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Biological Therapies for the Treatment of Cardiac Arrhythmias

Luca Boveri[1] • Barbara Colombi[1] • Silvia G. Priori[1,2,3*]

[1] Department of Molecular Cardiology, IRCCS Fondazione Salvatore Maugeri , Pavia, Italy
[2] Department of Cardiology, University of Pavia, Pavia, Italy
[3] Address for Correspondence: Molecular Cardiology, Fondazione Salvatore Maugeri , Via Maugeri 10/10A, 27100 Pavia, Italy
Corresponding author: * **spriori@fsm.it**

ABSTRACT

The large number of publications reporting advances in vector design, gene transfer protocols and stem cells manipulation prompted the expectation that these methods may become available to the clinicians. However, since the technological transfer from the bench to clinical practice demands that aspects such efficiency, long term effect of the therapy and its safety are elucidated, the development of the field has been slower than anticipated. The use of biological therapies in the management of cardiac arrhythmias to overcome the limitations of pharmacological, ablative and device treatments has attracted a strong interest from the arrhytmologists. Although the availability of such treatments in clinical cardiology, is still far ahead, various research groups are conducting preliminary but very encouraging investigations supporting the view that rhythm manipulation by biological means is a feasible approach at least in animal models. In this review we will discuss the approaches that have been outlined in the experimental laboratories for the control of rhythm disturbances. Three key strategies for biological therapies of cardiac arrhythmias will be presented: 1) the use of viral vectors to modulate molecular targets that are critical for the control of excitability (**gene delivery**), 2) the use of modified stem cells to re-create specialized structures such as the sinus node or the atrio-ventricular node (**cell graft**), 3) the application of post transcriptional modulation to control the production of substances that may attenuate the arrhythmogenic potential of different diseases (**expression modulation**). We will analyse the pros and cons of these approaches and will conclude by discussing the unmet needs and the challenges in this field.

Keywords: cell therapy, electrophysiology, gene therapy
Abbreviations: AAV, Adeno-associated vector; **AV node**, atrio-ventricular node; **BMC**, bone marrow cell; **EB**, embryonic body; **EP**, electrophysiology; **ES cell**, embryonic stem cell; **miRNA**, micro interference RNA; **RNAi**, RNA interference; **shRNA**, short-hairpin RNA; **siRNA**, small interference RNA; **SR**, sarcoplasmatic reticulum; **USMD**, ultrasound microbubble destruction

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INTRODUCTION

Seventeen years after the first successful attempt to introduce an exogenous genetic element to the rat's heart (Lin *et al.* 1990) the development of powerful vectors for the cardiac delivery of transgenes has provided a major impulse to the field of biological therapy for heart diseases. Several laboratories around the world have engaged into this fascinating new research area in the attempt to devise new cures for heart diseases ranging from heart failure to ischemic heart disease to cardiac arrhythmias. In this review we will focus on the use of molecular strategies for the treatment of cardiac arrhythmias.

Is there a need for biological therapy for rhythm disturbancies?

Cardiac arrhythmias represent one of the most common cau-

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ses of morbidity and mortality worldwide. Current management of cardiac arrhythmias relies on pharmacological therapy, ablation and the implantation of electronic devices such as pacemakers and defibrillators. Despite the important advancements that these techniques have provided, there are unresolved issues that provide a rational for exploring other therapeutic approaches. For example while antiarrhythmic drugs are used to limit recurrences of arrhythmias and are predominantly effective in the control of supraventricular arrhythmias, their role in the prevention of life threatening ventricular arrhythmias is extremely limited. Similarly ablation is a curing procedure for diseases such as Wolff Parkinson White syndrome, it is still unable to cure more complex conditions such as atrial fibrillation. Finally the use of devices such as implantable defibrillators and pace makers that are the only means of treating bradyarrythmias and improving survival of patients at risk of ventricular fibrillation, still suffers of several limitations related to the lifespan of leads and batteries, the need of repeated surgical interventions for device replacements and, last but not least their cost.

Clearly it is still impossible to predict whether biological approaches to anti arrhythmic treatments will be able to overcome the current limitations and to provide advantages as compared to conventional treatments, however we believe that it is time to investigate the potential for development of this new field with an open mind and an unbiased attitude (Priori *et al.* 2000).

The challenges of gene delivery to the heart

A limiting step when considering gene therapy for cardiac diseases is related to the difficulty of obtaining an efficient transgene delivery *in vivo*.

In order to exert its action on the target tissue a transgene has to reach the specific organ in a biologically relevant quantity, it should be expressed at the appropriate level and for enough time to impact its target tissue. Since cardiac myocytes are post mitotic cells they are not prone to transfection both *in vitro* and *in vivo* and this is one of the reasons why the development of gene therapy for cardiac diseases has developed at a slower pace than gene therapy for other organs. To overcome these limitations different vector systems are being tested to improve the efficiency of gene transfer to the heart and they will be discussed below.

Which vectors for gene delivery into the heart?

We will review this topic by presenting viral vectors and non-viral vectors that have been used for gene transfer into the heart. Whenever data are available we will discuss studies carried out in the attempt to modify the electrophysiological substrate of the heart.

Viral vectors

Viruses are intracellular parasites that replicate exploiting the host cells machinery for the production of their own proteins. Viruses can be engineered to carry exogenous genetic information while limiting their disruptive action on the host cells to become a suitable mean of gene transfer. Different viruses can be used for gene delivery. We will briefly discuss the pro and cons of the viruses that have been more commonly used for gene therapy in the heart focusing on the vectors that seems to be more promising for achieving safe and effective transgene delivery into cardiac myocytes.

