

## Introduction

# Toward a Universal Platform for Autologous Stem Cell Gene Therapy

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Adult/Postnatal stem cells provide for life-long cell replacement in tissues and organs. They have an inherent ability for self-renewal and differentiation into multiple cell types that are characteristic of their tissue of origin, and have been identified in an increasing number of tissues/organs even in brain and myocardium in which cell turnover was reputedly absent. They are thus the obvious targets of both long-term and transient-regenerative gene therapy, whereby they either provide for life-long transgenic cell turnover (e.g., gene-corrected hematopoietic stem cells in SCID patients) or are mobilized/recruited in a regenerative process (e.g., vasculogenesis in patients incurring critical limb ischemia). In both cases, their inherent homing ability is essential to their therapeutic repopulation dynamics into appropriate niches (long-term engraftment) and tissues (cell turnover), culminating in synergistic combinations aimed at magnifying their homing/regenerative/differentiative potential. Importantly enough, the homing ability of relevant stem/progenitor cells is a unique opportunity to substitute stem cell-mediated delivery for transient topical gene therapy both for regenerative medicine (e.g., aforementioned vasculogenesis) and for cancer therapy. Indeed, as “never-healing wounds”, tumors mobilize neovascularizing and connective-mesenchymal stem cells that are indispensable to their growth, thereby prompting stem cell-mediated tumor-targeted gene therapy protocols for maximized efficiency and minimized off-target side effects. Unexpectedly, neural stem/progenitor cells have been found to have such a tumor-homing propensity that they are currently used to track disseminated metastatic cells. Therapeutic homing is thus the driving force of our autologous stem cell gene therapy platform that comprises 1) a long-term gene therapy branch that relies on endonuclease-boosted gene targeting and cybridization, and 2) a transient regenerative/epigenetic/cancer gene therapy branch. Endonuclease-boosted gene targeting and *ex vivo* cybridization are true genomic homeostatic tools for gene repair/alteration (nuclear inherited diseases) and whole wild-type mitochondrial genome (mtDNA) transfer (mtDNA diseases including aging disorders),

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respectively, thereby eliminating random-integration hazards (dysregulations and adverse oncogenic events) that hamper current clinical gene therapy trials. The same holds true for emerging custom site-specific integrative gene therapy mediated by endonuclease-boosted gene targeting (mtDNA-independent acquired and degenerative/aging disorders). Transient regenerative gene therapy is a critical arm of our platform, since it is aimed both at driving regenerative medicine *sensu stricto* (wound healing and aging/degenerative pathologies) and at synergizing long-term gene therapy, thereby magnifying the repopulating/regenerative ability of transfected/transduced stem cells and cybrids (inherited/acquired disorders including mtDNA diseases). The transient epigenetic and cancer gene therapy arms are discussed in terms of 1) potential long-term inactivation/activation of endogenous genes through transient expression of promoter-specific siRNA and 2) breakthrough targeting of cancer stem cells for recurrence-free cancer therapy, respectively. Importantly enough, emerging adult/postnatal pluripotent stem cells that have an extensive/clonogenic *ex vivo* growth potential and are able to differentiate into cells of the three germ lineages (pluripotency) are presented as the ultimate autologous drive of our universal stem cell gene therapy platform, culminating in strategies aimed at selective *ex vivo* amplification of engineered stem cells and cybrids and at tackling both tissue-specific and multisystemic pathologies/regenerative needs.

**Keywords:** Autologous pluripotent stem cells; cybrid stem cells; cancer stem cells; tissue- and tumor-homing; endonuclease-boosted gene targeting; gene repair/alteration; targeted transgene integration; long-term and transient gene therapy.

## I. Therapeutic Homing as the Very Drive of Both Long-term and Transient-Regenerative Stem Cell Gene Therapy

Adult/Postnatal stem cells have both self-renewing and differentiative capabilities, thereby providing for life-long cell replacement in tissues and organs. In addition, many stem cells have inherent homing abilities that are instrumental in therapeutic applications (see below). They are thus the obvious target of both transient-regenerative and long-term gene therapy (see Bertolotti, 2001, 2003a and 2003b).

