Molecular biology and genetics in cardiovascular research: highlights of 2002

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ABSTRACT

In the future treatment of haemophilia B, a real breakthrough may be a strategy that uses site-specific genomic integration of a gene therapy vector to produce therapeutic levels of human clotting factor IX (FIX). A clinically relevant expression of plasma levels of FIX was noted for over 12 months. The strategy will be applicable for a broad range of therapeutic genes and tissues.
Following the concept that angiogenic growth factors could stimulate revascularisation, a highly interesting novel approach to the 'bio-bypass' has been presented that appears to have some unexpected advantages. It was demonstrated that specifically designed transcription factors can regulate gene expression *in vivo*.
Another important finding was that myocardial stress signals all appear to converge to a common downstream target, the class II histone deacetylases. In mice, hypertrophic stimuli proved to lead to the activation of a novel and so far unique cardiac HDAC kinase that phosphorylates the signal-responsive sites in class II HDACs. A major implication is that the cardiomyocytic HDAC kinase could well be a novel therapeutic target for the treatment of hypertrophy and heart failure.
And finally, Catherine Verfaillie and her group published a landmark paper demonstrating that pluripotent stem cells that have the potency to differentiate into most, if not all, somatic tissues can also be isolated from adult bone marrow.

INTRODUCTION

Nowadays, molecular biology and genetics are as much part of cardiovascular research as biochemistry or clinical epidemiology. Hence, selecting the top papers from the cardiovascular literature published in the year 2002 that involve 'a molecular biological or genetic approach' can only be done using subjective selection criteria which are, above all, based on personal perspective and taste. The four papers discussed have in common that they all describe a scientific breakthrough that was facilitated by the innovative use of molecular biology and that may well affect the design of experimental clinical protocols of the near future. Moreover, the concepts and implications put forward by these studies will be very likely applicable to molecular medicine in general.

SITE-SPECIFIC GENOMIC INTEGRATION PRODUCES THERAPEUTIC FACTOR IX LEVELS IN MICE

Olivares EC, Hollis RP, Chalberg TW, Meuse L, Kay MA, Calos MP. Nat Biotechnol 2002;20(11):1124-8
In October 2002 the gene therapy world was shocked by the news that leukaemia had developed in a three-year-old patient participating in a clinical trial for retroviral correction of severe combined immunodeficiency disease (SCID). This trial, led by Dr. Alain Fisher from the Necker Children's Hospital in Paris, had so far been one of the few real successes in gene therapy. SCID patients are deficient for the γc subunit of interleukin receptors and therefore lack T and NK lymphocyte function. In a land-

mark publication in *Nature* in 2000, the French group reported full correction of the immunodeficiency in two young SCID patients through stable transduction of bone marrow derived CD34-positive haematopoietic stem cells with a retroviral vector carrying an intact copy of the γc subunit gene.[1] Ten patients had been successfully treated with this vector[2] when the serious adverse event happened that had been identified as a potential risk associated with retroviral vectors. For a permanent correction of a genetic deficiency, the gene therapy vector has to integrate into the genomic DNA of the host cell. With the retroviral vector used, integration takes place in a random fashion and the risk of this method is that the strong constitutive viral promoter is accidentally positioned directly upstream of a potential oncogene. Analysis of the SCID patient's leukaemic T-cell clone showed that this was exactly what had happened as the retroviral vector had been inserted in the known T-cell leukaemia gene LMO-2 on chromosome 11.

A second gene therapy trial published in the year 2000 was reported in *Nature Genetics* by researchers from Pennsylvania.[3] Intramuscular injection of B type haemophilia patients with an integrating adeno-associated vector (AAV) coding for the human clotting factor IX (FIX) resulted into a clinically relevant expression of 5 to 7% of normal plasma levels of FIX for over 12 months. Gene therapy of haemophilia holds a lot of promise as only 1 to 5% of normal clotting factor levels are needed for a significant correction of the clotting deficiencies. Not withstanding this initial success, chromosomal integration of the used AAV virus remains a relatively random process with the associated risk of unwanted (in)activation of endogenous genes. Hence, the report by Olivares *et al.* in the November issue of *Nature Biotechnology* in 2002 may well represent a true breakthrough in the future treatment of haemophilia B as a strategy was presented that uses site-specific genomic integration of a gene therapy vector to produce therapeutic levels of FIX.[4] To obtain 'controlled' integration of the gene therapy vector the authors used a molecular biological trick that was learned from the *streptomyces* bacteriophage ϕC31. After injection into the host bacterium the phage DNA integrates into the host genome by recombination of a 40 base pairs long phage attP sequence with a specific complementary host sequence called the attB site (*figure 1*). The only factor needed for this recombination is the so-called integrase enzyme that is encoded for by the ϕC31 phage genome. Surprisingly, the integrase also works perfectly in eukaryotic cells such as mouse and human cells. In vitro studies showed that the chromosomes of these species contain a limited number of pseudo attB sites that are sufficiently homologous to the authentic streptomyces attB site to allow efficient integration of attP-site containing vectors, provided the ϕC31 integrase is present.[5] Olivares and co-