Adenoviral vectors

Adenoviruses are non-enveloped viruses that contain a linear double stranded DNA with inverted terminal repeats; they have a distinguishing icosahedral capsid. More than 40 adenoviral serotypes are actually recognized most of which can cause respiratory tract infection in humans, infecting a wide spectrum of post-mitotic cells from various tissues such as skeletal muscle, brain and heart.

Ease of production, high functional titres and broad target-cells tropism makes of the adenoviruses a suitable gene transfer vector (Leiden 2000; Mah *et al.* 2002).

Cardiomyocytes from different species have been shown to be prone to transduction by adenoviruses both *in vitro* as well as *in vivo* (Leiden 2000; Mah *et al.* 2002).

The wild type adenoviral genome is approximately 35 kb of which 30 kb can be replaced with allogenic DNA preserving the capability to infect host cells (Smith 1995; Verma *et al.* 1997).

Adenoviral vectors, thanks to their high transduction rate have been widely used for gene transfer into cardiac myocytes *in vivo* experimental studies (White *et al.* 2001). Due to several limitations it has become apparent that adenoviruses are not appropriate vectors when devising *in vivo* therapeutic strategies. First of all adenoviruses do not integrate their nucleic acids into the host genome rather they replicate as episomal elements. This feature guarantees lack of insertional mutagenesis but unfortunately it also causes a loss of the viral elements during cell division leading to a transient expression of the transgene. Furthermore adenoviruses elicit in the host a strong immune response that promotes the clearance of transduced cells further reducing the persistence of the transgene (Yang *et al.* 1994; Chirmule *et al.* 1999).

Finally human toxicity has been documented when infusion into the hepatic artery of an adenoviral vector caused the death of a patient in 1999. Since then search for better viral vectors has focused on the reduction of the immune response either by modification of adenoviruses or through the use of different viruses.

Lentiviral vectors

The ability of HIV-1 to infect terminally differentiated cells such as macrophage guided toward the use of lentivirus-derived vectors for gene delivery to cardiac myocytes (Lewis *et al.* 1992; Bukrinsky *et al.* 1993; von Schwedler *et al.* 1994). The ability to integrate into the genome and the large capacity of gene transferring make lentiviral vectors particularly attractive.

The main risk connected to lentiviral vector in a therapeutic perspective is related to the random integration of the proviral complex into the host genome exposing to the risk of insertional mutagenesis and of the activation of otherwise silent oncogenes.

In this respect an important observation was made by investigators who were able to show that integration-deficient lentiviral vectors (Lu *et al.* 2004; Saenz *et al.* 2004) their ability to induce a stable transduction *in vitro*. We are not aware of studies investigating the use of these vectors *in vivo* therefore it is not possible to elaborate on the potential clinical application of integration-deficient lentiviral vectors (Yanez-Muñoz *et al.* 2006).

Different groups (Rebolledo *et al.* 1998; Zhao *et al.* 2002; Bonci *et al.* 2003; Fleury *et al.* 2003) reported the efficacy of lentiviral vectors to induce transgene expression into neonatal/adult rat and adult rabbit cardiomyocytes *in vitro* as well as *in vivo*. Remarkably fetal human cardiomyocytes could be successfully transduced by lentiviral vectors. Overall these data point to the fact that lentiviruses are suitable vectors to be used when targeting cardiac cells however it remains questionable whether it will ever be possible to overcome the safety concerns remain a major limiting factor for their approval for clinical use.

Adeno-associated vectors

Adeno-associated vectors are considered among the most promising tools for gene delivery (Monahan *et al.* 2000) because of their low immunogenicity and long-term and sustained expression of the transgene. AAV belong to non-pathogenic human parvoviruses that can infect both dividing and non-dividing cells and have been utilized for heart targeting in different studies (Hoshijima *et al.* 2002; Xiao *et*

al. 2005). AAV are single stranded DNA viruses with two large open reading frames and Inverted Terminal Repeats sequences not autonomous in replication that therefore require a helper agent such as herpes virus or adenovirus. They can establish latent infection within the cells, either by site-specific integration into the host genome or by persisting in an episomal form. AAV-2 represents the more commonly used AAV serotype for experimental research. Today over 100 serotypes of AAV have been isolated from different animal species and some of them show a tropism for heart tissue.

Progresses in adeno-associated vector manipulation has allowed the engineering of hybrid vectors through the development of AAV vectors in which the capsid is composed of a mixture of subunits coming from different serotypes and AAV vectors in which the proteins of the capsid recapitulate properties of different wild type AAV vectors (Wu *et al.* 2006). One of the objectives of using genetically modified AAV is that of improving the tissue specificity of the vector. Accordingly it seems that some of the engineered AAV may be particularly effective in transferring exogenous genome into cardiac cells.

Recombinant adeno-associated vectors rAAV2/1 and rAAV2 are the most widely used AAV in experimental studies: recently it was reported (Pacak *et al.* 2006) that intravenous delivery of a rAAV2/1 vector engineered to deliver the *Gaa* gene encoding for the α-glucosidase protein in a transgenic mouse model of Pompe disease. The authors (Pacak *et al.* 2006) demonstrated that intravenous administration of the vector induced a 200-fold expression of the transgene that effectively induced correction of the enzymatic deficiency in the transgenic mice.