As illustrated by the first unequivocal successes for gene therapy, the selective engraftment and repopulating abilities of *ex vivo* gene-corrected hematopoietic stem cells (HSCs) have been instrumental in the clinical outcome of patients with X-linked severe combined immunodeficiency (X-SCID) and adenosine deaminase (ADA)-deficient SCID (Cavazzana-Calvo *et al.*, 2000; Aiuti *et al.*, 2002; Gaspar *et al.*, 2004). In these trials, efficient *ex vivo* retroviral transduction of bone marrow (BM) HSCs has been synergized to the BM niche-homing ability of these transduced stem cells and to the selective growth advantage of their lymphoid progenitor derivatives over mutant SCID cognates. The self-renewing and differentiative capabilities of engrafted gene-corrected stem cells thus secure long-term transgenic

lymphoid cell turnover (Hacein-Bey-Abina *et al.*, 2002; see Bertolotti, 2003a and 2003b) and, like in conventional BM transplantations, are expected to result in a life-long cure for these inherited diseases, thereby establishing the very paradigm of life-long therapeutic gene expression mediated by targeted autologous stem cells.

Stem cells are also the targets of transient gene therapy protocols, where they are mobilized and recruited in a regenerative process such as the formation of new blood vessels (see Bertolotti, 2001, 2002 and 2003c). Indeed, pioneering vascular endothelial growth factor (VEGF) gene therapy trials with topical transgene expression drove Isner and co-workers to the identification of circulating endothelial progenitor cells (EPCs or angioblasts) in peripheral blood (Asahara *et al.*, 1997) and to the demonstration that neovascularization in adult ischemic tissue is not restricted to angiogenesis (sprouting of endothelial cells [ECs] from pre-existing vessels; Folkman, 1971) but also involves vasculogenesis (Asahara *et al.*, 1997), where mobilization of BM EPCs (Takahashi *et al.*, 1999; Asahara *et al.*, 1999a) is increased by VEGF gene therapy (Asahara *et al.*, 1999b; Kalka *et al.*, 2000a and 2000c), culminating in the incorporation of EPCs into foci of neovascularization and in their differentiation into mature ECs (Asahara *et al.*, 1997 and 1999b; Kalka *et al.*, 2000a and 2000c). Importantly enough, mobilization of BM EPCs is correlated to the transient expression of the therapeutic VEGF transgene and subsequent transient increase in plasma VEGF (Kalka *et al.*, 2000a and 2000c), indicating that one of the main targets of VEGF gene therapy is indeed BM stem/progenitor cells, i.e., EPC precursors such as putative adult heman-gioblasts (precursors of both HSCs and angioblasts; see Reyes *et al.*, 2002), multipotent lineage-negative c-kit-positive ( $\text{lin}^-$  c-kit $^+$ ) stem cells (Orlic *et al.*, 2001) and/or pluripotent mesenchymal stem cells (MSCs)/pre-MSCs (Reyes *et al.*, 2002; Jiang *et al.*, 2002; Anjos-Afonso and Bonnet, 2007).

As shown by Isner and co-workers, a key feature of circulating EPCs is their propensity to home into ischemic tissues (Asahara *et al.*, 1997 and 1999b; Kalka *et al.*, 2000a and 2000c). Such a regenerative homing of EPCs prompted the development of 1) stem cell-mediated gene delivery to ischemic/neovascularizing tissues (Asahara *et al.*, 1997; Iwaguro *et al.*, 2002) and 2) autologous vasculogenic stem cell therapy. The first approach is a breakthrough for both regenerative medicine and cancer gene therapy (see below). The second approach was initiated on experimental models for both critical limb ischemia (Kalka *et al.*, 2000b) and myocardial ischemia (Kawamoto *et al.*, 2001; Kocher *et al.*, 2001), and then readily moved from bench to bedside because stem cell transplantation was already

a well-established medical practice (BM transplantation) (see: Matsubara, 2003; Brehm *et al.*, 2003; Assmus *et al.*, 2003; Bertolotti, 2003c).

Such a stimulation of the homing ability of EPCs to ischemic foci by transient topical gene therapy prompted us to devise a synergistic approach for long-term stem cell gene therapy, in which *ex vivo* protocols are combined with transient topical gene therapy in order to maximize the homing, regenerative and differentiative capabilities of the autologous therapeutic stem cells when they are returned to the patients (Bertolotti, 2001, 2003a and 2003b). The same synergistic combination with transient topical gene therapy applies to straight stem cell regenerative therapy too, as initially proposed for cardiovascular stem cell gene therapy (Bertolotti, 2002 and 2003c). Importantly enough, the intensive investigations that are aimed at unraveling the molecular mechanisms driving stem cell homing in a variety of niches and tissues/organs will be instrumental in the development of highly specific transient gene therapy protocols (see Bertolotti, 2003a and 2003b).

