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Application and optimization of a gene targeting strategy called Small Fragment Homologous Replacement for the treatment of the Spinal Muscular Atrophy

by

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A mio padre, mia madre,
mio fratello e le mie sorelle.

Non credo che la pratica della scienza
possa andare disgiunta dal coraggio e
che la scienza possa proporsi altro
scopo che quello di alleviare la
fatica dell'esistenza umana

Galileo Galilei
in *Vita di Galileo* di Bertold Brecht

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ABSTRACT

The majority of patients affected by spinal muscular atrophy (SMA) have deletion of the *survival of motor neuron 1* (*SMN1*) gene, but they retain a "nonfunctional" copy of the duplicate gene (*SMN2*) in their genome. *SMN2* produces defective SMN protein because of a C>T transition in exon 7, which causes the skipping of exon 7 during SMN mRNA maturation. Many attempts have been made to correct altered *SMN* gene expression and to increase the level of normal SMN protein, but to date an effective treatment for this disease has not been established. Small Fragment Homologous Replacement (SFHR) is a site-specific gene modification approach that has the potential to maintain the genomic organization necessary for expression. The target modification in the genome is mediated by small DNA fragments (SDFs) 400–800 bp in length. In this study we used SFHR to induce a T > C transition at codon 280 in exon 7 of the *SMN2* gene in order to produce an increase in functional SMN protein. SDFs were transfected *in vitro* into cells obtained from five human fetal chorionic villi of embryos, homozygous for the *SMN1* deletion, by either electroporation or microinjection. Transfected SMA cells showed an increase of up to 53% in full-length SMN mRNA compared with untransfected controls, as detected by real-time polymerase chain reaction. Consistent with the RNA data, immunocytochemistry and immunoblotting revealed a significant 2-fold increase in wild-type SMN protein. Furthermore, genotype and phenotype of transfected cells remained stable after several *in vitro* passages, demonstrating the stability of the correction over time

INTRODUCTION

Spinal Muscular Atrophy (SMA; OMIM 601627) is a common recessive autosomal disorder that results in destruction of motor neurons in the anterior horn of the spinal cord (Dubowitz, 1995). Motor neuron degeneration affects voluntary muscles and causes proximal symmetrical weakness and atrophy of the limbs and trunk. The *survival of motor neuron* (*SMN*) gene has been shown to be the SMA-determining gene (Lefebvre *et al*., 1995). Two SMN genes are present on chromosome 5q13: the telomeric or *SMN1* gene and the centromeric or *SMN2* gene (Melki *et al*., 1994; Lefebvre *et al*., 1995; Burglen *et al*., 1996). All forms of SMA are caused by homozygous loss of the *SMN1* gene as a result of gene deletion, conversion, or mutation. In about 95% of affected patients exon 7 of the *SMN1* gene is absent, and other, more subtle mutations have been identified in the remaining affected patients (Melki *et al*., 1994). *SMN2* copy number influences the severity of the disease (Campbell *et al*., 1997; McAndrew *et al*., 1997; Wirth *et al*., 1999; Feldkotter *et al*., 2002; Mailman *et al*., 2002). Patients with a milder form of SMA, type II or III, have been shown to have more copies of *SMN2* than do type I patients. Eleven nucleotide differences exist between *SMN1* and *SMN2* transcripts, but none of these differences leads to changes in the amino acid sequence (Monani *et al*., 1999). The critical difference between *SMN1* and *SMN2* is a C-to-T transition in the *SMN2* gene, which affects the activity of an exonic splicing enhancer (ESE), reducing the recognition of *SMN2* exon 7 by the spliceosome (Cartegni and Krainer, 2002). This results in the transcription of two isoforms, the full-length functional SMN protein and more predominant transcripts lacking exon 7 (Monani *et al*., 1999; Lorson and Androphy, 2000; Lorson *et al*.,

1999). The full-length *SMN* transcript produces a protein of 38 kDa that is present in both the cytoplasm and the nucleus with variable expression levels among the different tissues examined: greatest in the brain, spinal cord, and muscle and lowest in lymphocytes and fibroblasts (Coovert *et al*., 1997; Lefebvre *et al*., 1997). Nuclear SMN is concentrated in aggregates called Gemini of coiled bodies (gems; Liu and Dreyfuss, 1996). The number of gems per cell is drastically decreased in SMA patients, and can be used as a prognostic indicator of disease severity. The SMN protein acts within macromolecular complexes together with the SMN-interacting proteins, gemin 2, 3, and 4. The complex in the nucleus is involved in pre-mRNA splicing, ribosome production, and transcription (Fischer *et al*., 1997; Liu *et al*., 1997; Charroux *et al*., 1999, 2000), whereas that in the cytoplasm is involved in biogenesis of the spliceosomal UsnRNP (uridine-rich small nuclear ribonucleoprotein).

To date, no effective therapy exists to rescue the motor neuron degeneration of SMA. However, the discovery of the SMA-determining gene, and of the differences between *SMN1* and its highly homologous copy *SMN2* in the human genome, has led to the development of novel therapeutic strategies for SMA. In this regard, *SMN2* provides a natural target for therapeutic intervention in almost all SMA patients and many attempts have been made to devise strategies to enhance the incorporation of exon 7 during mRNA splicing, or to increase the activity of the *SMN2* promoter (Hofmann *et al*., 2000; Zhang *et al*., 2001; Young *et al*., 2002; Brichta *et al*., 2003; Skordis *et al*., 2003; Andreassi *et al*., 2004). Several approaches are being explored to develop potential therapies for SMA, but no gene-targeting strategies have been successfully attempted. Gene targeting induces a site-specific chromosomal modification that leads to long-term and genetically

inheritable expression of the correct gene, regardless of its size. Moreover, direct conversion of genomic sequences preserves the integrity of the gene in such a way that the coding sequences and regulatory elements remain intact. The availability of better delivery methods, such as electroporation and microinjection, has made nonviral transfer an increasingly safer and more viable method for gene therapy. One of these oligonucleotide-based gene-targeting strategies, Small Fragment Homologous Replacement (SFHR), has been successfully used to correct or modify gene sequences responsible for some inherited disease *in vitro* and *in vivo* (Kunzelmann *et al*., 1996; Goncz and Gruenert, 1998, 2000, 2001; Goncz *et al*., 1998, 2001, 2002; Kapsa *et al*., 2001; Bruscia *et al*., 2002; Sangiuolo *et al*., 2002, 2005; Thorpe *et al*., 2002; Gruenert *et al*., 2003; Zayed *et al*., 2006 Goncz *et al*.2006).. In this study, we investigated the efficacy of SFHR to induce a T > C transition in exon 7 of the *SMN2* gene in order to rescue physiological level of *SMN* transcript and protein, after transfection by either electroporation or microinjection in human fetal cells obtained from embryos affected by SMA.