As for other viral vectors safety issues will have to be fully investigated before AAV viruses can be considered suitable for clinical use. Specifically it will be important to conduct careful assessments to quantify the risk of germ line transmission of the virus in humans (Nakai *et al.* 2003) based on the evidence that transient germ line transmission has been reported by some authors.

Non viral vectors

The injection of naked DNA into the myocardium represented the first successful attempt to introduce exogenous genetic sequences into cardiac myocytes, but because of the very low transfection efficiency and the advents of more powerful tools (Muller *et al.* 2007) the method was abandoned.

Lipoplexes (liposome-DNA complexes) (Lee *et al.* 2003) and polyplexes polymer-DNA complexes (Pitard *et al.* 2004) can be systemically injected without being degraded like naked DNA; however, they still cannot guarantee a sustained expression of the transgene or higher infection rate.

Overall the use of non-viral vectors is hampered by low transfection efficiency and short biological half-life and therefore it is unlikely to represent a method that can be envisioned for delivery of gene therapy for cardiac arrhythmias in which a high level of efficiency and a prolonged expression are needed.

How to deliver gene therapy into the heart to treat cardiac arrhythmias?

The selection of the gene delivery strategy is an important step when devising a biological therapy based on gene transfer. A fundamental choice that has to be made is to decide whether localized or global transfer of the transgene is preferred to achieve the therapeutic goal. In the heart viral vectors can be injected in the cardiac wall to achieve a localized delivery to small and targeted regions of the heart (usually 1 to 2 mm around the injection site) (Kass-Eisler *et al.* 1993) or they can be injected in the coronary arteries to achieve a more uniform distribution in the heart. The proper selection of the delivery strategy is critical to the achievement of the expected curative results minimizing the undesired consequences.

When using gene transfer to modulate cardiac excitability the electrophysiological characteristics of the heart need to be carefully considered. In order to avoid pro arrhythmic rather than antiarrhythmic effects it is important that the transgene does not alter the physiological distribution of ion channels that ensures uniform and coordinated impulse propagation across the myocardium. The complexity of the electrical propagation in the heart should be kept in consideration when designing strategies for gene therapy to treat arrhythmias. The delivery of exogenous DNA encoding ion channels could profoundly alter the physiological distribution of these proteins. In fact it is known that the distribution of ion channels is not uniform in the heart (Schram *et al.* 2002) and that this lack of uniform distribution is critical for the maintenance of the transmural gradient across the myocardial wall. The duration of the action potential and the shape of the action potential is remarkably different in epicardial, mid myocardial and endocardial cells (Antzelevitch *et al.* 2001). Such a diversity is caused by a specific distribution of ion channels in the different layers of the ventricular wall (Schram *et al.* 2002). Furthermore data are also available that demonstrate the existence of regional electrical differences also between the right and left chambers (Melnyk *et al.* 2005). These data point to the fact that intracoronary injection of a transgene might well be the optimal approach when there is a need to achieve a uniform expression of the "therapeutic" gene to the heart. However the same approach may be detrimental if applied to the induction of expression of ion channels without respecting the regional dishomogeneity of their distribution.

In this respect new methods of delivery may represent a promising approach when regional delivery of the transgene is desired.

Kikuchi *et al*. (2005) provided another contribution to the field of gene transfer into the myocardium by developping a novel gene painting strategy that consisted of the adenoviral vector engineered with the transgene of interest complexes to poloxamer gel. They showed that "painting the atria" with this complex (adenovirus plus poloxamer gel) method allowed to achieve uniform transmural gene transfer.

A technique recently approved by the FDA is represented by Ultrasound Targeted Microbubble Destruction (UTMD). The term "microbubbles" refers to second generation ultrasound contrast agents filled with gasses (Forsberg *et al.* 1994). Microbubbles can be loaded with plasmidic DNA or with viral vectors that remain trapped in the bubble and can circulate in the blood stream. The microbubbles can be ultrasonically destroyed when they reach the target zone allowing the spreading of the vector (Shohet *et al.* 2000; Bekeredjian *et al.* 2003; Chen *et al.* 2003; Bekeredjian *et al.* 2004; Wang *et al.* 2004; Howard *et al.* 2006). Few groups (Howard *et al.* 2006) reported an efficient cardiac delivery of USMD-adenovector system injected in the peripheral circulation: whether this method may prove suitable for the deliver of biological therapy for arrhythmias will have to be investigated.

Gene therapy to treat cardiac arrhythmias

In the last few years different strategies were devised to treat a variety of rhythm disturbances using gene transfer therapies. Overall it is possible to identify four areas in which gene transfer has been applied: 1) pacemaker dysfunctions 2) modulation of the AV node for rate control in atrial fibrillation 3) abbreviation of prolonged repolarization 4) molecular ablation. We will discuss the most relevant studies published on each topic (**Table 1**) providing a critical insight and commenting on the potential clinical applications.

Table 1 Gene therapeutic studies.