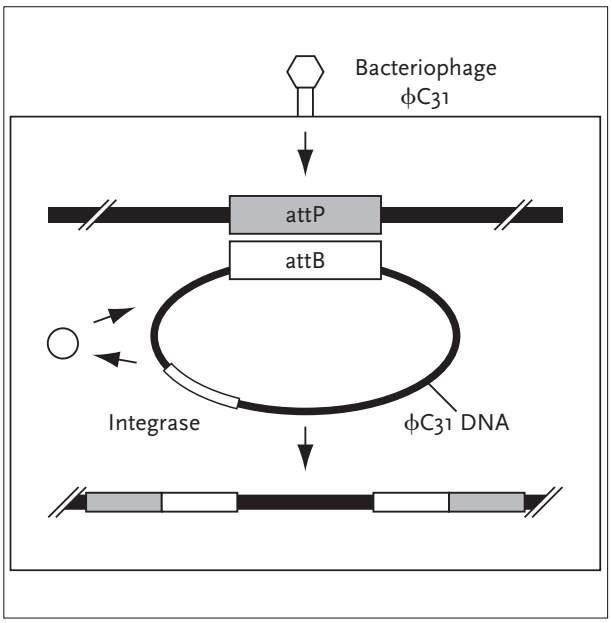

Figure 1

Schematic representation of the integration of bacteriophage ϕC31 DNA into the bacterial host genome

After injection of the ϕC31 DNA into the *Streptomyces* bacterium site-specific integration of the phage DNA into the host chromosome takes place through a ϕC31 integrase-dependent recombination between the attP sequence of the bacteriophage genome and the homologous attB sequence of the bacterial chromosome.

workers showed that simply adding an attP site to a FIX expression plasmid in combination with an expression vector for the ϕC31 integrase can lead to site-specific integration of the FIX vector into the mouse genome.

A simple high pressure/high volume injection of the naked plasmid DNAs into the mouse tail vein resulted in therapeutic levels of human FIX in the serum that were maintained for at least 250 days (*figure 2*). Surprisingly, as has been previously demonstrated, these hydrodynamic-based injections predominantly lead to transfection of the liver, the natural site of FIX expression. Control experiments confirmed that the FIX expression was derived from integrase-dependent vector insertion in two dominant pseudo attB sites present on mouse chromosome 2 and 11. As no genes have been shown to be present in these two loci and the same holds true for the human pseudo attB sites, these integration sites are expected to be relatively safe. Although one cannot exclude a certain degree of random integration of the used vectors, it is likely that the number of random integrations are greatly reduced. The authors note that the strategy used holds a strong potential for the treatment of haemophilia B and will also be applicable for a broad range of therapeutic genes and tissues. If the cellular expression of the non-self ϕC31 integrase is not associated with limiting immunological complications they may well be correct.