SPINAL MUSCULAR ATROPHY

Spinal Muscular Atrophy (SMA) is a heterogeneous group of disorders characterised by the degeneration of alpha motor neurons in the anterior horns of the spinal cord and the lower brainstem. SMA occurs in childhood with muscle weakness and respiratory failure. Muscle weakness is always symmetrical and associated with a marked decrease in deep reflexes, muscle fasciculation and hypotonia. The disease represents the most frequent inherited cause of infant mortality (Pearn *et al.*, 1978). Depending on the age of manifestation and achieved motor abilities, SMA has been classified into four different types. Type I patients (approximately 50% of all SMA cases) are never able to sit or stand unaided and usually die before the age of 2 years. Type II SMA patients are able to sit, but are never able to stand, whereas type III SMA patients are able to sit and stand, but mostly get wheelchair bound while muscle weakness is progressing (Munsat *et al.*, 1992). The mildest form of SMA, type IV, is defined as a slowly progressing disease, which typically occur after the age of 30 (Zerres *et al.*,1995). Regardless of SMA type, disease progression and life expectancy is strongly correlated to age of onset. Moreover, patients with SMA tend to have the greatest rate of loss of muscle power at disease onset (Zerres *et al.*,1995). Mutations of the Survival of Motor Neuron (SMN) locus, on chromosome 5q13, are responsible for the disease. In humans, the SMN locus contains an inverted duplication consisting of the telomeric SMN1 gene and the centromeric, highly homologous SMN2 gene. More than 95% of SMA patients are homozygously deleted for SMN1 (Lefebvre *et al.*, 1995), and the remaining cases (5%) show missense, nonsense or splicing mutations within SMN1 gene (Wirth *et al.*, 2000). All patients deleted for

SMN1 retain at least one centromeric copy of the gene, *SMN2*, which is unable to complement SMN1 because of a crucial mutation in an exonic splicing enhancer (Lorson *et al.*, 1998), leading to an alternative splicing phenomenon. In fact the SMN2 gene mainly produces a protein isoform, which has exon 7 deleted (delta7 SMN) (about 80%), and reduced amounts of full-length (FL-SMN) mRNA (about 20%). Delta7 SMN encodes for an unstable protein and consequently the insufficient levels of SMN protein in motor neurons represents the cause of the disease phenotype. To support this hypothesis, there is evidence of a clear inverse correlation between the severity of the SMA phenotype and the SMN2 copy number. SMN2 is the major modifier of SMA phenotype and the critical parameter being the amount of total SMN (both fulllength and delta7) it can produce (Thanh et al., 2005). In fact, the more SMN2 copies a patient has, the milder the phenotype (Burghes *et al.,* 1997; Feldkotter *et al.*, 2002; Helmken *et al.*, 2003). Other evidence sustaining this supposition comes from *Smn*$^{-/-}$ knockout mice. In fact introducing one or two copies of human SMN2 in this murine model makes it possible to improve the clinical phenotype, trasforming the embryonic lethal phenotype into a severe one (Hsieh-Li *et al.*, 2000; Monami *et al.*, 2000). SMN is a protein of 38 kDa involved in a series of pathways, the most essential of which is the housekeeping function in snRNP biogenesis and spliceosome assembly (Liu *et al.*, 1997; Pellizzoni *et al.*, 1998) and the neuron-specific function in RNA transport along the axons (Rossoll *et al.*, 2002; Zhang *et al.*, 2003). The SMN gene does not have an exclusively neuronal expression or function, but it is ubiquitously expressed, with only few tissue and developmental differences (Yong *et al.*, 2004; Will *et al.*, 2001). Within a single cell, the protein is present in both the cytoplasm and the nucleus, where it is concentrated in two sub-

structures that often overlap, Cajal bodies and Gems (Rossoll *et al.*, 2002; Zhang *et al.*, 2003; Gabanella *et al.*, 2005; Wan *et al.*, 2005). Gems contain high levels of factors involved in transcription and RNA processing. At least seven additional proteins (from Gemin2 to Gemin8) are stably associated with the SMN protein in large macromolecular complexes (Carissimi *et al.*, 2006; Gubitz *et al.*, 2004). The number of gems inversely correlates with disease severity, with type I patients showing few or even no gems (Coovert *et al.*, 1997; Patrizi *et al.*, 1999) while there is an increase in type II and III patients. For this reason the number of gems per cell drastically decreases in SMA patients, and represents a useful prognostic indicator of disease severity. Also the localisation of the gems correlates with the phenotype, whereas in affected cells the gems are localised more within the cytoplasm than in the nuclei compared to wild type cells (Liu *et al*., 1997; Lefebvre *et al.*, 1997). However the precise function of SMN is not yet known.

Furthermore, it is not quite clear why SMA motor neurons are the only cell type for which SMN expression levels are unadequate for their normal activity. To date, no effective therapy exists which is able to rescue the motor neuron degeneration of SMA patients. However, the discovery of the SMA-determining gene, and of the differences between *SMN1* and its highly homologous copy *SMN2* in the human genome, has led to the development of novel therapeutic strategies for SMA. To this aim, *SMN2* provides a natural target for therapeutic intervention in almost all SMA patients. The general hypothesis is that increasing full-length SMN protein levels should have a positive impact on the onset and progression of SMA disease. This goal can be reached by increasing the expression of SMN, or changing the splicing pattern of the SMN2 gene or even stabilizing the SMN protein. The therapeutical approach could be pharmacological or genetic. Moreover the

development of stem-cell therapies has great potentiality, since this recent developments in stem cell differentiation and transplantation.

GENE TARGETING

The genome-based approaches are aimed to an *in situ* permanent correction of defective endogenous gene (gene targeting approach). These procedures are clearly distinct from the cDNA-based strategies that utilize viral cDNA-based methods by relying on non-viral DNA delivery vehicles. Furthermore, they are preferable to the delivery of such exogenous non-integrating vectors, transiently expressing wild type versions of non functional genes or their cDNA (gene augmentation approach).

Gene therapy has very straight-forward goals to effectively correct the pathogenic phenotype resulting from genetic mutation(s) and to insure that the therapeutic strategy is safe for the patient.

The Gene Therapy approaches can be broadly divided into viral and non-viral gene transfer technology. Viral vectors take the advantage on the facile integration of the gene of interest into the host and high probability of its long–term expression but are plagued by safety concerns vehicles. The main risk of retrovirus-mediated gene transfer is insertional mutagenesis resulting from random retroviral integration. In fact, random integration has been reported in viral gene delivery to increase the possibility of gene disruption, including disruption of genes involved in cell cycle or tumour suppression

(Remus *et al.*, 1999; Muller *et al.*, 2001), and as a result gene targeting strategies have being received increasing attention.