Transgene delivered	Model	Vector used	Site of injection/type of perfusion	Report	Reference
β-2-adrenergic receptor	Porcine/murine	Plasmid	Right atrium injection	Heart rate increasing	Edelberg *et al.* 1998, 2001
HCN-2	Canine	Adenoviral	Left bundle branch injection	Rhythm originating from the left ventricle (during vagal stimulation)	Plotnikov *et al.* 2004
HCN-2	Canine	Adenoviral	Left atrium injection	*In situ* pacemaker activity	Qu *et al.* 2003
HCN-2/ Mutant HCN-2 (E324A)	Canine	Adenoviral	Left bundle branch injection	Pacemaker activity	Bucchi *et al.* 2006
Mutated HCN-1 (HCN-1-ΔΔΔ)	Guinea pig/porcine sick sinus model	Adenoviral	Left ventriculum injection/Left atrium	Pacemaker activity	Tse *et al.* 2006
Kir2.1AAA (dominant negative form)	Guinea pig	Adenovirus	Left ventricular cavity perfusion + aortae cross clamping	Pacemaker activity	Miake *et al.* 2003
Mutated Kv1.4 (R447N, L448A, R453I G528S)	Guinea pig	Adenovirus	Left ventriculum	Pacemaker activity	Kashiwakura *et al.* 2006
$G\alpha_{i2}$	Porcine	Adenovirus	AV nodal artery infusion	Slowed heart rate during atrial fibrillation	Donahue *et al.* 2000
$G\alpha_{i2}/cG_i$ (constitutive active mutant)	Porcine (persistent AF)	Adenovirus	AV nodal artery infusion	cG_i showed a grater decrease in heart rate compared to wild type	Lee *et al.* 2002
Small G protein Gem	Guinea pig	Adenovirus	AV nodal artery infusion	Slowed AV nodal conduction/ shortened QTc interval. Reduced heart rate during fibrillation	Murata *et al.* 2004
Kv1.5	Transgenic mouse (LQTS – Kv1DN)	Adenovirus	Intramyocardial injection (basal part of free LV)	Shortened action potential, EAD's eliminated, shortened QT interval, decreased dispersion of repolarization, increased heart rate	Brunner *et al.* 2003
Kv1.5	Transgenic mouse (LQTS – Kv1DN)	rAAV	Intramyocardial injection (basal part of free LV)	Shortened action potential, EAD's eliminated, long term expression	Kodirov *et al.* 2003
KCNH2-G628S (dominant negative KCNH2)	Porcine (model with inducible postinfarct VT)	Adenovirus	Focal perfusion to infarct scar borders	Block of the ventricular arrhythmias	Sasano *et al.* 2006
SERCA1 and Kir2.1	Guinea pig	Adenovirus	Intramyocardial injection	Abbreviated repolarization	Ennis *et al.* 2002

Gene therapy for the treatement of pacemaker dysfunctions

The fascinating concept of recreating a "biological pacemaker" that could replace "electronic pacemakers" has been pursued with enthusiasm by several groups that have contributed to test different strategies in a variety of experimental models. We will follow a chronologic approach to describe the developments in this field.

The first proof of concept that it is possible to modulate sinus node function by gene transferring dates back to the studies of Edelberg (Edelberg *et al.* 1998, 2001) who in 1998 and subsequently in 2001 showed that exogenously administered plasmid constructs containing the cDNA of the human β adrenergic receptors was able to increase the sinus rate in the murine and porcine hearts. The authors envisioned that these studies could lead to an innovative molecular therapy for rate dysfunction such as that observed in sick sinus syndrome patients.

Few years later the group of Marban at John Hopkins (Miake *et al.* 2003) demonstrated the possibility of inducing ectopic pacemaker function by using an adeno viral vector engineered to deliver a genetically modified Kv2.1 construct in which the amino acids 144-146 located in the pore forming region of the channel were substituted to create a dominant negative suppression of iK1 density. The observed phenotype at high degree of Ik1 suppression was that of inducing spontaneous firing in ventricular myocytes (i.e. creating an idioventricular rhythm). Obviously this study has to be seen as a proof of concept of the use of gene transfer for inducing an idioventricular rhythm more than an actual strategy that should be pursued for the development of gene therapy. In fact it is known that suppression of Ik1 may induce prolongation of the action potential as observed in patients with Andersen syndrome (Plaster *et al.* 2001) and therefore the proarrhythmic potential of this approach is most likely greater than the anticipated benefit.

The most logical approach when attempting to recreate an artificial pace maker structure is that of transferring the gene that encodes for the pace maker channel that conducts the If current. This strategy was tested in the pioneering work of Qu *et al*. (2003) who provided a major advancement into the field of biological pacemaker when in 2003 they were able to develop a biological pacemaker using an adenoviral construct to delivery *in vivo* an HCN family isoform (HCN2). Two are the advancements that this study provided: on one side the authors proved the feasibility of successfully recreating a pacemaker by using the physiological current that regulates pacemaker function and on the other side they successfully faced the challenge of local delivery in the left atrial appendage. Remarkably this study demonstrated that transient expression of the construct that HCN2 overexpression provides an if current that is sufficient to drive the heart thus preserving cardiac excitation during vagally induced sinus arrest. However, before a clinical use of this approach is envisioned, several challenges remain to be addressed such as to overcome the limitation of transient expression, to ensure that the biological pace maker may be modulated by the authonomic nervous system to recapitulate a physiological behaviour.

Recently Bucchi *et al*. (2006) as well as Tse *et al*. (2006) reported successful studies in which the pacing function was achieved by transferring genetically modified HCN-constructs in order to provide features that may be more beneficial to recipients of the construct. Once more, beside the results obtained in the specific study, it is important for the purpose of this review, the relevance of the concept that in light of developing gene transfer strategies to treat cardiac arrhythmias it may be possible to envision the development of ion channels with kinetics tailored ad hoc to maximise the therapeutic benefit.