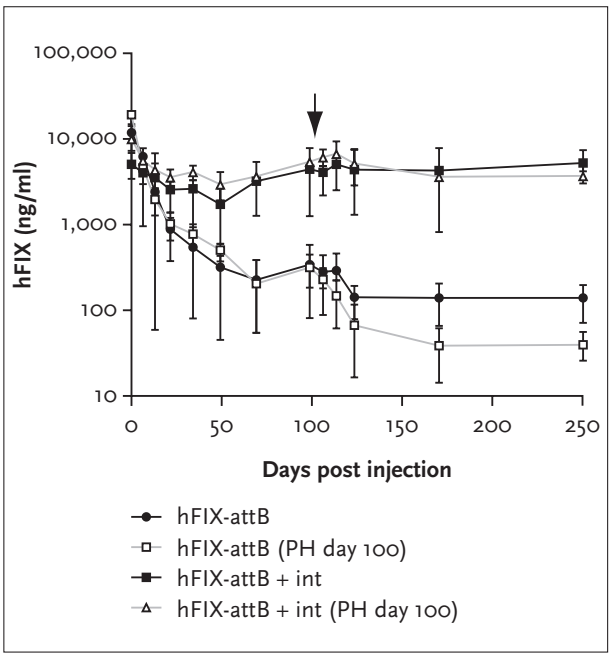

Figure 2

Therapeutic levels of human FIX persist after partial hepatectomy[4]

Mice received a large-volume tail vein injection of 25 g of hFIX-*attB* plasmid alone (circles) or with 25 g of integrase expression plasmid (squares). Two-thirds partial hepatectomies were performed on the indicated groups 100 days after injection (indicated by the vertical arrow). Persistence of expression after partial hepatectomy indicates stable integration of the FIX vector into the mouse genome.

INDUCTION OF ANGIOGENESIS IN A MOUSE MODEL USING ENGINEERED TRANSCRIPTION FACTORS

Rebar EJ, Huang Y, Hickey R, Nath AK, Meoli D, Nath S, Chen B, Xu L, Liang Y, Jamieson AC, Zhang L, Spratt SK, Case CC, Wolffe A, Giordano FJ. Nat Med 2002;8(12):1427-32

Gene therapy aimed at increasing tissue perfusion by stimulating the formation of neovascularisation or collateral formation is slowly but steadily developing from the experimental stage to a serious option for treatment of peripheral and cardiac ischaemic vascular disease.[6] The concept that angiogenic growth factors could stimulate revascularisation in patients (therapeutic angiogenesis) was first explored by Dr. Jeffrey Isner's group from St. Elizabeth's Medical Centre in Boston, USA. Direct injection of a purified DNA vector coding for vascular endothelial cell growth factor (VEGF) in the leg of patients with critical peripheral ischaemic vascular disease led in several cases to an improvement in rest pain, healing of ischaemic ulcera and increased vascularisation of the treated leg to an extent that was beyond expectations.[7] These seminal observations led to a true 'gold rush' for the identification of the optimal angiogenic factor and delivery strategy for the clinical application of this, both clinically and commercially, highly significant therapy. In the *Nature Medicine* of November 2002 a highly interesting novel approach to the 'bio-bypass' was presented that appeared to have some unexpected advantages.[8] To increase local levels of VEGF, not DNA or a viral vector coding for VEGF but a 'designer transcription factor' highly specific for endogenous 'natural' VEGF gene was infected. Transcription factors can turn on genes by binding to the gene promoter sequence that lies upstream of the coding sequences of the gene. They typically have two domains, one to bind the promoter of the target gene in a sequence-specific fashion and a second 'effector' domain that activates the transcription of the gene by RNA polymerases. One of the most common DNA binding motifs is the so-called zinc finger domain that is present in over 700 different human genes. A zinc finger domain spans about 30 amino acids and typically binds three base pairs of a double-strand DNA sequence. As these zinc fingers are often modular (e.g. three consecutive zinc fingers bind a sequence of nine base pairs) DNA-binding factors are evolved that selectively bind promoter sequences. The combined use of techniques for mutagenesis and selection, such as 'bacteriophage display', has facilitated the generation of collections of zinc finger motifs that can bind nearly all possible three base-pair sequences. Statistically, a specificity of 16 base pairs is more then sufficient to target a single site in the entire human genome. Based on this concept 'polydactyl zinc finger proteins' have been designed and developed that indeed were shown to be selective and effective activators of gene transcription.[9]

The study by Rebar *et al.* discussed here was intended to test whether these engineered transcription factors are effective *in vivo* using a polydactyl zinc finger designed to regulate the VEGF gene. Injections of an adenoviral vector encoding the novel transcription factor into mouse tissues induced the expression of the zinc finger protein and stimulated angiogenesis and markedly accelerated the healing of experimental wounds. Moreover, the neovasculature resulting from the zinc finger protein was functional and not hyperpermeable in contrast to novel vessels produced in the same model after expressing VEGF from an endogenous cDNA vector (*figure 3*). The VEGF gene codes a number of splice variants and recent data suggest that only the natural combination of these splice forms elicit the formation of physically mature neovasculature.[10] The hypermeability could thus be explained as being the result of the expression only on a single VEGF splice form. This study demonstrated that specifically designed transcription factors can regulate gene expression *in vivo* and, moreover, emphasises the importance of proteomics in that there is more involved than just the sequence of a gene, it is how that sequence is used to generate the protein(s) encoded by that gene.

