomyocyte proliferation.8 Clinical trials with the epidermal growth factor–like domain of NRG in heart failure have demonstrated safety and efficacy,91,92 and another NRG molecule, glial growth factor-2, is currently in clinical trials for heart failure93 and has shown efficacy in a preclinical model.94 The nature of the interactions between the cognate receptor of NRG in the heart, ErbB4, and its preferred signal induction partner, ErbB2, may provide an opportunity for exploitation via protein engineering. Our group hypothesized that ErbB receptor interactions, which are prerequisite to signaling, could be biased away from the most commonly induced partnerships by receptor–ligand affinity interactions imposed by a bivalent ErbB ligand.95 Bivalent NRG (NN) was shown to induce differential signaling compared with NRG95 and was shown to have superior cardioprotective efficacy compared with the epidermal growth factor-like domain of NRG in a mouse model of doxorubicin-induced cardiomyopathy.96 Further exploration of this strategy may reveal a benefit of protein engineering for NRG in cardiac repair and regeneration.

**Vascular Endothelial Growth Factor and Fibroblast Growth Factor**

Because of their well-characterized mitogenic effects on endothelial cells in many contexts, vascular endothelial growth factor97 and fibroblast growth factor,98 as well as their splice isoforms and variants, have been examined extensively for therapeutic vascularization in the heart during the past ~25 years.99,100 However, negative outcomes of early clinical trials with both fibroblast growth factor102 and vascular endothelial growth factor103 have stalled progress of therapeutic approaches designed to promote selective receptor activation, such as protein engineering techniques to augment these therapeutic strategies. Antibody-based therapeutic approaches and other protein therapies for immunomodulation continue to emerge as potential treatments for atherosclerosis.104 More recently, mimetic peptides and monoclonal antibodies have been developed to inhibit PCSK9 (proprotein convertase subtilisin/kexin type 9) to lower cholesterol levels.105 All of the aforementioned approaches have promise, and the further application of protein engineering techniques to augment these therapeutic strategies could potentially enhance their efficacy.

**Therapeutic Frontiers in Protein Engineering**

Despite the implementation of some protein therapeutics for cardiovascular therapy, many limitations still remain. The ability to target delivery of a protein, or any drug, directly to the heart would minimize both the required dose and undesirable off-target effects; however, this ability remains mostly beyond our current reach. In general, proteins have often been considered poor drug candidates because of their low oral bioavailability and lack of long-term stability and other characteristics; this applies to proteins for cardiovascular therapy as well. Many drug discovery professionals, especially those with chemistry training, are taught a common mantra: proteins are not drugs. The protein engineering revolution in biotechnology has helped to change this perception, as protein drugs for numerous applications—notably in cancer therapy—are now on the market and in the clinic. However, this revolution has, for the most part, occurred outside of the realm of cardiovascular medicine. The existing cardiovascular therapeutics created via protein engineering noted demonstrate only a fraction of the potential of the field. Innovative and exciting protein engineering approaches are currently being developed, with many already having been applied to noncardiovascular therapeutics. Application of these burgeoning technologies to cardiovascular medicine, for example, to enable heart-specific targeted drug delivery, to enhance protein stability and circulation times, or to
promote direct stimulation of specific pathways to promote cardiomyogenesis via a therapeutic protein, may enhance the utility of protein-based cardiac therapy.

**Molecular Display**

Molecular display encompasses techniques that present molecules—typically peptides or proteins—on the surface of a cell, virus, or other host entity. This approach enables a linkage of genotype and phenotype of the displayed molecule, as its coding information is hybridized with that of the carrier. Thus, selective enrichment is possible through successive propagation steps under specified conditions—a process known as panning—and large polypeptide libraries can be screened for desired properties or interactions (Figure 1).

The concept of molecular display arose after George Smith’s seminal report of phage display, the display of a foreign protein on the surface of filamentous phage. Since then, a number of platform approaches for molecular display have been developed to enable high throughput screening of protein interactions. Technologies such as cell-surface display, ribosomal display, mRNA display, and others have allowed for directed evolution of proteins to refine activity and stability. Phage display is the most commonly used and is popular in the biotechnology industry, having been used in the development of a number of protein drugs, especially antibodies. Beyond directed evolution, phage display has been used for panning of biological and tissue samples—known as biopanning—to select for peptide ligands that enable tissue-specific homing and enhanced tissue retention of drugs or drug carriers.