Recently the observation that retroviral gene transfer induced T cell leukaemia in two children of ten patients treated for typical X-linked severe combined immunodeficiency (SCID-X1) has raised significant safety concerns for traditional gene strategies (Williams *et al.*, 2003; Hacein-Bey-Abina *et al.*, 2003, 2003a). Hacein-Bey-Abina and colleagues showed retrovirus vector integration in proximity to the *LMO2* proto-oncogene promoter, leading to aberrant transcription and expression of *LMO2,* suggesting that retrovirus vector insertion can trigger deregulated premalignant cell proliferation with unexpected frequency, most likely driven by retrovirus enhancer activity on the *LMO2* gene promoter. Accordingly, the American Food and Drug Administration (FDA) placed on "clinical hold" all active gene therapy trials using retroviral vectors and suspended the enrolment of new patients in clinical trials that involve the use of retroviruses. Furthermore in the 2005, another clinical trial reports the failure of retroviral gene therapy in 2 such patients affected by a severe combined immunodeficiency (SCID-X1, c-deficiency), despite effective gene transfer to bone marrow CD34+ cells, suggesting that there are intrinsic host-dependent restrictions to efficacy (Thrasher *et al.*, 2005).

Non-viral vectors (naked DNA fragments and plasmid DNA), although less efficient at introducing and maintaining foreign gene expression, have the profound advantage of being non-pathogenic and non-immunogenic.

Above all, gene targeting strategies retain the integrity of the target gene in terms of relationship between the protein coding sequences and gene-expression. By preserving the integrity of the targeted gene, the relationship between the coding sequences and regulatory elements remains intact and

the corrected gene would still undergo the cell-specific regulatory elements. Therefore, targeted gene would be expressed at physiological levels in the appropriate cell type. Consequently, cell-specific expression is not altered.
It is therefore mandatory to develop vectors with an improved safety profile and/or transfect cells in ex vivo, followed by checking them carefully by genetic analysis before infusing back into the patient.
At the present time, the recent availability of better delivery methods (e.g. electroporation and microinjection) has made the non-viral gene transfer an increasingly more important and viable method for gene therapy. Therefore if further developed, gene targeting strategies will gain a higher capacity of correction and will lead to fewer mutagenic side effects than do methods that randomly insert genes into the genome (Sullenger *et al.*, 2003).

SMALL FRAGMENT HOMOLOGOUS REPLACEMENT (SFHR)

SFHR is a gene repair strategy that involves the introduction of small DNA fragments (SDFs) (up to 1 kb) into cells. These SDFs involve homologous exchange between their sequences and the endogenous (genomic or episomal) ones, resulting in phenotypic changes. The technique has been used to modify endogenous genomic DNA in both human and mouse cells. After entering the cells, the fragment pairs with its genomic homologue and replaces the endogenous sequence with the exogenous fragment through an, as yet, undefined mechanism (Gruenert *et al.,* 1998, 1999); (Yanez *et al.*, 1998), that probably involves similar pathways to those of homologous recombination and/or uncharacterized pathways of DNA repair (Goncz *et al.*, 2000). It is likely that SFHR functions by targeting and replacement,

involving the necessary proofreading and annealing to homologous target regions, with subsequent strand invasion, and exchange of genetic material.

Typically, small DNA fragments are utilized double stranded although in 2004 Kamiya's group reported a very significant enhancement of the SFHR-mediated gene correction efficiency using a sense ssDNA fragment. In this model, an inactivated Hyg-EGFP fusion gene was utilized in a gene correction assay, obtaining a 12-fold enhancement in gene correction (Tsuchiya *et al.*, 2004).

Genotype and phenotype analyses have shown specific modification of disease causing genetic loci and suggested that it has potential as a therapeutic modality for the treatment of inherited disease, if not with a single treatment, with repeated applications. Different kinds of genomic mutations have been altered by using this approach, suggesting a broad range of utility in terms of target genes and cell types able to support SFHR. Moreover the SFHR technique appears to be effective both *in vivo* and *in vitro*. In fact, genetic modification of several kinds of cells was reported, including mouse embryonic stem cells (Gruenert *et al.,* 1998; Goncz *et al.*, 2000, 2001; Kapsa *et al.*, 2002). The recent application of this strategy to stem cell and demonstration that these cells can be efficiently targeted would mean that a single treatment could correct a genetic defect within a given organ for the lifetime of the patient, using an ex vivo strategy (Goncz *et al.*, 2000,2006; Hatada *et al.*, 2000; Gruenert *et al.,* 1998; Zayed *et al.*, 2006). By targeting gene repair to stem cell populations, it is possible that long-term correction might be achieved through clonal expansion.

Different efficiency values of SFHR-mediated modification were reported. This variability can depend from purity of the synthetized olionucleotides and transfection efficiency, specific for each cell line (Schindelhauer *et al.*,

2002). In this way any improvement in gene delivery will be fundamental to the success of gene repair, especially for somatic gene repair in vivo where transfection efficiencies are considerably lower than in vitro. The efficiency of transfection could be increased using a different way of delivery of SDF into cell nuclei. Recent studies have reported the successful use of microinjection techniques in progenitor stem hematopoietic cells-HPCs, human lung sarcoma-HT1080, and immortalized lung epithelial-16HBE14o- and primary human fibroblasts (Goncz *et al.*, 2001,2006; Schindelhauer *et al.*, 2002). This technique, together with an innovative electroporation protocol, might overcome the inefficient nuclear delivery of DNA that was observed using chemical delivery vehicles. Another factor surely influencing SFHR efficiency is represented by the absence of a selection mechanism to accurately define the genetic changes induced by this strategy. For this purpose a subcloning of the transfected cells by using the limited dilution technique could be attempted (Bruscia *et al.* 2002), demonstrating the stability of the modification over multiple generations.