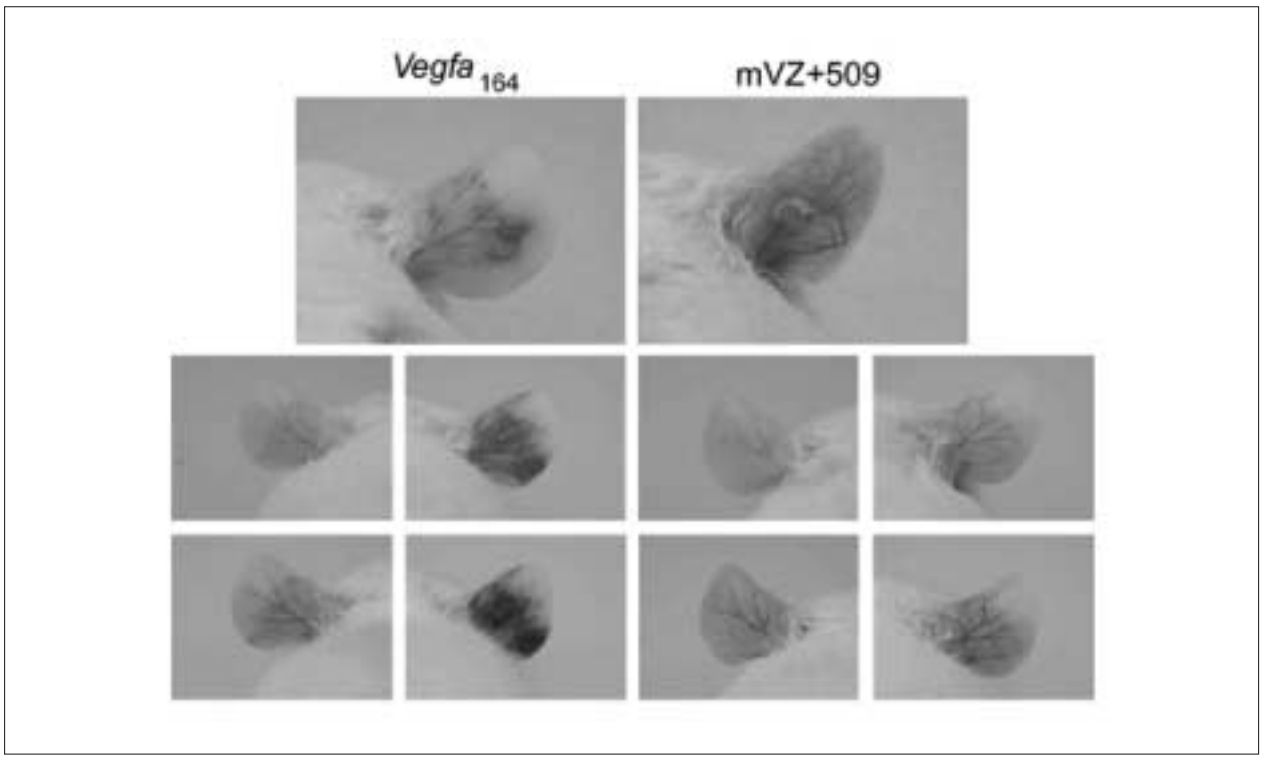

Figure 3

VEGF-activating ZFP expression induces angiogenesis in the mouse ear[8]

Subcutaneous injection of adenovirus encoding the polydactyl zinc-finger mVZ+509 that activates the endogenous VEGF gene results in visible neovascularisation after three days. Angiogenesis stimulated by mVZ+509 (top and middle right) does not produce a hyperpermeable neovasculature as determined by Evans blue dye extravasation (bottom right). The neovasculature induced by *Vegfa*$_{164}$ adenovirus transduction (left) shows spontaneous haemorrhage (middle) and Evans blue extravasation (bottom).