In addition to phage display, other display technologies have emerged and may have relevance for the development of cardiovascular therapies in the near future. Introduced by Wittrup et al., yeast surface display enables, among other things, high-throughput quantitative library screening via fluorescent-activated cell sorting and has been used to evolve extremely high-affinity antibodies and peptides that have facilitated molecular targeting, for example, to promote high-resolution vascular imaging. Cell-free protein evolution methods such as ribosome display or mRNA display are not encumbered by transformation or expression limitations and also allow rapid evolution of high-affinity binding proteins. Overall, molecular display technologies are generally mature methods for selection and refinement of protein characteristics, which have significant potential to promote cardiovascular protein therapies.

**Engineered Protein Scaffolds**

Antibodies have been the most successful protein therapeutics to date. However, antibodies have weaknesses as therapeutics, including limited tissue penetration because of their large size. To address this and other issues, a number of engineered protein scaffolds (EPS) have been developed. For protein engineering, EPS comprise a minimal polypeptide framework that can be based on either an immunoglobulin or a nonimmunoglobulin molecule. The framework is typically monomeric without disulfide bonds or glycosylation sites and is usually highly stable and readily soluble. The framework is typically monomeric without disulfide bonds or glycosylation sites and is usually highly stable and readily soluble. EPS are also typically able to be easily expressed in a host organism and have surface-accessible residues that allow incorporation of sequence diversity and subsequent selection via molecular display.

A number of EPS have moved beyond the initial development stage toward clinical use. Among the most

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**Figure 1. Molecular display.** A protein or peptide with specific properties can be derived via molecular display after multiple rounds of selection. Polypeptide libraries can be made or purchased and either inserted into molecular hosts such as phage or yeast or appended to carrier molecules such as mRNA or ribosomes. The schematic is representative of phage or cell-surface display, where proteins are displayed on the surfaces of organisms that are subsequently exposed to various substrates, which serve as selection criteria. In the example, protein-mediated binding of the organism with a specific substrate followed by washing away of nonspecific binders and elution enable amplification of those organisms displaying proteins with the desired properties. Repetitive screening with additional or more stringent selection criteria enables ultimate isolation of an optimized protein sequence based on phenotype, which can then be linked with genotype after extraction of genetic material from the organism. Thus, molecular display facilitates discovery of information needed to design a protein with specific properties evolved to match nearly any chosen selection criteria.
well-developed are the following: Adnectins, derived from type III fibronectin domains; Anticalins, derived from lipocalin; Kunitz domains, derived from Kunitz-type protease inhibitors; DARPs, derived from ankyrin repeat proteins; and avimers, derived from the A-domain of low-density lipoprotein receptors. Ecallantide (Kalbitor), a rationally designed Kunitz domain, was approved in 2009 for clinical use to treat hereditary angioedema and could potentially be used in cardiothoracic surgery as a replacement for aprotinin. Many other EPS are being tested for myriad applications, and as molecular targets for cardiac repair and regeneration continue to be defined, EPS have the potential to play an important role in future development of cardiovascular therapeutics.

Noncanonical Amino Acids

The concept of incorporating ncAAs (also referred to as unnatural amino acids, non-natural amino acids, and artificial amino acids) site-specifically into proteins offers stunning potential: an avenue to attach any chemical functional group of interest to a protein, allowing for coupling with other proteins or molecules such as fluorophores or small-molecule drugs using straightforward, efficient, and tunable chemical reactions. Although several variations exist, the general approach involves aminoacylation of a tRNA that has an incorporated suppressor anticodon with an ncAA. This chemical modification facilitates site-specific ncAA incorporation during mRNA translation at a nonsense codon site, which can be inserted (site-specifically) in a gene of interest using standard gene synthesis or molecular biology techniques (Figure 2). This development of ncAA technology was pioneered by Schultz et al., and this group and many others have contributed to important advances that have made it robust for imparting desired functionality to proteins for therapeutic applications. For example, ncAA incorporation enables site-specific PEGylation of therapeutic proteins to enhance evasion of the immune system and to promote longer circulation times. This approach also allows for insertion of molecular staples—hydrocarbon linkers that can stabilize protein secondary structure in part by physically preventing entropic structural relaxation—into proteins to improve stability. This strategy could be extended via expressed protein ligatin techniques to fuse other engineered proteins for similar purposes. Using a related process developed by Tirrell et al., residue-specific ncAA incorporation of interferon β-1b was PEGylated for improved pharmacological properties.

The ncAA site-specific incorporation has further been used to generate bispecific antibodies and thus could be adapted to the creation of bivalent NRG molecules that might stimulate cardiomyogenesis. As ncAA technology continues to evolve, its potential for application toward cardiovascular therapy, which is already promising, should only be enhanced.