Recently in this field an interesting debate has emerged and different groups have discussed which is the best strategy to devise a biological pacemaker. As the use of HCN genes seems to have the advantage of using the physiological pacemaker channel for therapeutic purposes, it may also have limitations. Some authors (Ulens *et al.* 2001; Brewster *et al.* 2005) in fact have suggested that the transfer of HCN channels may be less succesful than anticipated because heteromultimerization of the construct with the native HCN subunits may lead to unpredictable efficiency of the therapy. In this respect, Kashiwakura *et al*. (2006) recently delivered through an adenoviral vector DNA encoding for a genetically engineered variant of the Kv1.4 channel into the left ventricular wall of guinea pig. The expression of the transgene was able to elicit an idioventricular rhythm in response to metacholine induced bradycardia thus revamping the interest in the concept of ad hoc designed synthetic channels to be used as biological pacemakers.

Modulation of the AV node for rate control in atrial fibrillation

Another interesting line of investigation in the field of genetic therapy for cardiac arrhythmias is represented by the modulation of AV nodal conduction. In patients with atrial fibrillation slowing of atrio-ventricular conduction is one of the therapeutic strategies (Wyse *et al.* 2002) A comparison of rate control and rhythm control in patients with atrial fibrillation that is usually accomplished with the use of drugs such as beta blockers or calcium antagonists. The idea that genetic therapy could allow permanent slowing of AV conduction has stimulated investigators to test molecular strategies for AV nodal modulation.

Donahue *et al*. (2000) through a perfusion of the atrioventricular nodal artery delivered an adenovector encoding G-α-i-2 subunit to a porcine heart. The overload of G-α-i-2 mimicking the effect of a β adrenergic antagonist suppressed atrio-ventricular conduction and slowed heart rate of the treated animal and during atrial fibrillation the ventricular response rate decreased significantly without developing complete heart block. Similar results were obtained by the same group (Murata *et al.* 2004) that overexpressed ras-related small G protein Gem in the heart of Guinea Pig by adenoviral mediated gene transfer to AV node.

Overall these studies support the concept that local delivery of different transgenes in the AV node is an appealing strategy to control by non pharmacological means heart rate response in atrial fibrillation.

Abbreviation of prolonged repolarization

The availability of transgenic animal models of long QT syndrome has provided the opportunity to test the use of gene transfer to shorten repolarization. The first study that proved the feasibility of this approach was performed by Brunner *et al*. (Brunner *et al.* 2003; Kodirov *et al.* 2003) who used a transgenic mouse model overexpressing a truncated K channel and tested the hypothesis that gene transfer of wild type Kv 1.5 gene would reconstitute the 4 amino pyridine component of IK slow. As hypothesized that gene transfer of Kv1.5 with an adenoviral vector was able to show transient therapeutic effect that resulted in shortening of the action potential duration and of the QT interval with disappearance of early after depolarizations and arrhythmias. The authors used the same model in a subsequent study in order to test whether they could achieve long term expression of the transgene and reversal of the phenotype using an adeno-associate viral vector.

The results were quite encouraging as at six months after infection the electrophysiological properties of the transgene were still present.

These studies represent an important contribution and they support the view that gene transfer to the entire heart may be able to prevent arrhythmias caused by an abnormal genotype.

Isolation of arrhythmic foci in the ventricle

Very recently Sasano *et al*. (2006) provided a challenging and fascinating study when they envisioned a gene transfer strategy with the ambitious objective of performing "molecular ablation" of ventricular tachcyardia. The rational behind the approach that they developed is that of encircling the border zone of myocardial infarction with transgene that would locally prolong ventricular repolarization thus creating an exit block that would impair propagation of extrasystolic beats to the ventricle. The transgene that was used to induce a local prolongation of repolarization was a mutant HERG gene presenting the G628S mutation that has been identified in Long QT Syndrome patients and therefore is very well known to prolong QT interval. After adminsitration of the transgene in the border zone of the infarct the inducibility rate of Ventricular tachycardia dropped form 100% to zero. This study is very important because it opens to the use of viral gene transfer to the prevention of sudden death in patients with ischemic heart disease.

THE NEW HORIZON: STEM CELL THERAPY

Different types of stem cells have been used as a substrate to devise therapeutic strategies for heart diseases. Somatic, embryonic and adult stem cells have been used in experiments targeted to recover contractility into necrotic or dysfunctional areas of the myocardium (the so called "myocardial regeneration"). Much more limited is the availability of data concerning the use of stem cells for the treatment of arrhythmias. Nonetheless the successful results reported provide a proof of concept that cell therapy provides a feasible strategy to treat arrhythmias. Before discussing the available experience on the use of cell therapy for the treatment of arrhythmias, we will review some basic concepts about the different types of stem cells that can be used for therapeutic purposes.

Embryonic stem cells

Embryonic stem cells are pluripotent cells that are present only in embryonic tissue and can differentiate into several cell types including cardiomyocytes. Although few of the key pathways and signals that mediate cellular differentiation have been recognized (Lev *et al.* 2005), some factors and compounds have been identified that can be used in culture to induce and enhance stem cells differentiation towards a cardiomyocytes lineage (Xu *et al.* 2002; Passier *et al.* 2005). When cultured *in vitro*, embryonic cells organize in a three dimensional structure called "*embryonic body*" that provides a source of cells that, when exposed to appropriate conditions, may show evidence of cardiac lineage differentiation (pacemaking, atrial, ventricular, Purkinje-like) (He *et al.* 2003) with specific marker expression (GATA-4, Nkx2.5 and MEF-2) (Kehat *et al.* 2001) and ultrastructure organization (Snir *et al.* 2003).