CLASS II HISTONE DEACETYLASES ACT AS SIGNAL RESPONSIVE REPRESSORS OF CARDIAC HYPERTROPHY

Zhang CL, McKinsey TA, Chang S, Antos CL, Hill JA, Olson EN. Northwestern Medical Centre Texas. Cell 2002;110:479-88

The adult myocardium responds to stress signals by hypertrophic growth, a process central to the development of heart failure. The nature of this stress signal can be quite diverse and include cardiac pressure overload, hypertension, myocardial infarction and autocrine and paracrine signalling pathways involving angiotensin II, endothelin and adrenergic signalling. Despite the diversity of these stress signals they all lead to the same outcome, the development of myocyte hypertrophy as a result of activation of a foetal cardiac gene programme.[11] In August 2002 a paper was published in the journal *Cell* by Dr. Eric Olsen's group from Texas Southwestern Medical Centre who for the first time showed that myocardial stress signals all appear to converge to a common down-stream target, the class II histone deacetylases.[12] Histone acetyl transferases (HATs) and histone deacetylases (HDACs) control gene expression through association with gene specific transcription factors. When HATs are recruited to genes by transcriptional activators they promote gene activation by acetylating nucleosomal histones. This results in a relaxed chromatin structure that facilitates transcription. In contrast, HDACs deacetylate histones leading to condensed chromatin and gene repression. The Olsen group showed that, in mice, hypertrophic stimuli such as thoracic aortic banding lead to the activation of a novel and so far unique cardiac HDAC kinase that phosphorylates the signal-responsive sites in class II HDACs like *HDAC9*. Mutants of the latter that can not be phosphorylated act as dominant negative repressors of cardiomyocyte hypertrophy and foetal cardiac gene expression *in vitro*.

Hence, the common pathway to hypertrophy laid out by this paper is the following. In the mature, differentiated cardiomyocytes the foetal cardiac gene programme is suppressed by gene repressor mediated recruitment of HDACs that keep the chromatin condensed and not accessible for the transcriptional machinery. However,

cardiac stress signals activate a kinase that leads to HDACs phosphorylation and inactivation. Subsequently, chromatin acetylation by HATs open up the chromatin associated with the foetal cardiac gene programme facilitating the expression of these genes and the progression of hypertrophy (*figure 4*). The importance of this pathway is dramatically demonstrated using *HDAC9* knock-out mice that indeed turn out to be hypersensitive to cardiac stress and, in three weeks, develop hypertrophic hearts with a 105% increase in left ventricular mass (*figure 5*).
A major implication of this study is that the cardiomyocytic HDAC kinase could well be a novel therapeutic target for the treatment of hypertrophy and heart failure. As the authors point out, current therapies target the early steps in hypertrophic signalling pathways such as cell surface receptors, calcium channels or the β-adrenergic receptors system. Although not yet characterised, the HDAC kinase appears to be a common denominator of these pathways and therefore a drug target of high potential.