## **2. Stem/Progenitor Cells as Tissue- or Tumor-homing Vectors for Targeted Transient Regenerative/ Cancer Gene Therapy**

### **2.1. Autologous Stem/Progenitor Cells as Tissue-Homing Vectors for Targeted Transient Regenerative Gene Therapy**

The homing ability of EPCs has been pioneered by Isner and co-workers as a substitute for topical VEGF gene therapy in order to overcome autologous stem cell scarcity in aged patients through an increase of the *in vivo* revascularizative action of adult EPCs. Indeed, in this experimental trial on nude mice, *ex vivo* expansion and subsequent adenoviral transduction with a VEGF transgene strongly increased both the *in vitro* proliferative index and the *in vivo* revascularizative action of adult human EPCs. VEGF transduction was such a powerful booster that it reduced the effective therapeutic dose of EPCs to one thirtieth of its original value (Iwaguro *et al.*, 2002; Asahara *et al.*, 2002). The relative contribution of VEGF-induced angiogenesis, VEGF-induced mobilization/vasculogenesis of endogenous EPCs and transplanted-EPC-mediated vasculogenesis remains to be evaluated. Importantly enough, in this case homing does not apply to stem cells that are bound to engraft in their specific niche for life-long transgene expression (see HSCs in above SCID trials), but to progenitors cells that are bound to the tissue in which they incorporate through differentiation and in which

they will incur basic cell turnover (see Iwaguro *et al.*, 2002). Under these conditions, transient expression of the transgene is granted because adenoviral transgenes do not integrate into host chromosomal DNA and are thus lost by dilution through the last bursts of EPC divisions that precede final differentiation.

Such a transient stem cell gene therapy has obviously many applications in regenerative medicine, and is part of the transient regenerative gene therapy arm of our universal platform (Bertolotti, 2007; see below). It has been pioneered with EPCs, but also with MSCs and with neural stem cells (NSCs) that might work as progenitors and/or real stem cells (see below).

## **2.2. Autologous and Immortalized Stem/Progenitor Cells as Tumor-homing Vectors for Targeted Cancer Gene Therapy**

Like train servicing a combat unit, angiogenesis/vasculogenesis is essential both to most regenerative processes and to tumor growth/development. As expected, mobilization of BM EPCs to the tumor bed has been shown to be essential to tumor angiogenesis/vasculogenesis and growth (Lyden *et al.*, 2001). In fact, the formation of tumor stroma closely resembles wound healing and is so tightly associated to cancer pathological growth that tumors are seen as “never-healing wounds” (Dvorak, 1986). Increased neovascularization and turnover/proliferation of connective stromal cells in tumors appear thus to rely on both EPCs and MSC regenerative potential, thereby paving the way to EPC-mediated (Ferrari *et al.*, 2003) and MSC-mediated targeted cancer therapy (Studený *et al.*, 2002). The propensity of both EPCs and MSCs to home into the tumor bed is the very drive of an emerging EPC- and MSC-based strategy for targeted delivery of therapeutics and oncolytic viruses to primary and metastatic tumors (see: Ferrari *et al.*, 2003; Moore *et al.*, 2004; Hall *et al.*, 2007; Pereboeva and Roth, this volume).

Importantly enough, neural stem/progenitor cells (NSCs) have been found to display extensive tropism for pathology (Aboody *et al.*, 2000) and, in experimental models, appear to be fairly efficient for targeted cancer gene therapy both for intracranial gliomas and for a variety of primary and metastatic tumors (see Yip *et al.*, 2006; Aboody *et al.*, 2006; Shah, 2007; Neujbauer *et al.*, this volume). On the other hand, CD34<sup>+</sup> HSCs have been successfully used to target leukemias in another experimental system (Carlo-Stella *et al.*, 2006 and this volume).

For cancer therapy, we are dealing with a transient gene therapy protocol even though armed stem cells can be transduced with an integrating vector. Therefore, the main point is to have an efficient homing into the tumor

beds or nodules in order to topically deliver a transient transgene product, thereby maximizing the therapeutic effect and minimizing off-target side effects. Such a strategy is well fitted to autologous stem cells that are easy to obtain for *ex vivo* expansion and transfection/transduction (MSCs, EPCs and possibly circulating CD34<sup>+</sup> HSCs), but cannot apply to autologous NSCs and many other tissue-stem cells. For these reasons and for the sake of standardization and easiness, off-the-shelf cell lines or preconditioned heterologous stem/progenitor cells are currently favored over autologous stem/progenitor cells (see: Hall *et al.*, 2007; Neujbauer *et al.*, this volume). This is why cancer stem cell gene therapy has not been formally included in our autologous stem cell gene therapy platform (see below).