The lack of a detailed knowledge of the mechanism of action of the SDF at the molecular level sets a limit to a precise reproducibility of this strategy. The efficiency of modification is very variable and depends from different conditions. In fact the transfection protocol, the kind of fragment, its length and also its concentration and the chromatin structure, which changes throughout the cell cycle, may be key factors underlying these variations in efficiency. These hypothesis prompted us to systematically investigate SFHR-mediated gene repair process during various phases of the cell cycle using different kinds of SDF. To this purpose, we design and constructed an *"assay system"* that can be used to optimize SFHR protocol *in vitro* in order to improve the SFHR efficiency in eukaryotic cells. A mutated copy of

EGFP gene were stably integrated within immortalized embryonic fibroblasts (MEF), obtained from Sma mice model ($Smn^{-/-}$; SMN2). Different parameters such as the concentration, the length and the nature of the *wt*SDF, but most importantly different phases of cell cycle (G0/G1-S-G2/M) were tested to understand which was the exact mechanism underlying the SDF-mediated integration within genomic DNA. The efficiency of modification was quantified by Flow cytometry analysis, measuring the percentage of EGFP-positive cells obtained. Transfection protocol was optimized by nucleofection (Nucleofector Amaxa) and the SDF for the corrected EGFP sequence were transfected into the GFPneg cell line. While in phase G0/G1 the efficiency of correction was estimated about 0.01%, this value double in phase S (0.02%). The best results was achieved after synchronizing the cells in G2 phase and transfecting $7{,}5x10^6$ molecules of SDF/cell. In fact, during this phase the efficiency of correction increased 10 times, up to 0.1%. We also tested the effect of the methylation status of the "corrective" fragment, comparing PCR-amplified SDF versus plasmid-digested one. The latter gave best results in terms of recombination efficiency thanks to its higher methylation grade. In fact, transfecting plasmid-digested SDF in G2 synchronized cells the percentage of fluorescent cells increased 50 times respect to the standard condition. After cell sorting and several doublings the stability of genomic modification SDF-mediated was assessed by Southern blot and microscopy analyses. In conclusion, this study allows us a better comprehension of SDF molecular way of action (G2 phase and homologous recombination) and consequently open up new perspective for considering SFHR as a gene targeting strategy applicable to the ex vivo protocols (Filareto *et al.* manuscript in preparation).

The SFHR technique has been successfully applied to modify mouse genomic loci, as the *Cftr* (Cystic fibrosis transmembrane conductance regulator) gene, and the *dystrophin* gene, both in vitro and in vivo. Phenotypic and genotypic changes have been demonstrated for the *Cftr* gene, while only genotypic modifications have been shown in *mdx* mice (Kapsa *et al.*, 2002). A study by (Goncz *et al.*, 2001), has demonstrated the importance of the delivery during the application of SFHR-mediated protocols. The authors used the SFHR technique to introduce in the genome of a wild-type mouse a 3bp deletion within the *cftr* gene. SDFs were transferred in normal mouse lung via intratracheal instillation, after complexation with four different transfecting agents. Detectable levels of sequence alteration were observed, but the data suggests a gradient of these DNA vehicles in terms of reproducibility and specifically, the artificial viral envelope (AVE) gave better results than LipofectAMINE, dimethyl-dioctadecyl-ammonium-bromide (DDAB) and SuperFect. This study underscored the difficulties in extrapolating results from the *in vitro* to *in vivo* settings. A similar approach was used in our laboratory to introduce the same modification in mouse embryonic stem cells (D3) using cationic liposome as the SDFs transfection vehicle (manuscript submitted). Confocal microscopy was carried out to track the entry of Cy-5 labelled SDFs (red) into the nuclei (green). The 3 bp deletion (TTT) was detected at the DNA and mRNA level, after amplicon cloning, to avoid PCR artefacts. Modification efficiency of *cftr* locus was quantified at the mRNA level by using real-time PCR, resulting in more than 5 % of transfected cells. In addition, we demonstrated that a mutated gene product was obtained using this technique. This high frequency of selective change in ES cells using

SFHR is encouraging for the future development of therapeutic protocols based on cell therapy.

The Mdx mouse model of Duchenne muscular dystrophy (DMD), (Kapsa et al., 2002, 2001) was also repaired by using SFHR. A nonsense mutation in the dystrophin locus was targeted in both primary myoblast cultures and by direct injection of affected muscle (tibilias anterior). In vitro and in vivo application of a wild-type SDF (603bp) was used to mediate a T to C conversion in exon 23 of the dystrophin gene. Different conditions in the lipofectamine complex enhanced the efficiency of SFHR-mediated modification in vitro. Conversion was observed at both the DNA and RNA levels. The conversion of mdx to wild-type sequence in vitro was about 15 % by PCR analysis, although there was no detection of normal dystrophin protein. In vivo the correction efficiency was up to 0.1 % in the tibialis anterior of male *mdx* mice, but again there was no evidence of gene expression at either the transcript or protein level. It was suggested that the disparity between the genomic repair and protein expression was possibly due to toxicity of the transfected agent on myoblasts, or a delay in protein expression. The correction in myoblasts from mdx mice persisted at least 28 days in culture and up to 3 weeks in vivo. These genomic conversion frequencies were lower than those reported for chimeraplasty, which did result in protein expression (Rando *et al.*, 2000; Bartlett *et al.*, 2000), after direct injection into muscles of *mdx* mice. Two weeks after single injections into tibialis anterior muscles, the maximum number of dystrophin-positive fibers in any muscle represented 1–2 % of the total number of fibers in that muscle. The expression appears to be stable until ten weeks after single injections.

More recently the SFHR technique has been successfully applied to modify the *DNA-PKcs* gene responsible of the SCID mouse (Zayed *et al*. 2006) and the hβ-globin gene (Goncz *et al.* 2006). The human β-globin (β^A -globin) sequence has been modified into the sickle *β*-globin (β^S-globin) sequence. A DNA fragment (559 bp) homologous to β^S-globin sequence was microinjected into the nuclei of $lin^-/CD38^-$ hematopoietic cells. Individual microinjections delivered from 2.5 x 10^2 to 1 x10^4 SDFs per cell. In 72 separate experiments, site-specific conversion (β^A -globin -> β^S -globin) was observed in 42% of the experiments when DNA and RNA were analyzed 2–5 weeks postinjection. The conversion frequency generally ranged from approximately 0.2 to 3%, as determined by assuming a minimal conversion frequency of one cell per experiment showing SFHR-mediated modification. The conversion frequency in one experiment was assessed by evaluating the cloned polymerase chain reaction (PCR) products generated from the amplification of genomic DNA harvested 24 days postinjection. The frequency of β -globin alleles converted from β^A-globin -> β^S-globin in this experiment was determined to be ~13%. When sickle cell patient-derived lymphoblastoid cells were transfected with wild-type, β^A-globin SDFs, conversion of the β^S-globin to the β^A-globin sequence was observed up to 10 days posttransfection.

Zayed et coll. have also shown that such gene targeting strategy can occur in murine severe combined immune deficiency. Using a T cell thymoma line derived from severe combined immunodeficient (SCID) mice with a point mutation in the gene encoding the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), they have shown that short DNA fragments (SDFs; 621 bases) can provide genotypic and functional correction of these cells.