Futhermore, a spontaneous beating activity of cardiac derivates from embryonic stem cells was evidenced *in vitro*, as well as *in vivo* after transplantation (Kehat *et al.* 2004; Xue *et al.* 2005).

Even when the problems of developing ethically acceptable renewable sources of embryonic stem cells committed

to cardiac lineage are solved, several aspects related to the transplant in humans of ES cells will have to be carefully addressed such as 1) the immunological aspects that may lead to transplant rejection, 2) the risk of tumours development (especially teratomas) and 3) last but not least the important issue of proarrhythmic action of cells that may establish abnormal electrical coupling with the adjacent myocytes.

Adult stem cells

Adult stem cells are present in different tissues; these cells are already committed to develop into a limited number of cell types. Bone marrow is the principal and most accessible source of stem cells. Four different subsets of stem cells can be found in bone marrow: hematopoietic, endothelial stem/precursors, mesenchymal and multipotent adult progenitor cells. Several laboratories have been able to show that when stem/progenitor cells (BMCs) are engrafted into the myocardium they develop into cardiac myocytes and establish electrical coupling with adjacent tissue.

Albeit there is still little understanding on the true potential for myocardial regeneration through the use of adult stem cells, their clinical use has proceeded quite rapidly and few phase I/II clinical study on adult stem cells were conducted and showed an encouraging safety profile. However, only data on a limited follow up and on a limited number of patients are available and clearly this field should be still considered at a very early stage of development in which both efficacy and safety still have to be defined.

Somatic cells

About half of the cells presents in the heart are not myocytes and the largest population is represented by fibroblasts. Recently the interest of investigators has been attracted by the possibility of exploiting genetically modified fibroblasts to modulate excitability of cardiac tissue. These approach is made possible by the evidence that fibroblasts and cardiac myocytes may establish electrophysiologic coupling (Rook *et al.* 1992; Fast *et al.* 1996). Furthermore at variance with myocytes, fibroblasts may be genetically manipulated to achieve targeted electrophysiological properties that in principle may allow the development of customized cells for treatment of different arrhythmias. Although *in vivo* experiments are not yet available the use of fibroblast is certainly a highly promising novel strategy for the application of cell therapy to the treatment of cardiac arrhythmias.

Cell therapy to treat cardiac arrhythmias

Cell therapy has been introduced in cardiology with the primary objective of obtaining myocardial regeneration therefore the use of cellular therapy for the treatment of arrhythmic diseases is still in its infancy (**Table 2**).

We have outlined in the previous section that the development of biological therapies for the treatment of bradyarrhythmias using gene transfer has met with very encouraging results. Preliminary data have been provided to support the view that exogenously implanted cells showing intrinsic automaticity may provide a novel strategy for the development of a biological pacemaker.

Kehat and colleagues (Kehat *et al.* 2004) were possibly the first to report that subepicardial injection of spontaneously beating cells derived from embryonic bodies into the ventricle of pigs with complete AV block was able to induce a spontaneous rhythm that showed a positive chronotropic response upon exposure to isoproterenol administration thus recapitulating a physiological pacemaker function. Similar results were also obtained by Xue *et al*. (2005) using a different strategy. These authors injected in subepicardial region of the left ventricle of guinea pigs cardiac myocytes derived from human embryonic stem cells and demonstrated that these cells were able to functionally integrate with native myocytes and to induce rhythmical electrical activity able to spread from the injection site to the adjacent cardiac tissue.

More recently successful attempts to use genetically modified adult stem cells to generate biological pace makers has been reported. Potapova *et al*. (2004) used mesenchymal stem cells overexpressing *HCN2*, i.e. the gene encoding for the ion channel that conducts the pacemaker current I_f. When these genetically modified stem cells were co-cultured with neonatal rat ventricular myocytes they were able to induce a 70% increase in the beating rate as compared to control myocytes. The same cells were injected into the left ventricular wall in dogs and immunohistochemical analyses showed that the grafted cells were able to couple with cardiac myocytes through gap junctions. When sinus arrest was induced in the animals, the grafted cells were able to produce a ventricular rhythm that propagated to the heart increasing heart rate by 30% as compared to the rate of control animals.

A novel approach to the use of cellular therapy has been exploited by Feld *et al*. (2002). These authors tested the concept that transfected engineered fibroblasts could provide a novel strategy to manipulate cardiac excitability. Their *in vitro* study exploited genetically engineered fibroblasts expressing the Kv 1.3 potassium channel engrafted into primary cultures of neonatal rat myocytes. The study proved that engineered fibroblasts may establish electrical

Table 2 Cell therapeutic studies.