Enabling Methods for Drug Delivery

Protein engineering techniques have begun to be used for the development of proteins and peptides that enable more effective and efficient small-molecule, protein, and nucleic acid delivery across biological barriers targeted to specific tissues. This is a critical consideration because effective cardiovascular protein therapies will almost certainly require targeted or localized delivery to the heart. Some of these strategies have already been mentioned or implied, such as the functionalization of a therapeutic protein or a microcarrier or nanocarrier with a targeting sequence derived by molecular display or from an EPS. This general concept has also been applied to enable

![Figure 2. Incorporation of noncanonical amino acids (ncAAs) into proteins.](image-url)
gene delivery with enhanced tissue specificity. In addition, Dowdy et al. developed the concept of protein transduction domain–containing fusion proteins for protein delivery. In this method, the protein of interest is fused with a protein transduction domain, also referred to as a cell-penetrating peptide, such as that derived from the human immunodeficiency virus transactivator of transcription protein. This allows, among other things, proteins to cross biological barriers that would normally restrict them because of size and charge considerations (Figure 3). Protein transduction domains have also been used in gene delivery. A conceptually similar fusion protein approach whereby the protein of interest is fused with an antibody or peptide that is transported across a biological barrier, such as the blood–brain barrier, by receptor-mediated transport has also been explored and is sometimes referred to as the molecular Trojan horse approach. Some of these techniques have already been applied to facilitate targeted delivery to the myocardium, and continued expansion of these and other efforts will likely prove critical to the ultimate success of protein-based cardiovascular therapies.

Enabling Technologies for Cell-Based Therapy

In addition to providing an alternative for cell-based cardiac therapy, protein engineering can be used to augment this therapeutic approach. Cardiac cell transplantation has been limited by poor localization of injected cells, an ≈90% death rate for cells within 1 week of the injection or implantation, and, in the case of stem cells, uncontrolled cell proliferation or differentiation after transplantation. Some of these limitations have been overcome in the most recent clinical trials; however, the efficiency and efficacy of cell-based cardiac therapy can still be improved. Our group has used a therapeutic approach utilizing molecularly designed self-assembly peptide scaffolds combined with engineered proteins to create a microenvironment for improved engraftment and regenerative potential of transplanted cells for myocardial regeneration. In this case, self-assembly occurs through electrostatic interactions between heterospecific complementary amino acid sequences; a peptide scaffold is engineered to express one sequence, and the therapeutic protein entity is engineered to express the complement. This approach allows for nearly any chosen growth factor(s) to be controllably displayed or delivered along with cells, which may bind or otherwise interact with the peptide scaffold. In this way, differentiation and engraftment of transplanted cells can be improved. Moreover, the starting materials are easy to synthesize and purify on a large scale. This and other methods for enabling cell-based therapies for the heart provide yet another therapeutic frontier for cardiovascular protein engineering.

Conclusion

Protein engineering is a powerful approach with untapped potential for cardiovascular therapeutic development. As previously stated, approximately half of new drugs are proteins, and many of these therapeutics were created via protein engineering. The application of this technology to therapeutic development should only increase in the future; yet, no engineered protein drugs are currently being routinely applied for treatment of cardiovascular diseases, perhaps because of deficiencies in our current understanding of the molecular mechanisms of cardiac repair and regeneration, which is still evolving (the authors direct the reader to several extensive recent reviews on the topic). From the standpoint of therapeutic development, 2 phenomena that contribute to myocardial repair and regeneration have been the focus of much of the effort: vascularization and cardiomyogenesis. Although the fundamental processes that constitute vascularization—angiogenesis, arteriogenesis, and vasculogenesis—are relatively well-defined, the mechanistic

Figure 3. Protein engineering methods to enable drug delivery. A number of techniques within the realm of protein engineering could be used to enhance delivery of small molecules, proteins, and nucleic acids to the heart. Examples shown include the targeting of a nanocarrier to a cell type of interest (eg, cardiomyocytes, endothelial cells) by a peptide molecule derived via molecular display and the enhancement of cellular internalization of a therapeutic entity via fusion with a protein transduction domain (PTD). siRNA indicates small interfering RNA. (Illustration credit: Ben Smith).
underpinnings of cardiomyogenesis remain the subject of contro-
versy. Hopefully, advances in the molecular cardiology field and
resolution of fundamental controversies within it will facilitate a
leap forward for cardiovascular medicine through increased use of
engineered protein therapeutics.

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paid consultant for the company, and serves on the company’s Board of
Directors. Provasculon has interests in stromal cell–derived factor-
1 therapy, an area related to the topics discussed in this review. Dr
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Jay and Lee  Protein Engineering for Cardiac Repair  941


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