Double-stranded SDFs (dsSDFs) or single-stranded SDFs (ssSDFs) were designed to span the wild-type sequence of exon 85 in the DNA-PKcs gene and part of the 3' and 5' flanking intron regions. SCID cells were nucleofected with both single- and double-stranded wild-type SDF sequences. Corrected cells were selected on the basis of protection from radiation hypersensitivity that occurs as a consequence of the SCID mutation. Correction was mediated by both SDF forms (double and single stranded). These results have indicated that SDFs can correct point mutations by HR with the possibility of harnessing ionizing radiation (IR) as a selection method to eliminate noncorrected cells and enrich for corrected SCID radioresistant cells.

DELIVERY SYSTEMS

Certainly one of the deepest effects on the targeting ratio is the method of delivery of the vector DNA. Aware of that, scientists attempted different gene-transfection methods during the past years and parallel studies, meant to increase the efficiency of the most promising ones, were carried on. At the present time the three most common techniques still are liposome-mediated delivery, microinjection and electroporation, therefore now most of the efforts have shifted to improving their efficacy both for *in vitro* and *in vivo* applications.

CATIONIC-LIPOSOME DELIVERY

The cationic-liposome based gene delivery, first reported by Felgner in 1987 (Felgner *et al.*, 1987), allows transfer of genetic material trough the cell-membrane and its subsequent release into the cytoplasm. Once inside, the DNA is thought to pass into the nucleus, maybe by physical association with the chromatin during mitosis or by crossing the nuclear membrane. Initially the negatively charged DNA is electro-statically bound to the liposome in a lipocomplex that is able to associate with the cellular membrane (negatively-charged) and is then up-taken by the cell. Although both the up-take and the release mechanisms have not been completely clarified yet, it has been claim that, maybe, endocytosis and/or lipocomplex fusion with the membrane permit the liposome entrance into the cell, and that once inside the DNA could be released by mixing of the lipid membranes with the liposomes and the consequent disruption of the latter (Wiethoff, 2003)

Cationic liposomes (CL) are wildly used as delivery system in mammals (Gao, 1995), although they show a general high toxicity to cells. In fact, CLs are membrane active elements, that is to that they can hamper the membrane functions and equilibrium (Xu, 1996).

This was recently confirmed by transfection experiments on salmonid cell lines of hepatocyte and macrophage origin (Romoren *et al.*, 2004). Those observations lead to the conclusion that differences in the transfection efficiency and in CL toxicity-levels between the cell lines had to be mainly addressed to their different membrane compositions, although in the above mentioned cell lines the CL cytotoxicity increased with the increasing lipid concentration.

Furthermore, in general, different results suggest that the DNA/liposome mixing ratio significantly affects the intracellular trafficking of plasmid DNA complexed with the cationic liposomes (Sakuraia *et al.*, 2000). Nevertheless liposome transfer has several advantages: they can be used both for *in vitro* and *in vivo* transfections, are non –immunogenic, can transfer any type of nucleic acids and their use and preparation is relatively simple.

MICROINJECTION

Another way of physically introducing DNA into a cell is through microinjection that consists in directly injecting foreign DNA into cells. A cell positioned under a microscope, is manipulated to a blunt capillary and the DNA or RNA is inserted into the cytoplasm or nucleus.

Microinjection has been successfully used with large frog eggs, cultured mammalian cells, mammalian embryos, and plant protoplasts and tissues.

Examples of its application to transfer in the cells molecules capable of either transitorily compensate mutated gene or directly repair mutations on the chromosomal DNA, is reviewed by Davis et al. In particular, the work focuses on glass needle-mediated Micro-injection as a method for the delivery of genetic material into blood stem cells (Davis *et al.*, 2000).

An illustration of the use of the microinjection technique was reported in a recent study where the efficiency of correction of two differently designed chimeraplasts were compared (Goncz *et al.,* 2002). These constructs were evaluated for their ability to correct a point mutation in the gene encoding

recombinant enhanced green fluorescent protein (eGFP) that rendered the protein non fluorescent. To realize this, a plasmid encoding this mutant eGFP gene and a chimeraplast were co-introduced directly into the nuclei of primary fibroblasts by microinjection (Tran *et al.*, 2003)

ELECTROPORATION

Electroporation is an efficient method that can be used both *in vitro* and *in vivo* to transfer physiologically the gene into the cell without complicated preparations.

This technique is based on the application of external electric fields that results in transient, reversible breakdown of the cell membrane. During the process, the membrane becomes extremely conductive and as a consequence of the current passage inside the cell, is achieved formation of pores, trough which small molecules are transferred into the cytosol.

Developments over the past decades have led to sophistication of equipment and optimization of protocols; among all the different successful innovation, the most interesting one is the electroporation of small molecules delivered directly inside the nucleus, using the new Amaxa Nucleofector technology that is able to introduce the genetic material directly into the nucleus. This reduces assay time to a couple of hours versus 24 to 48 hours for standard transfection methods that use liposome mediated delivery. Ideally the system is matched for selected primary cell populations (including hematopoetic cells, chondrocytes, cardiomyocytes, endothelial cells,

keratinocytes, stem cells, neurons, and DC cells). Furthermore it is not restricted to DNA only but it can also be used for transferring siRNA (small interference RNA) into cells, especially primary cells. The only drawbacks to the machine are that not optimized cell systems require a protocol optimization and in general it must be used trial and error on each of the programs provided. Very recently the nucleofector method was exploited to transfect different cell lines. It showed a transfection frequency higher than 50% used in nature killer (NK) cells (Maasho *et al.*, 2004; Trompeter *et al.*, 2003), 60% in dendritic (DC) cells (Lenz *et al.*, 2003). Transfection of primary cells and stem cells, a problem in the laboratory routine since most methods working effectively for cell lines in culture fail to transfect them, has been achieved as well by Hamm (Hamm *et al.,* 2002).

MATERIALS AND METHODS

Small DNA fragment design and synthesis

Fragment was generated by polymerase chain reaction (PCR) of human genomic DNA wild type at the *SMN1* locus and lacking the *SMN2* gene. Primers used to generate a small DNA fragment (SDF) homologous to the human *SMN1* sequence were located within introns 6 and 7, respectively (Fig. 1A). The sense primer sequence was SMN1F (5'-AGTTGTGGGATTGTAGGCATG-3') and the antisense primer sequence was SMN1RBsmI (5'-GCATTCTAGTAGGGATGTAGA-3'). The reverse oligonucleotide was designed to incorporate a base change at base pair position 497 of the amplified fragment. The point modification together with the *SMN1* sequence allowed the incorporation of a unique *Bsm*I restriction site that can be used as a marker to verify SFHR-mediated specific correction (Goncz and Gruenert, 1998; Goncz *et al*., 1998). PCR was performed (annealing temperature, 55°C) in a total volume of 50 μl, using 1.5 U of *Pfu* DNA polymerase, 20 pmol of each primer, and 300 ng of genomic DNA. The SDF was cloned into a plasmid vector for large-scale production and, before use, always gel and ethanol purified (DNA gel extraction kit; Millipore, Bedford, MA).