### **2.3. Cancer Stem Cells as Breakthrough Targets for Stem Cell-mediated Gene Therapy and Other Cancer Therapies**

Cancer stem cell gene therapy has a double meaning. It covers either the aforescribed approach in which armed stem cells are used for tumor-targeted delivery of a transgene or an oncolytic virus (see above), or emerging gene therapy approaches aimed at targeting cancer stem cells. However, we favor a third option conceptualizing an emerging breakthrough approach based on armed tumor-homing stem cells to specifically target cancer stem cells, thereby opening a promising avenue for recurrence-free cancer therapy.

Cancer stem cells are indeed the driving force of cancer (or at least of many of them), where genetic/epigenetic alterations have culminated in uncontrolled self-renewal and tumorigenesis either in tissue stem cells or in some of their progenitor/differentiated derivatives (see: Pearce and Bonnet, this volume; Piccirillo and Vescovi, this volume; Shackleton *et al.*, this volume; Patrawala and Tang, this volume). As a rare subset of the tumor, cancer stem cells are the only drive of tumor initiation/propagation and, upon transplantation, have been shown to recapitulate the hierarchical clonogenic differentiating/differentiated organization of the original tumor cell population (Bonnet and Dick, 1997; Reya *et al.*, 2001). Cancer stem cells have been identified in leukemia (Lapidot *et al.*, 1994; Bonnet and Dick, 1997) and in an increasing number of solid tumors such as breast cancer (Al-Hajj *et al.*, 2003), brain cancer (Singh *et al.*, 2003 and 2004; Galli *et al.*, 2004), prostate cancer (Collins *et al.*, 2005; Patrawala *et al.*, 2007), colon cancer (O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007) and pancreatic cancer (Li *et al.*, 2007).

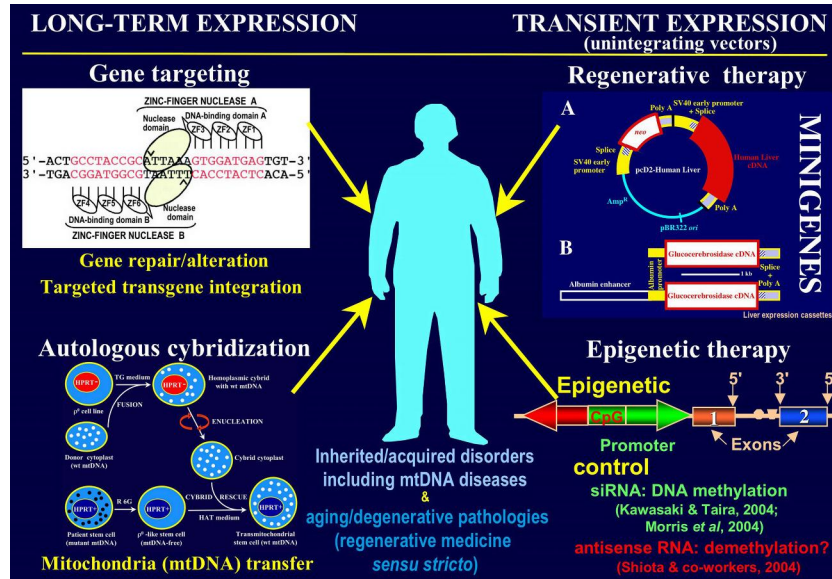
Cancer stem cells have the exclusive ability to reinitiate tumors (tumor-initiating cells) and are thus the very targets of a tantalizing

progression-free, recurrence-free therapy. Intensive investigations are thus aimed both at their identification in an exhaustive number of tumor types (Clarke *et al.*, 2006) and at developing cancer stem cell-specific therapeutic regimens (see: Piccirillo and Vescovi, this volume; Patrawala and Tang, this volume; Bao *et al.*, 2006; Piccirillo *et al.*, 2006; Jin *et al.*, 2006; Krause *et al.*, 2006). In this respect, we believe that cancer stem cell gene therapy protocols capable of efficiently driving a stem cell-mediated destruction of specific cancer stem cells while sparing their normal tissue-stem cell counterparts will soon be available.