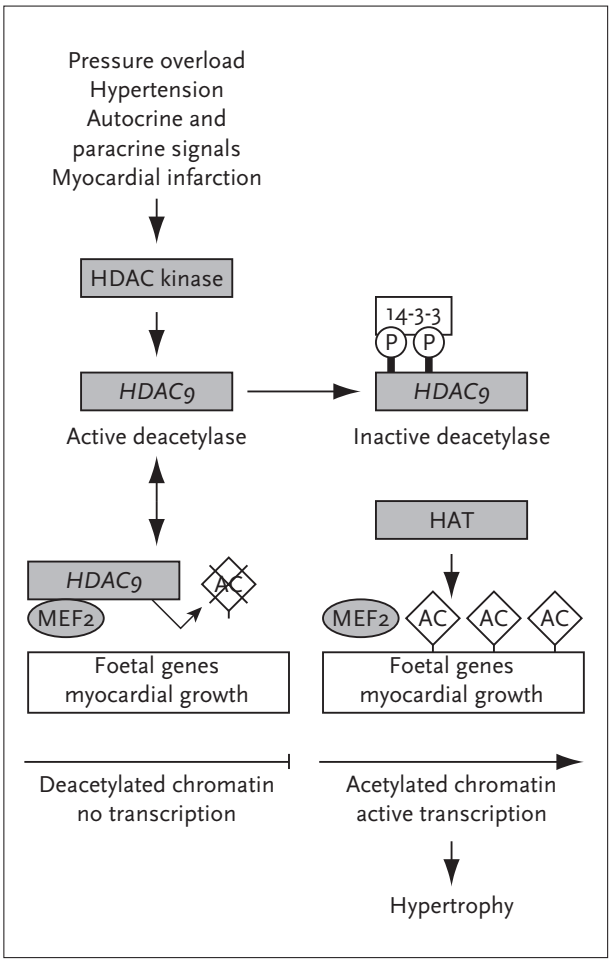

Figure 4
Repression of cardiac hypertrophy by class II HDACs
Class II HDACs associate with MEF2 and inhibit hypertrophy and the foetal gene programme. Stress signals stimulate an HDAC kinase that phosphorylates (P) HDACs at two conserved serine residues. When phosphorylated, HDACs bind 14-3-3, dissociate from MEF2, and are exported from the nucleus. Upon release of HDACs, MEF2 is free to associate with histone acetyltransferases (HATs) and to activate downstream target genes that drive a hypertrophic response.

PLURIPOTENCY OF MESENCHYMAL STEM CELLS DERIVED FROM ADULT MARROW

Jiang, et al. Nature 2002;418:41-9
In 2002 stem cells were one of the themes on central stage. Animal studies showing the potential of stem cells to grow new insulin-producing cells to treat diabetes[13] or dopamine-producing nerve cells to reverse the symptoms of Parkinson's disease[14] have delivered the proof of principle for future stem cell therapies. Nevertheless most studies were performed with embryonic stem cells. These cells, which can be obtained from the inner cell mass of the blastocyst, are pluripotent and can be cultured in high numbers. Although embryonic stem cells have been isolated from humans[15] ethical considerations and, more practically, the immunological incompatibility of the stem cells with the genetic makeup of the potential patients may limit their use. In the July 2002 issue of *Nature* Catherine Verfaillie and co-workers published a landmark paper demonstrating that pluripotent stem cells that have the potency to differentiate into most, if not all, somatic tissues can also be isolated from adult bone marrow.[16] This cell, termed the multipotent adult progenitor cell or MAPC, maintains its stem cell properties for over 80 population doublings and can be obtained from mesenchymal bone marrow cultures from different species including humans irrespective of the age of the donor. Previous studies had shown that, *in vitro*, MAPC can differentiate into multiple cells from the mesenchymal lineage[17] to endothelial cells[18] and even hepatocytes.[19] If the MAPC were truly pluripotent cells *in vivo*, these cells could be an ideal source for stem cell based therapies as these cells could be derived from the patient's own bone marrow, thereby avoiding tissue rejection. In the July paper the group demonstrated that when injected into an early blastocyst, single MAPCs contribute to virtually all somatic cell types. As the injected MAPC were obtained from a mouse donor carrying the traceable genetic marker *LacZ*, in the mice generated from injected blastocysts, MAPC derived tissues can be identified by a blue colour that develops after X-gal staining (*figure 6*).
This paper has inspired a large number of researchers all over the world to also try to isolate the MAPC or similar pluripotent adult cells as they may be an ideal source for therapy of inherited or degenerative disease.