Cell culture

Primary cell cultures from human placental villi were established from five normal and five SMA embryos after transabdominal chorionic villus sampling (CVS) at week XII of pregnancy (Zahed *et al*., 1988) from normal

women and women at 25% risk of SMA who were undergoing prenatal molecular diagnosis. Informed consent from patients was obtained. Cells were grown in Chang medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (FBS), 1% Lglutamine, and 1% penicillin–streptomycin. After about 10 days of culture cells grown from the explants of the villi were detached from the dish, using a standard EDTA–trypsin solution (Sigma-Aldrich, St. Louis, MO) and subcultured in fresh medium for an additional 2–4 days as indicated, before transfection. In some experiments, after transfection, cells were propagated several times (>40 passages).

Cell transfection by microinjection

Injection was performed into cells cultured for at least 48 hr from the first subculture and at about 30% confluency. In each experiment, about 1000 cells were microinjected (Table 1) under a Nikon Eclipse TE 300 inverted microscope, equipped with a Narishige MO-188NE micromanipulator (Narishige International, East Meadow, NY) and a FemtoJet microinjector (Eppendorf, Hamburg, Germany). Microinjection needles had a 1.0 ± 0.2 ±m outer tip and an inner tip diameter of 0.5 μm (Femtotips; Eppendorf). In preliminary experiments, to establish the efficiency of the injection process in delivering molecules into the nucleus and cell viability after injection, SDFs were injected together with rhodamine B isothiocyanate–dextran 10S (RITC–dextran, MW 10,200; Sigma-Aldrich). Observation under a fluorescence microscope revealed that up to 85% of cells remained intact after microinjection and between 77 and 90% of these showed nuclear fluorescence (Table 1).

In each experiment up to 1000 cells were microinjected with 0.7 μl of SDF (~24 ng), corresponding to ~4 x10^7 molecules of SDF per cell. SDF concentration has been exactly calculated with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Cells were then grown and expanded for various periods as indicated, harvested, and stored for analysis. Untransfected cells harvested after the same amounts of time in culture were used as controls.

TABLE 1. MICROINJECTION CONDITIONS FOR HUMAN FOETAL TROPHOBLAST

Sample	*Cells plated*	*Cells injected*	*Viable injections*
CVS01-Mi 01	700	531	389 (73%)
CVS01-Mi 03	1000	976	800 (82%)
CVS01-Mi H_20	700	515	400 (77%)
CVS02	750	630	478 (75%)
CVS03	900	751	600 (80%)
CVS04	850	670	570 (85%)
CVS05	910	790	630 (79%)

The growth rate of rhodamine 3 isothiocyanate-dextran 10s (RITC-Dextrane) - injected, SDF-injected and water-injected controls was similar; 1 population dobling every 24 hours. *Abbreviation*: Mi, microinjection.

Cell transfection by electroporation

Nucleofection (i.e., transfection of DNA directly into the nucleus) of human CVS cells in culture was performed according to the optimized protocol for adult human dermal fibroblasts (NHDF-Adult) developed by Amaxa Biosystems (Cologne, Germany). Human CVS cells were grown in flasks (25 cm2; Corning, Corning, NY) until they reached 80% confluence, corresponding to approximately 5.6 $x10^5$ cells per flask. At this time cells were detached with EDTA–trypsin and resuspended in 100 μl of Nucleofector solution (Amaxa Biosystems), to which 3 μg of pEGFP or 3 µg of SDF-SMN1 (~1.0 $x10^7$ molecules of SDF per cell) was added. Samples were nucleofected with the Amaxa apparatus. To assess the best nucleofection conditions (in terms of transfection efficiency and cell survival), we tested various Amaxa programs (U-23, P-22, G-16, and A-24), using pEGFP DNA. After electroporation, cells were immediately plated, expanded for various periods of time, harvested, and stored for analysis.

The number of SDF per cell was calculated as follows (Maurisse *et al.*2006):

$$(MWbp)(N)/NA = Y \text{ g/SDF},$$

where MWbp = 660 amu/base-pair (bp) = 660 g/mole bp, N = the number of bp/SDF (491-bp), NA = 6.022 x 10^{23} molecules/mole (Avogadro's number).

Therefore,

Y = (660 g/mole bp) (498-bp/SDF molecule)/(6.022 x 10^{23} molecules/mole) = 5.46 x 10^{-19} g/SDF

Thus, 10^7 SDF/cell added 10^6 cells = 10^{13} SDF or (10^{13} SDF) (5.46 x 10^{-19} g/SDF) = 5,46 μg of wt-SDF/10^6 cells.

Analysis of DNA from transfected cells

Genomic DNA from transfected cells was amplified with two oligonucleotide primers (HDNAF and HDNAR) located outside the SDF region. This amplicon (694 bp) was gel purified (DNA gel extraction kit; Millipore) and used as a template for an amplification-refractory mutation system (ARMS)-PCR using hSMN7WT/hSMN7Comm and hSMN7MUT/hSMN7Comm primers, specific for *SMN2* and *SMN1* sequences, respectively (Fig. 1B). The presence of the *Bsm*I restriction site has been demonstrated to be a marker of the SDF-induced recombination event. The reaction was run on a 6% polyacrylamide gel.

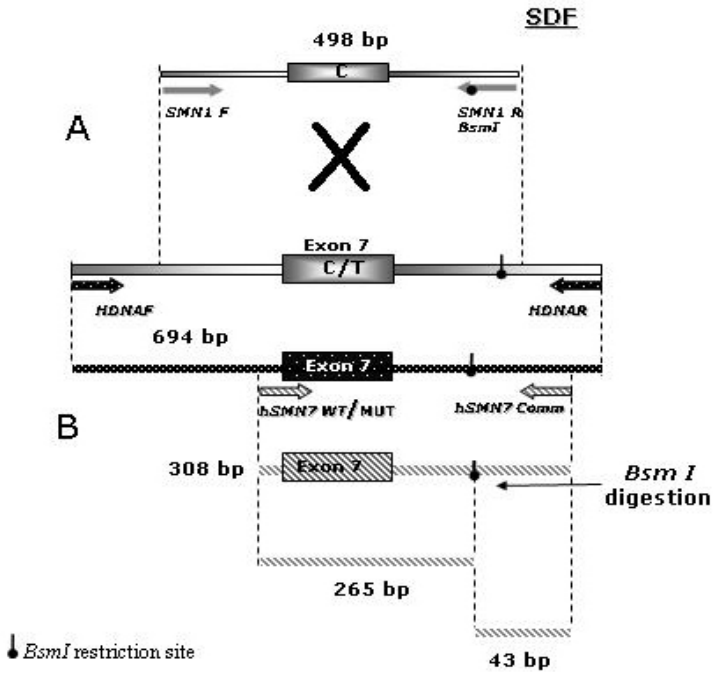

FIG. 1. (A) SDF design and synthesis using two primers (SMN1R *BsmI* and SMN1F) one of which contains a mutation that inserts a *BsmI* site. (B) Analysis of genomic DNA consists of two successive amplification rounds. The first was done using two primers (HDNAF/HDNAR) located outside of SDF and gave a 694 bp-fragment, and the second was developed with an internal allele specific primer ($hSMN_7WT$/ hSMN7Comm - $hSMN_7MUT$ /$hSMN_7Comm$) producing a 308bp-fragment whose specificity was checked by *BsmI* enzymatic restriction, releasing two bands of 265 and 43 bp respectively.