### **3. Therapeutic Homing and Autologous Stem Cells as the Main Drive of Our Stem Cell Gene Therapy Platform**

As schematized in Fig. 1, autologous stem cells are the universal drive of our stem cell gene therapy platform (Bertolotti, 2007). They are the targets of both long-term and transient gene therapy protocols that culminate in synergistic combinations (Bertolotti, 2001, 2003a and 2003b). As discussed above, therapeutic homing drives both long-term engraftment of gene-corrected autologous stem cells into their original niche when they are returned to the patient (e.g., CD34<sup>+</sup> HSCs into the BM for life-long supply of transgenic lymphoid cells in the aforescribed SCID patients) and tissue-specific integration of stem/progenitor cells that have been mobilized by transient gene therapy (e.g., EPCs in transient topical VEGF gene therapy in the aforementioned critical limb ischemic patients). It also drives the tissue-specific migration of transgenic stem cells aimed at transient topical regenerative gene therapy (e.g., aforescribed EPC-mediated VEGF gene therapy for experimental critical limb ischemia) or the tumor-targeted armed stem cell delivery of transgene products for maximized efficiency of transient cancer gene therapy (e.g., Aboody *et al.*, 2000; Studeny *et al.*, 2002; Shah *et al.*, 2005; Carlo-Stella *et al.*, 2006; Komarova *et al.*, 2006). As discussed above, cancer stem cell gene therapy has not been formally included in our autologous stem cell gene therapy platform because, for the sake of standardization and easiness/availability, off-the-shelf cell lines or pre-conditioned heterologous stem/progenitor cells are currently favored over autologous stem/progenitor cells (see: Hall *et al.*, 2007; Neujbauer *et al.*, this volume).

As schematized in Fig. 1, the long-term gene therapy branch of our platform also has a transmitochondrial arm aimed at tackling mitochondrial DNA (mtDNA) diseases (Bertolotti, 2005). In this case, autologous



**Fig. 1.** Stem cell gene therapy: a universal platform (Poster Exhibition of June 2, 2005, ASGT 8th Annual Meeting, Saint-Louis: Bertolotti, 2005a). A schematic overview of our universal platform in which autologous stem cells are the targets of both long-term (left: endonuclease-boostered gene targeting and cybridization; Bertolotti, 2004a and 2005) and transient (right: regenerative medicine and epigenetic therapy\*; Bertolotti, 2000c, 2001 and 2003b) gene therapy protocols culminating in synergistic combinations (Bertolotti, 2001 and 2003a). Cancer stem cell gene therapy has not been included because off-the-shelf stem cell lines or preconditioned heterologous stem/progenitor cells are currently favored over autologous stem/progenitor cells for tumor-targeted stem cell gene therapy (see text). Autologous adult/neonatal stem cells include emerging multipotent/pluripotent stem cells with an extensive *ex vivo* growth potential (e.g., MAPCs, MIAMI cells, hBMSCs and maGSCs; see text), umbilical cord derivatives and potential ES-like cells that might arise from intensive cell reprogramming investigations (see Bertolotti, 2001). Such a universal platform is aimed at eliminating hazardous random integration of therapeutic DNA, at reversing inherited diseases by re-establishing wild-type genomic homeostasis, and at tackling most pathologies through stem cell repopulation dynamics into appropriate niches (long-term engraftment) and tissues (cell turnover). \*Although the experimental work of Kawasaki and Taira is under fraud suspicion investigations (Fuyuno and Cyranoski, 2006), siRNA-mediated transcriptional gene silencing in human cells has been also described by Morris *et al.* (2004) and more recent data (see text); on the other hand, siRNA-mediated gene activation has now been shown to occur too (Li *et al.*, 2006; Janowski *et al.* 2007). (Based on Fig. 1 of: Bertolotti R., *Gene Therapy and Regulation*, 3: 1–14 (2007), with permission of WSPC.)

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transmitochondrial stem cells obtained through *ex vivo* cybridization (see below) are returned to the patient like a conventional long-term gene therapy trial (e.g., aforescribed SCID trials). However, in this case, one of the first potential targets might be the heart instead of the BM, and efficient engraftment of relevant cybrid stem cells might need the synergistic magnification of their homing/repopulating ability by the transient regenerative gene therapy arm of our platform (Bertolotti, 2005 and this volume).

Importantly enough, transient regenerative gene therapy is a critical arm of our platform since it is aimed both at driving regenerative medicine *sensu stricto* (healing disorders and aging/degenerative pathologies) and at synergizing long-term gene therapy, thereby magnifying the repopulating/regenerative ability of autologous native *ex vivo* expanded stem cells, transfected/transduced stem cells and cybrids.