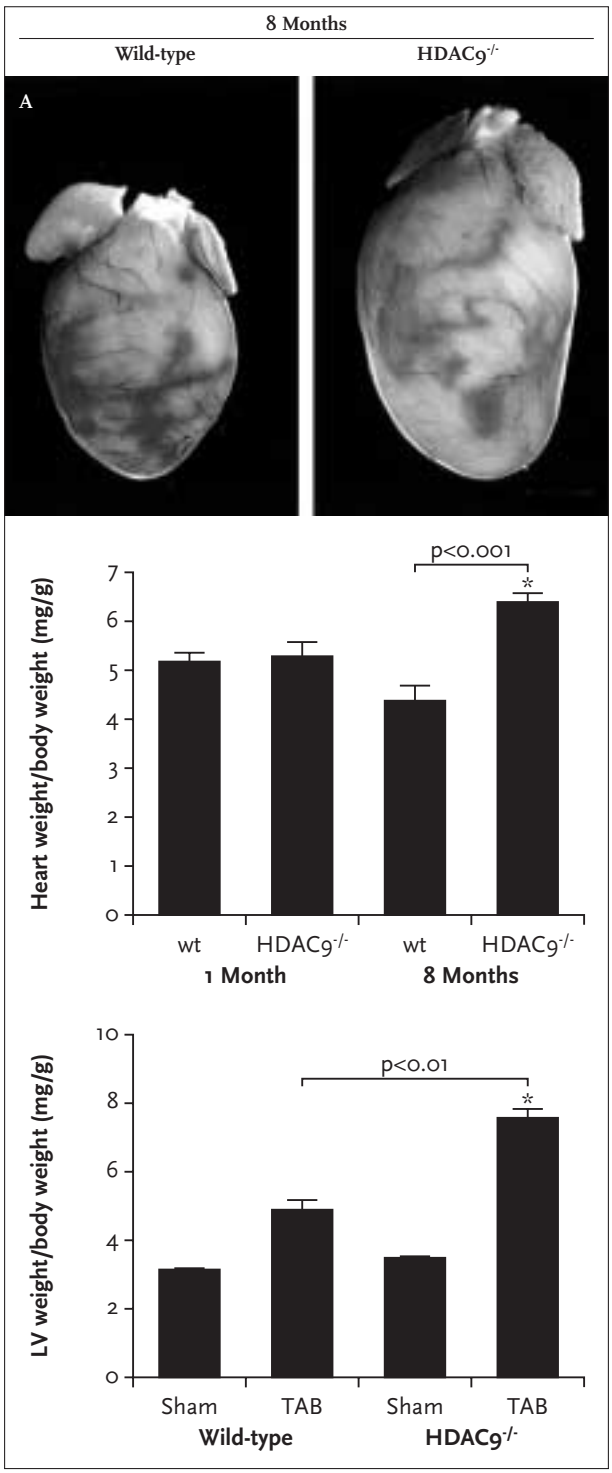

Figure 5

Cardiac hypertrophy in HDAC9 mutant mice[12]

(A and B) *HDAC9* mutant mice and wild-type littermates were sacrificed at one and eight months of age and heart weight-to-body weight ratios were determined. Values represent the mean ± standard deviation (SD). N=5. Scale bar equals 2 mm. (C) Hypersensitivity to TAB. Six-to-eight-week-old mice were subjected to thoracic aortic banding (TAB) or to sham operation. Twenty-one days later, animals were sacrificed and the ratios of left ventricular (LV) mass-to-body weight were determined. At least five mice of each genotype were analysed. Values represent the mean ± SD.