Quantitative expression analysis of SMN transcripts

mRNA was reverse-transcribed to cDNA according to protocols provided with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA USA). 50 µl of 2X RT Master Mix (2X RT buffer, 2X dNTP mixture, 2X random primers, 5U of MultiScribe RT) was added into each tube containing 50 µl of RNA sample (500ng-1500ng). Incubation conditions were 10min at 25° C and 2 hours at 37°C. Real time RT-PCR

was performed on a TaqMAN ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA USA) as previously reported with further modification (Andreassi *et al.*, 2004).

The relative level of expression was calculated from the ratio of SMN1 cycle threshold (Ct) to SMN2 Ct values, both of which were normalized to the control transcript β 2 macroglobulin Ct value using the formula 2 x [ΔCt (SMN1)- ΔCt (SMN2)], in which ΔCt represents the difference in Ct values between the wild-type transcript and the delta7 transcript. All PCR reactions were performed in triplicate.

Western blot analysis

Electroporated or microinjected cells were grown to confluency, rinsed in phosphate-buffered saline (PBS), and scraped from the culture flask in ice-cold lysis buffer (50 m*M* Tris-HCl [pH 7.4], 150 m*M* NaCl, 1% Triton X-100, 1% sodium deoxycholate, 100 m*M* NaF, 0.1% sodium dodecyl sulfate [SDS]) containing freshly added protease inhibitor cocktail (Sigma-Aldrich). Samples were analyzed for protein concentration by Bradford protein assay (Bio-Rad, Hercules, CA). Thirty micrograms of protein resuspended in Laemmli buffer was electrophoresed on a 10% SDS–polyacrylamide gel and subsequently electrotransferred to a nitrocellulose membrane, in accordance with standard procedures. Membranes were incubated in blocking solution (PBS, 3% bovine serum albumin [BSA], 0.1% Tween 20) overnight at 4°C and then incubated with anti-SMN polyclonal antibody, diluted 1:250 in blocking solution, for 1 hr at room temperature. The antibody was produced in rabbit, using 250 _g of purified

peptide (Inbios, Pozzuoli, Italy), the sequence of which corresponds to exon 7 of *SMN*, as reported (Hsieh-Li *et al*., 2000).

The membranes were washed in PBS containing 0.1% Tween 20 and incubated with the secondary antibody (anti-rabbit IgG conjugated to horseradish peroxidase) for 1 hr at room temperature. After extensive washes the immune complexes were revealed by autoradiography, using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences/GE Healthcare, Piscataway, NJ). Blots were reprobed with a mouse polyclonal antiactin antibody, diluted 1:1000 (Sigma-Aldrich). Western blot quantification was performed by scanning the autoradiographs, using a VersaDoc imaging system (Bio-Rad).

Immunofluorescence analysis

Cells grown on glass coverslips were rinsed in PBS and fixed in methanol for 5 min at _20°C. Samples were then rinsed for 30 min at room temperature in PBS containing 3% BSA and 0.1% Triton. Primary antibodies against SMN protein (H195; Santa Cruz Biotechnology, Santa Cruz, CA), pancytokeratins (C-2562; Sigma, St. Louis, MO), and vimentin (V6630; Sigma) were added (diluted 1:150) and applied to the coverslips overnight at 4°C. After extensive washes in PBS, cells were incubated for 1 hr in the dark in the presence of the appropriate secondary antibodies (Molecular Probes, Eugene, OR), diluted 1:800. Last, samples were treated with Hoechst (diluted 1:2000 in PBS) for 5 min to identify nuclei and mounted with a solution of 50% glycerol in PBS. Fluorescence was observed under a Zeiss Axioplan microscope, equipped with a _100 objective.

Negative controls included cells incubated with IgG of the same species as the primary antibodies instead of the primary specific antibodies.

Statistical analysis

Real-time PCR data were analyzed by analysis of variance (ANOVA) (SPSS version 10.0; SPSS, Chicago, IL). For gem number studies, variance between the means was analyzed by paired *t* test analysis (SPSS version 10.0). A *p* value less than 0.05 was considered statistically significant.

RESULTS

Molecular analyses of transfected cells for SMN *transcripts*

Genotypes of the cultured cells obtained from normal and SMA fetal villi were confirmed by DNA diagnostic tests. The latter showed deletion of the *SMN1* locus and retention of the *SMN2* locus. SMA cells were then transfected by either electroporation or microinjection and analyzed in comparison with untransfected cells harvested after the same number of cell doublings. Figure 2A shows the setting of the optimal transfection protocol on CVS01 samples, using electroporation or microinjection. Only one of the four electroporation programs tested, specifically G-16, led to high transfection efficiency (95%) associated with high viability of cells (75%). In fact, G-16 electroporated cells revealed an increase of about 18% in the amount of full-length *SMN* and a 2.1-fold increase in the ratio of full-length *SMN1* to *SMN2* (Δ7) isoforms compared with untreated cells. Conversely, microinjection experiments showed that the amount of full-length *SMN* mRNA increased up to 53% compared with untransfected cells, whereas the full-length: Δ7 isoform ratio increased up to 3.11- fold (Fig. 2A). No modification in transcript levels was observed in cells electroporated according to the U-23, A-24, and P-22 programs and in water-microinjected cells.

These results were confirmed successively in four independent samples of villi transfected under the optimal conditions selected (Fig. 2B).With the exception of CVS05 (see below), microinjection always gave better results than electroporation, underlining the importance of the route of delivery of SDFs into cells. After electroporation, the full-length SMN transcript

increased up to 17% and the full-length: Δ7 ratio reached a value of 2 in CVS04. Microinjected CVS04 cells also gave the maximum value of 20% full-length SMN transcript increase and a 2.29 *SMN1*:_7 ratio (Fig. 2B). In CVS05 cells the situation seemed to be reversed; in fact, the electroporated sample showed an increase in full-length mRNA up to seven times than seen in the microinjected sample. All samples (CVS01 to CVS05) were previously tested for DNA genomic modifications and were positive by *Bsm*I analysis, except for samples electroporated according to U-23, A-24, and P-22 (data not shown).