Cell type	Transgene	Delivery/injection site	Model	Report	Reference
Human fetal atrial cardiomyocytes (included sinus nodal cells)	–	Free wall left ventricle	Porcine (AV node ablated)	Stronger idioventricular rhythm in treated pig	Lin *et al.* 2005
Neontal atrial cardiomyocytes (included sinus nodal cells)	–	Free wall left ventricle	Porcine (AV node ablated)	Stronger idioventricular rhythm in treated pig	Cai *et al.* 2006
Fetal canine atrial cardiomyocytes	–	Free wall left ventricle	Canine (X-linked muscolar dystrophy-AV node ablated)	Electrical and mechanical coupling between allogenic and donor cardiomyocytes	Ruhparwar *et al.* 2002
In vitro differentiated Human embryonic stem cells (from EB's)	–	Subepicardial injection	Porcine (complete AV block)	Spontaneous rhythm/response to Isoproterenol	Kehat *et al.* 2004
In vitro differentiated Human embryonic stem cells (from EB's)	–	Subepicardial injection	Guinea pig	Rhythmical electrical activity able to spread from the injection site to the adjacent cardiac tissue	Xue *et al.* 2005
Mesenchymal stem cells	HCN-2	Left ventriular wall	Canine	Increased heart rate	Potapova *et al.* 2004
Fibroblast	Kv1.3	–	*In vitro* experiment: neonatal myocytes	Multiple local conduction blocks	Feld *et al.* 2002

coupling with myocytes and demonstrated that they were able to modify electrical properties of the surrounding tissue. Specifically the experiments were able to induce conduction blocks that modified the spreading of electrical activation in the cellular culture. These experiments remind of the approach used by Sasano *et al*. (2006) who isolated by viral gene transfer of mutant HERG channel an arrhythmic foci in the ventricle to achieve a "molecular ablation" of the arrhythmogenic foci. Although experiments *in vivo* are still lacking, the innovative work of Feld *et al.* (2002) and co-workers suggests that cellular therapy may be envisioned for the treatment of different types of arrhythmias beyond impaired pacemaker function.

POST TRANSCRIPTIONAL MODULATION: IS THERE A ROLE IN ELECTROPHYSIOLOGY?

RNA interference (RNAi) is a fundamental cellular mechanism for silencing gene expression, originally elucidated in *Caenorhabditis elegans* (Fire *et al.* 1998). This post transcriptional regulation process is physiologicallly activated when small molecules of RNA (micro interference RNA or miRNA) pair with a complementary messenger RNA with a subsequent degradation of the double stranded ribonucleic acids.

The discovery of the mechanism of RNA-mediated-interference (RNAi) has made a major impact in the scientific community and the exploitation of this physiological mechanism for new applications has developed at an extremely rapid pace.

Few years ago, Elbashir and colleagues (Elbashir *et al.* 2001) showed that introducing chemically synthesized sequence (small interfering RNA - siRNA), mimicking the role of miRNA into mammalian cells, can mediate effective and specific silencing of gene expression: this observation paved the way to the use of gene silencing to study gene function and to investigate its potential therapeutic role.

Before envisioning a therapeutic use of siRNA the issue of effective delivery *in vivo* needs to be achieved. In order to exert their silencing action siRNA have to reach the cytoplasm of cells. Furthermore due to their rapid elimination by kidney filtration (because of their small size) and to their degradation by endogenous serum RNase the half-life of siRNAs *in vivo* is short it varies from seconds to minutes (Soutschek *et al.* 2004) and the efficacy if exogenously administered siRNA rapidly is reduced upon every cell division and therefore fades with time.

In terminally differentiated cells such as neurons and macrophages (Mah *et al.* 2002b; Omi *et al.* 2004), siRNA-mediated silencing may last for several weeks, in rapidly dividing cells such as tumour cells, the siRNA activity can persist for 3-7 days (Holen *et al.* 2002). To overcome the limitation of transient efficacy of siRNA in light of therapeutic applications, several investigators have used different viral vectors, such as adenovirus and lentivirus (Li *et al.* 2003,. 2005) to deliver, in particular to hardly to transfect cells, molecular precursors of siRNA. An important advantage of these methods is represented by the opportunity to co-express a reporter gene facilitating the tracking of the transduced cells.

One of the most popular approach based on the delivery of siRNA precursors uses shRNA (Short-hairpin RNA) (Kennerdell *et al.* 2000) that can be delivered with viral vectors and can be processed by the target cells into the active siRNA. Furthermore, an interesting tool is represented by the use of inducible promoters that allow to drive a regulated expression of the interfering RNA (Miyagishi *et al.* 2002).

POST TRANSCRIPTIONAL MODULATION TO TREAT CARDIAC ARRHYTHMIAS

The initial studies exploring the possibility of using siRNA in cardiac myocytes were performed in neonatal cultures (**Table 3**) and have the primary objective of testing the feasibility of siRNA transfer in cardiac cells and the achievement of silencing of the gene of interest. Neonatal cardiomyocytes do not present the ion channel expression patterns and current densities typical of adult cardiac myocytes, however despite this limitation the model can still be considered an acceptable tool to test the therapeutic efficacy of "molecular ablation" of different candidate targets and an useful instrument to highlight proteins functions and interactions.

In light of the above considerations, different groups assessed the responsibility of different endogenous proteins in cell calcium uptake and sequestration by the sarcoplasmatic reticulum. Since Phospholamban (PLB) could be considered a potential target for a therapeutic approach in heart failure given its role in inhibiting SR Ca^{2+}-ATPase 2 (SERCA2) Ca^{2+} uptake, Watanabe *et al.* (2004) investigated the possibility of restoring the normal Ca^{2+} uptake in neonatal cardiac myocytes of rat in which SERCA2 protein level was decreased. Interestingly, PLB siRNA restored Ca^{2+} uptake affinity: this evidence is consistent with the data presented by another group (Fechner *et al.* 2007) in which an adenoviral vector transcribing shRNA against PBL was used to infect primary neonatal rat cardiomyoytes modulating the active sequestration of Ca^{2+}.

Overall most of the published scientific works that report the use of neonatal cardiomyocytes culture as a target for molecular ablation of intrinsic cellular molecule exploit this model for the understanding of cellular pathways focusing their attention on the mechanism conducting the excitation-contraction phenomenon and have not focused on the opportunity of identifying therapeutic targets for heart rhythm disturbances.