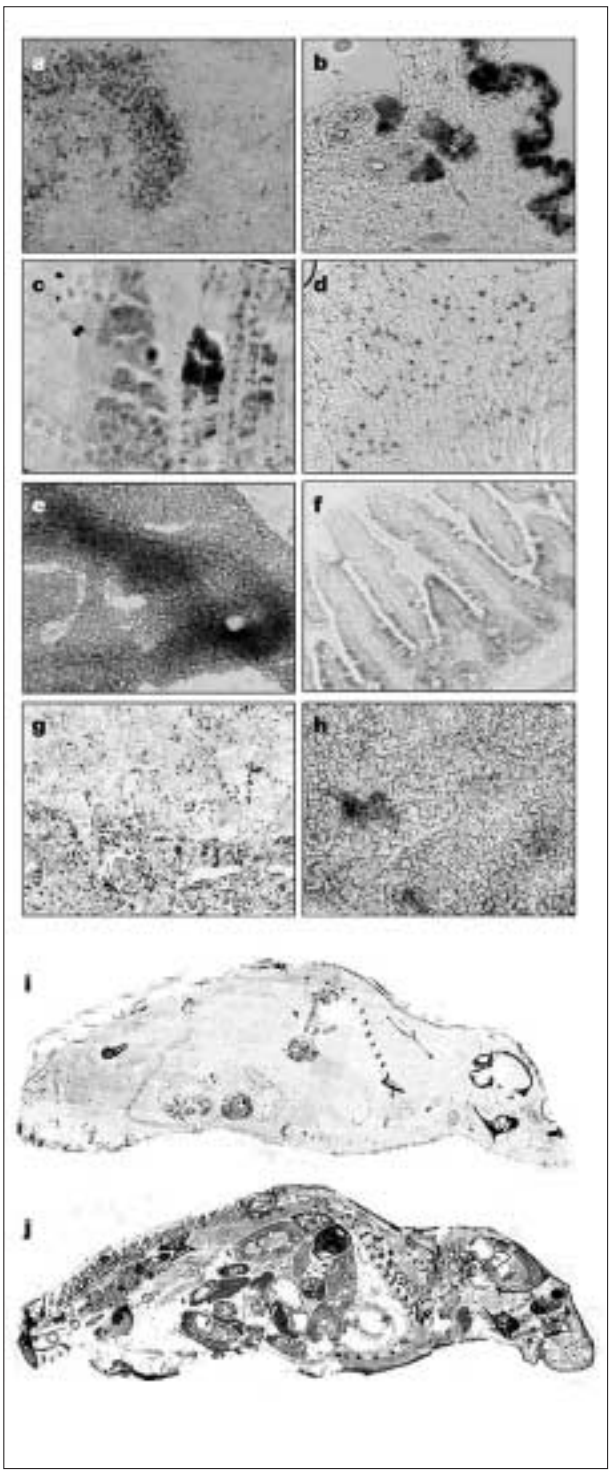

Figure 6

Chimaerism detection by X-gal staining and anti-gal staining in animals generated from blastocysts micro-injected with a single ROSA26 MAPC[16]

a-h: Images from X-gal-stained individual organs from a 45% chimeric mouse, determined by Q-PCR for Neo on tail clip. Tissue sections were from: brain (a), skin (b), skeletal muscle (c), myocardium (d), liver (e), small intestine (f), kidney (g) and spleen (h). i + j: Images from an X-gal-stained section through a mouse that was not chimeric (i) or was 45% chimeric (j). Magnification 20x.

REFERENCES

1. Cavazzana-Calvo M, Hacein-Bey S, Saint BG de, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science 2000;288(5466):669-72.
2. Hacein-Bey-Abina S, Fischer A, Cavazzana-Calvo M. Gene therapy of X-linked severe combined immunodeficiency. Int J Hematol 2002;76(4):295-8.
3. Kay MA, Manno CS, Ragni MV, et al. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. Nat Genet 2000;24(3):257-61.
4. Olivares EC, Hollis RP, Chalberg TW, Meuse L, Kay MA, Calos MP. Site-specific genomic integration produces therapeutic Factor IX levels in mice. Nat Biotechnol 2002;20(11):1124-8.
5. Groth AC, Olivares EC, Thyagarajan B, Calos MP. A phage integrase directs efficient site-specific integration in human cells. Proc Natl Acad Sci USA 2000;97(11):5995-6000.
6. Isner JM. Myocardial gene therapy. Nature 2002;415(6868):234-9.
7. Isner JM, Pieczek A, Schainfeld R, et al. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. Lancet 1996;348(9024):370-4.
8. Rebar EJ, Huang Y, Hickey R, et al. Induction of angiogenesis in a mouse model using engineered transcription factors. Nat Med 2002;8(12):1427-32.
9. Liu Q, Segal DJ, Ghiara JB, Barbas CF III. Design of polydactyl zinc-finger proteins for unique addressing within complex genomes. Proc Natl Acad Sci USA 1997;94(11):5525-30.
10. Grunstein J, Masbad JJ, Hickey R, Giordano F, Johnson RS. Isoforms of vascular endothelial growth factor act in a coordinate fashion To recruit and expand tumor vasculature. Mol Cell Biol 2000;20(19):7282-91.
11. Chien KR. Stress pathways and heart failure. Cell 1999;98(5):555-8.
12. Zhang CL, McKinsey TA, Chang S, Antos CL, Hill JA, Olson EN. Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. Cell 2002;110(4):479-88.
13. Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R, McKay R. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science 2001;292(5520):1389-94.
14. Kim JH, Auerbach JM, Rodriguez-Gomez JA, et al. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. Nature 2002;418(6893):50-6.
15. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282(5391):1145-7.
16. Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002;418(6893):41-9.
17. Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, Verfaillie CM. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood 2001;98(9):2615-25.
18. Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM. Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest 2002;109(3):337-46.
19. Schwartz RE, Reyes M, Koodie L, et al. Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. J Clin Invest 2002;109(10):1291-302.