#### **4. Autologous Pluripotent Stem Cells: Toward a Universal Stem Cell Gene Therapy Platform**

Using the therapeutic homing ability of adult/postnatal stem cells, we have thus designed an autologous stem cell gene therapy platform aimed at tackling most diseases (Bertolotti, 2005 and 2007). As schematized in Fig. 1, the long-term gene therapy branch comprises a gene targeting arm aimed at tackling 1) nuclear inherited diseases through gene repair/alteration and 2) mtDNA-independent acquired/degenerative/aging disorders through custom site-specific integration of therapeutic transgenes (see below). The long-term gene therapy branch also has a cybridization arm in which autologous transmitochondrial stem cells are used to treat all mtDNA diseases (Bertolotti, 2005). On the other hand, as emphasized above, the transient gene therapy branch comprises a regenerative arm for regenerative medicine *sensu stricto* and for the synergistic magnification of the homing/regenerative/differentiative potential of *ex vivo* engineered stem cells from the long-term gene therapy arm (see above), and an epigenetic arm for long-term inactivation/activation of endogenous genes through transient expression of promoter-specific siRNAs and antisense RNA (see below). In addition, the transient gene therapy arm of our stem cell gene therapy platform includes a potential cancer gene therapy arm for stem cell-mediated, tumor-targeted delivery of transgenic products or oncolytic viruses (see above). Some of the main features of our platform are detailed below.

## 4.1. Gene Targeting and Cybridization as Genomic Homeostatic Tools

### 4.1.1. Endonuclease-boosted gene targeting

Pioneering gene therapy clinical trials relied on retrovirus-mediated transduction of transgenes encoding the wild-type product of the disease genes (Blaese *et al.*, 1995; Kohn *et al.*, 1995; Bordignon *et al.*, 1995). Such an approach culminated in the early 2000s with the first unequivocal successes for gene therapy (Cavazzana-Calvo *et al.*, 2000; Aiuti *et al.*, 2002; Gaspar *et al.*, 2004). Unfortunately, this therapeutic breakthrough is plagued by adverse oncogenic events resulting from the fact that retroviral vectors integrate at random into host chromosomal DNA (Hacein-Bey-Abina *et al.*, 2003b; Baum, 2007). Random integration of transgenic DNA into host chromosomal DNA is an insertional mutagenesis event that can hit a cancer-prone gene (e.g., oncogene, tumor suppressor or DNA-repair gene), thereby promoting a true long-term carcinogenesis hazard as experienced by patients from the first unequivocal successful gene therapy trial (Cavazzana-Calvo *et al.*, 2000; Hacein-Bey-Abina *et al.*, 2003b). In this trial, in which four out of eight successfully treated X-SCID patients incurred retroviral vector-mediated random-insertional T cell lymphomas, the first lymphoma case occurred almost three years after successful therapeutic gene transfer (Hacein-Bey-Abina *et al.*, 2003a) and the fourth case a few months ago (Baum, 2007).

Although a theoretically low-probability risk, random insertional oncogenesis is a true safety hazard of retroviral vectors. In addition, random integration into host chromosomal DNA does not provide for optimal transgene expression/regulation, a major concern when, unlike with SCID patients, we deal with a tightly regulated function (see Bertolotti, 1998 and 2000a). Site-specific integrating vectors are thus under intensive investigations, and are now emerging for both viral and nonviral gene therapy protocols (see: Olivares and Calos, 2003; Allen and Samulski, 2003; Wilkinson *et al.*, 2005; Urabe *et al.* and Fraefel *et al.*, this volume). However, these site-specific integrating vectors lack chromosomal target flexibility and might thus incur differentiation background trouble if one wishes to target a broad spectrum of tissue stem cells. Unlike these approaches, integration of exogenous DNA mediated by gene targeting is driven by homologous recombination with chromosomal DNA (see Capecchi, 1989) and has thus no target restriction, since the integration site is not specified by the DNA-binding domain of a protein but by the very DNA sequence of the targeting vector that is homologous to target chromosomal DNA. Gene targeting mediates flexible DNA

exchanges between chromosomal DNA and transfecting/transducing DNA, thereby providing the means to modify at will the sequence of target chromosomal DNA (Capecchi, 1989). Gene targeting stands thus not only as the ultimate process for site-specific (i.e., targeted) transgene integration, but also for gene repair/alteration (Bertolotti, 1996 and 1999). Therefore, clinical gene targeting is discussed both in terms of gene repair/alteration for inherited diseases/anti-viral therapy and in terms of custom site-specific integrative gene therapy for acquired/degenerative and aging disorders (Bertolotti, 1999, 2000a and 2004a).