Table 3 Post transcriptional modulation studies.

Gene	Vector	Model	Report	Reference
PBL	Hemagglutinating virus of Japan	Neonatal rat cardiac myocytes (with decreased SERCA2 level)	Restored Ca^{2+} uptake affinity	Watanabe *et al.* 2004
SERCA2	Adenovirus	Neonatal cardiac myocytes	Increased NCX, TRPC4, TRPC5 and related transcriptional factors	Seth *et al.* 2004
NCX1	Adenovirus	Neonatal cardiac myocytes	Compensatory effect of sarcolemmal Ca^{2+} pump expression	Hurtado *et al.* 2005
PLB	Adenovirus	Neonatal rat cardiomyocytes	Modulation in active Ca^{2+} sequestration	Fechner *et al.* 2007
Kir 2.1	Adenovirus	Adult rat cardiac myocytes	Reduced membrane potential (1st experiment on ACM)	Rinne *et al.* 2006
PUMA	Adenovirus	Mouse neonatal cardiomyocytes/ primary rat cardiomyocytes	Block of ER stress induced apoptosis	Nickson *et al.* 2007
Trx-1	shRNA	*In vivo*/heart	(study of drug evaluation)	Malik *et al.* 2006
Kir 2.1	pGenesil1	Rat cardiomyocytes	The vector can infect	Lei *et al.* 2005
FLIP	Plasmid	Neonatal cardiomyocytes	Increased level of apoptosis	Davidson *et al.* 2003
ZnT-1	siRNA	Neonatal cultured myocytes	Prevention of a rapid pacing and inhibition of LTCC	Beharier *et al.* 2007
β1-AR	Liposomes	*In vivo*: systemic injection	Reduction in cardiomyocytes apoptosis in myocardial infarction	Arnold *et al.* 2007

Even if adult cardiac myocytes culture represent the best *in vitro* approach to assess the efficacy of a treatment several aspects make gene silencing in such cells a more challenging task than the delivery into neonatal cells.

One critical point has already been discussed in the section dedicated to gene transfer and relates to the difficulty of infecting terminally differentiated non replicating adult myocytes thus we will not repeat here the same considerations that also apply to the use of viral vectors to transfer siRNA. A further challenge posed by transfer of siRNA into adult myocytes related to the fact that in adult myocytes there is a delay in gene silencing as compared to the time course observed in neonatal cells. While a complete gene silencing in neonatal cardiac myocytes could be assessed after 24 hours on the mRNA and in 48 hours on the protein level (Watanabe *et al.* 2004), in adult cardiac myocytes a comparable effect is achieved in 6 days, with the same working conditions failed to silence the gene in adult cardiac myocytes. The delay in the silencing seems to be related to post mitotic characteristics of adult myocytes these cells that resemble what occurs in cultured neurons (Sago *et al.* 2004). Obviously the fact that silencing occurs over a prolonged time raises concerns as the viability of adult myocytes *in vitro* over prolonged time periods is low.

Rinne *et al*. (2006) were able to obtain successful shRNA-mediated silencing of the Kir2.1 gene in adult isolated rat cardiac myocytes at 6-8 days after transfer of shRNA.

In this set of experiments the authors obtained a reduction of the Ik1 current density by 90% thus proving the concept that gene silencing can be achieved in adult cardiac myocytes with the use of adenoviral vectors

The next challenge to be addressed before the use of siRNA for therapeutic purposes in the heart is that of attempting *in vivo* delivery: only few in vivo studies have been reported so far (**Table 3**). In this respect the study by Arnold *et al*. (2007) is particularly relevant. These authors explored the possibility to use gene silencing to reduce β-1 adrenergic receptors in order to create a highly selective β-1 adrenoreceptor blocking treatment. They delivered by systemic injection liposomes-complexed-siRNAs targeted to the mRNA of β-1-Adrenergic receptors and successfully induced a reduction of the expression of β-1 but not β-2 adrenoreceptors. Remarkably animals treated with siRNA showed a significant reduction of blood pressure lasting 12 days and resulting in a decrease of cardiac hypertrophy. Furthermore administration of siRNA before induction of myocardial infarction significantly improved cardiac function. This study is important as it proves the feasibility of selective gene silencing *in vivo* using systemic delivery of siRNA and it also demonstrated the occurrence of a short term clinical benefit of siRNA treatment.

FUTURE DIRECTIONS

The application of biological therapies to cardiac diseases is a growing area of research as demonstrated by the fact that different approaches spanning for viral vector-mediated gene transfer to grafting of engineered cells to silencing of target genes are actively being investigated in many laboratories around the world.

In this review we presented and discussed the advancements made in the development of therapeutic strategies for the treatment of arrhythmias. In spite of the complexity of modulating the electrical properties of the heart and the risk of unintentionally creating a pro-arrhythmic substrate, several investigators have proposed interesting approaches that are rapidly moving from *in vitro* to *in vivo* testing. The issues of duration of the effects of biological therapies and the safety concerns remain the most challenging issues to be addressed before human studies can be envisioned.

Overall the field has produced important studies that have demosntrated the feasibility of molecular manipulation of electrophysiological properties for therapeutic purposes. Although few years will elapse before patients benefit of molecular electrophysiology we believe that similarly to the introduction of devices and ablation, biological therapies will represent the next revolution in clinical electrophysiology.

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