Conventional gene targeting is overwhelmed by random integration and is inefficient unless a double-strand break (DSB) hits target chromosomal DNA (see Bertolotti, this volume). Emerging DSB-boosted gene targeting relies on chimeric zinc-finger endonucleases (ZFNs) and customized homing endonucleases that create site-specific DSBs (see Bertolotti, 2007). Optimization of zinc-finger DNA-binding domains culminates now in targeting efficiencies compatible with clinical single-base correction: homologous recombination mediates strand exchanges between chromosomal and transfecting therapeutic DNA under negligible random integration of both therapeutic DNA and ZFN vectors (Urnov *et al.*, 2005). Hot-off-the-press data show that the same holds true for targeted integration of up to  $\approx 8$  kb transgenes (Moehle *et al.*, 2007), thereby opening a promising custom site-specific integrative gene therapy era in which therapeutic transgenes can be integrated at selected hazard-free locations for optimized regulated expression.

Endonuclease-boosted gene targeting is thus a breakthrough for clinical gene therapy (see Bertolotti, 2004a and this volume). However, customization of both ZFNs and homing endonucleases for clinical applications is still a tedious and time-consuming process, thereby limiting their availability (see Bertolotti, 2007 and this volume). In addition, either with ZFNs or emerging custom homing endonucleases, efficiency is excellent when the distance between the target bases and the DSB is short (less than  $\sim 50$  bp), but quickly drops when the distance increases (Elliott *et al.*, 1998; Miller *et al.*, 2005; Porteus, 2006). Such a limitation is a major hurdle for the clinical management of most multi-base mutations. In addition, it pinpoints the personalized facet of current endonuclease-boosted gene repair since most sites of a disease gene have to be targeted with specific sets of designed nucleases. Therefore, current strategic choices focus on polyvalent ZFNs/homing endonucleases that target mutation hotspots and nearby mutation sites. In order to reach full clinical potentialities, vectorization of therapeutic DNA

might need optimization (see Bertolotti, 2004a and 2004b). An alternate approach is related to our autologous cybridization arm (see below).

#### 4.1.2. *Cybridization: transmitochondrial stem cells for mtDNA diseases*

Due to the current lack of effective techniques for intramitochondrial genetic engineering, gene targeting is not amenable to mtDNA and wild-type mtDNA sequence cannot be restored in mtDNA mutants. Therefore, we have devised a strategy in which autologous transmitochondrial stem cells are generated *ex vivo* and then returned to the patient as in standard long-term stem cell gene therapy (Bertolotti, 2005). Current approaches rely on nuclear transgenes for allotopic expression of mtDNA-encoded proteins (e.g., Owen *et al.*, 2000; Manfredi *et al.*, 2002; Guy *et al.*, 2002) or for mutant mtDNA cleavage/methylation (e.g., Srivastava and Moraes, 2001; Tanaka *et al.*, 2002; Minczuk *et al.*, 2006), culminating in the mtDNA-free mitochondria concept in which all mtDNA-encoded proteins are converted into allotopic gene products (Zullo, 2001). Unlike these approaches, stem cell cybrid gene therapy can accommodate all types of mtDNA mutations and is not hampered by random-integration hazards and transgene dysregulations associated with current long-term nuclear gene therapy (Bertolotti, 2005).

In this approach, patients' stem cells are cured from their mtDNA content by a transient growth period in the presence of rhodamine 6G and then repopulated by wild-type mitochondria through fusion with relevant enucleated cells (cytoplasts), thereby generating cybrids which are true transmitochondrial stem cells (Bertolotti, 2005). Together with their differentiating/differentiated progeny, cybrid stem cells are thus expected to convert their gain in energy metabolism efficiency into a selective *in vivo* growth advantage over resident mtDNA mutant cognate cells. Like the nuclear  $\gamma$ C transgene in the seminal X-linked SCID trial (Cavazzana-Calvo *et al.*, 2000), wild-type mtDNA could thus drive effective therapeutic repopulation dynamics of autologous engineered stem cells and differentiated derivatives into appropriate niches and relevant tissues/organs of patients, respectively. Importantly enough, magnification of this repopulating/regenerative capability will be under the control of the transient regenerative gene therapy arm of our stem cell gene therapy platform (see above).

#### 4.1.3. *Genomic homeostasis and hazard-free custom site-specific integrative gene therapy*

The aforescribed approaches are thus ideal to tackle inherited diseases, since the mutant nuclear genome can be repaired by gene targeting and

the wild-type mitochondrial genome (mtDNA) substituted for its mutant counterpart by cybridization, thereby restoring a perfect wild-type genomic homeostasis in both cases (Bertolotti, 1996, 2005 and 2007). Such an approach eliminates dysregulations and oncogenic hazards that hamper random integration of conventional or allotopic transgenes into host chromosomal DNA (Bertolotti, 1998 and 2005). Importantly enough, for other long-term stem cell gene therapy applications (mtDNA-independent acquired and degenerative/aging disorders), the hot-off-the-press gene targeting breakthrough achievement that culminated in high-efficiency integration of an  $\approx 8$  kb transgene (Moehle *et al.*, 2007) opens a promising custom site-specific integrative gene therapy era in which therapeutic transgenes can be integrated at selected hazard-free locations for optimized regulated expression.

#### **4.2. Epigenetic Therapy: Potential Long-Term Gene Inactivation/Activation Through Transient siRNA Gene Therapy**

The epigenetic arm of our transient gene therapy branch is aimed at long-term inactivation/activation of endogenous genes through transient expression of promoter-specific siRNAs (Morris *et al.*, 2004; Li *et al.*, 2006; Janowski *et al.*, 2007). Transcriptional gene silencing mediated by siRNAs is tightly sequence-specific (Morris *et al.*, 2004; Castanotto *et al.*, 2005; Suzuki *et al.*, 2005; Ting *et al.*, 2005; Weinberg *et al.*, 2006; Han *et al.*, 2007), thereby necessitating potential patient personalization and, possibly, allele adjustment in heterozygote patients. On the other hand, siRNA (Li *et al.*, 2006; Janowski *et al.*, 2007) and noncoding antisense RNAs (Imamura *et al.*, 2004) are also amenable to converse epigenetic endogenous gene activation. Whether long-term gene silencing (Suzuki *et al.*, 2005) or activation (Janowski *et al.*, 2007) can be achieved under current conditions is not yet clear, and may be associated with efficient promoter methylation (Suzuki *et al.*, 2005).

#### **4.3. Emerging Pluripotent Stem Cells as Potential Universal Drives for Tissue-Specific and Multisystemic Gene Therapy**

Emerging adult/postnatal multi/pluripotent stem cells such as MAPCs (multipotent adult progenitor cells; Jiang *et al.*, 2002), MIAMI cells (marrow-isolated adult multilineage inducible cells; D'Ippolito *et al.*, 2004), hBMSCs (human bone marrow multipotent stem cells; Yoon *et al.*, 2005) or maGSCs (multipotent adult germ stem cells; Guan *et al.*, 2006) have an extensive/clonogenic *ex vivo* growth potential, and are therefore amenable to drastic

selective growth conditions. They are thus instrumental in our mitochondrial stem cell production platform for the selective cybrid rescue step (Bertolotti, 2005 and this volume). Such an extended *in vitro* growth potential also opens a promising avenue for emerging selective gene targeting, whereby relevant engineered autologous stem cells are selected/sorted and amplified *ex vivo* in order to compensate for nonclinical gene targeting frequencies incurred when the mutation track is too long or too far from the endonuclease target site (Bertolotti, 2006 and 2007; see Bertolotti, this volume).

Importantly enough, the breakthrough ability of the aforementioned adult/postnatal stem cells to differentiate into cells of the three germ lineages (pluripotency) is an important parameter in the development of our universal autologous stem cell gene therapy platform, since it opens exciting avenues to tackle all tissue-specific pathologies and tissue regeneration needs with a single stem cell population (Bertolotti, 2007; see Serafini and Verfaillie, this volume). In this respect, the pioneering transplantation of MAPCs as a substitute for tissue-specific stem cells (HSCs) with full functional reconstitution of the hematopoietic system stands as a promising proof of concept (Serafini *et al.*, 2007). However, one of the most exciting features of autologous pluripotent cells is that they should be amenable to multisystemic diseases such as progeria or most of the mtDNA diseases (see Bertolotti, this volume). Importantly enough, in order to take full advantage of the extraordinary potential of these cells culminating in multisystemic applications, magnification/optimization of their homing, regenerative, and differentiative abilities will exhaustively depend on the transient regenerative arm of our universal platform. Therefore, thanks to synergistic combinations between the different arms of our autologous stem cell gene therapy platform, we believe that both tissue-specific and multisystemic pathologies/regenerative needs (including mtDNA diseases and aging disorders) can be tackled through pluripotent stem cell repopulation dynamics into appropriate niches (long-term engraftment) and tissues (cell turnover) (Bertolotti, 2005 and 2007).

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