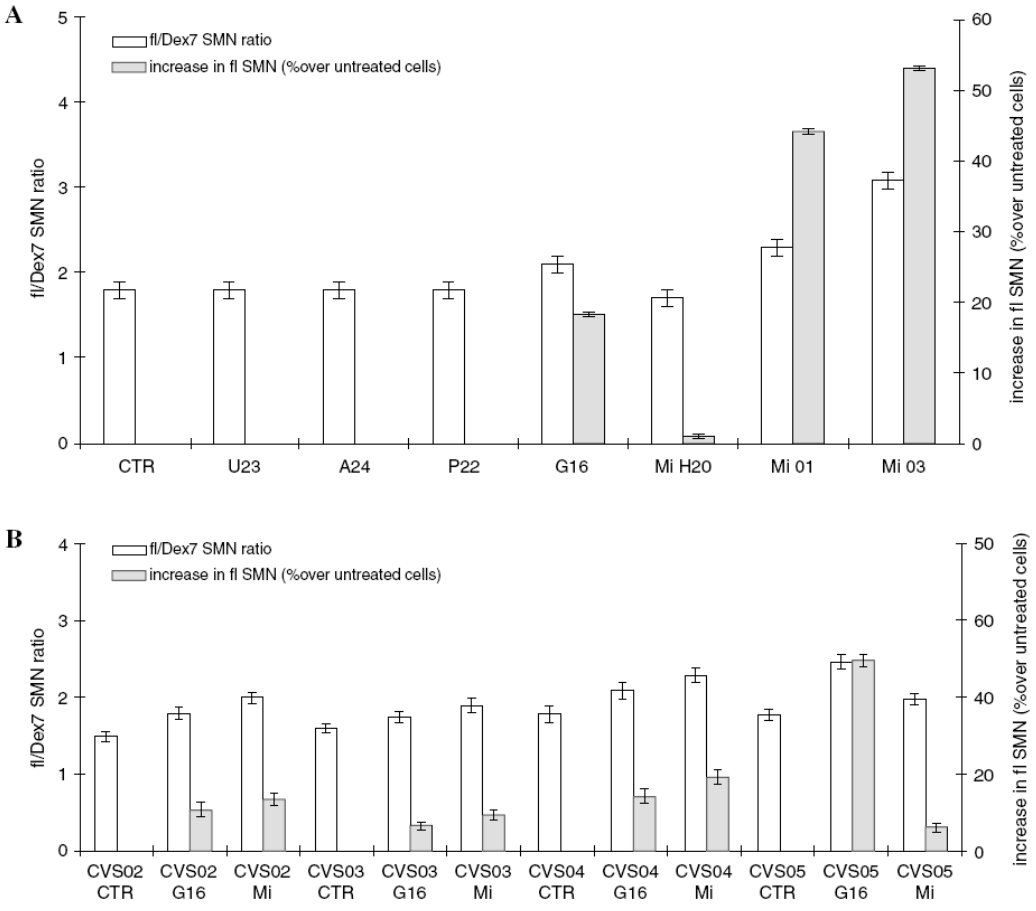

FIG. 2. Quantitative analysis of full length and delta7 SMN mRNA and protein nuclear gem number in CVS01 cells transfected with several electroporation and microinjection protocol (A) and in all other cultured cells (CVS02, CVS03, CVS04, CVS05) transfected by the G16 electroporation program or microinjection (Mi) (B). White bars represent the full-length/delta7 SMN ratio for each transfected sample (left axis). Grey bars represent the increase of SMN full-length amount in transfected cells (right axis). Black triangles indicate the percent increase in of nuclear gems (right axis). CTR = untransfected sample; P22, U23, G16 and A24 indicate different electroporation protocols. Mi01 and Mi03 are two independent microinjected samples. MiH_20 corresponds to a sample microinjected with water. All values obtained from treated cells represent the mean of at least three independent experiments performed in triplicate and were significantly different from those obtained with untreated cells. Bars indicate SD. Samples were always compared at the same number of cell doublings. Gems counts were performed on three different cover slips and two operators evaluated each sample. P value <0.05 was considered statistically significant.

SMN protein expression in transfected cells

We first analyzed cells from wild-type villi and untransfected SMA cells by immunocytochemistry (Table 2). As previously described, the SMN protein was found both in the cytoplasm and in the nucleus of cells as gems (Liu and Dreyfuss, 1996). Unlike wild-type cells, however, in which many gems are localized to the nucleus, in SMA cells few gems were present in the nucleus. In such cells most of the gems were localized in the cytoplasm, likely because of inefficient import of the Δ7 SMN isoform into the nucleus (Liu *et al*., 1997; Patrizi *et al*., 1999). We predicted that corrected SMA cells should produce more full-length SMN protein and, therefore, should have an increased number of gems in the nuclei (Di Donato *et al*., 2003). After transfection, cytoplasmic and nuclear gems were counted and compared between untransfected and transfected SMA cells from all samples of cultured villi (Table 2).

TABLE 2. GEMS COUNTS OF FOETAL TROPHOBLASTS FROM HUMAN PLACENTAL VILLI

Individual	*Phenotype*	*Total N. Cells*	*Nuclei with Gems %*	*Gems/100*	*Cells with cytoplasmic signal %*
CVS - 1	Normal	309	70	1947	0,09
CVS - 2	Normal	301	70	1651	1,04
CVS - 3	Normal	212	70	1483	1,05
CVS - 4	Normal	291	69	1820	0,35
CVS - 5	Normal	200	68	2044	0,35
Untransfected					
CVS - 01	SMA 1	235	42	240	10,09
CVS - 02	SMA 1	213	59	773	8,05
CVS - 03	SMA 1	326	53,9	693	9,02
CVS - 04	SMA 1	234	54,4	247	11,00
CVS - 05	SMA 1	270	61	521	10,03
Transfected					
CVS - 01	SMA-Mi 01	357	48	363	9,04
	SMA-Mi 03	342	53	546	7,09
	SMA-G16	330	59	543	8,04
CVS - 02	SMA-Mi	215	70	1172	5,08
	SMA-G16	193	70	1127	6,01
CVS - 03	SMA-Mi	331	70	1251	4,06
	SMA-G16	330	70	1126	5,05
CVS - 04	SMA-Mi	310	67	597	7,00
	SMA-G16	300	65	385	6,05
CVS - 05	SMA-Mi	299	64	599	7,09
	SMA-G16	305	65	1181	6,06

Gems counts were performed on three different cover slips and two operators evaluated each sample. P value<0,05 was considered statistically significant.

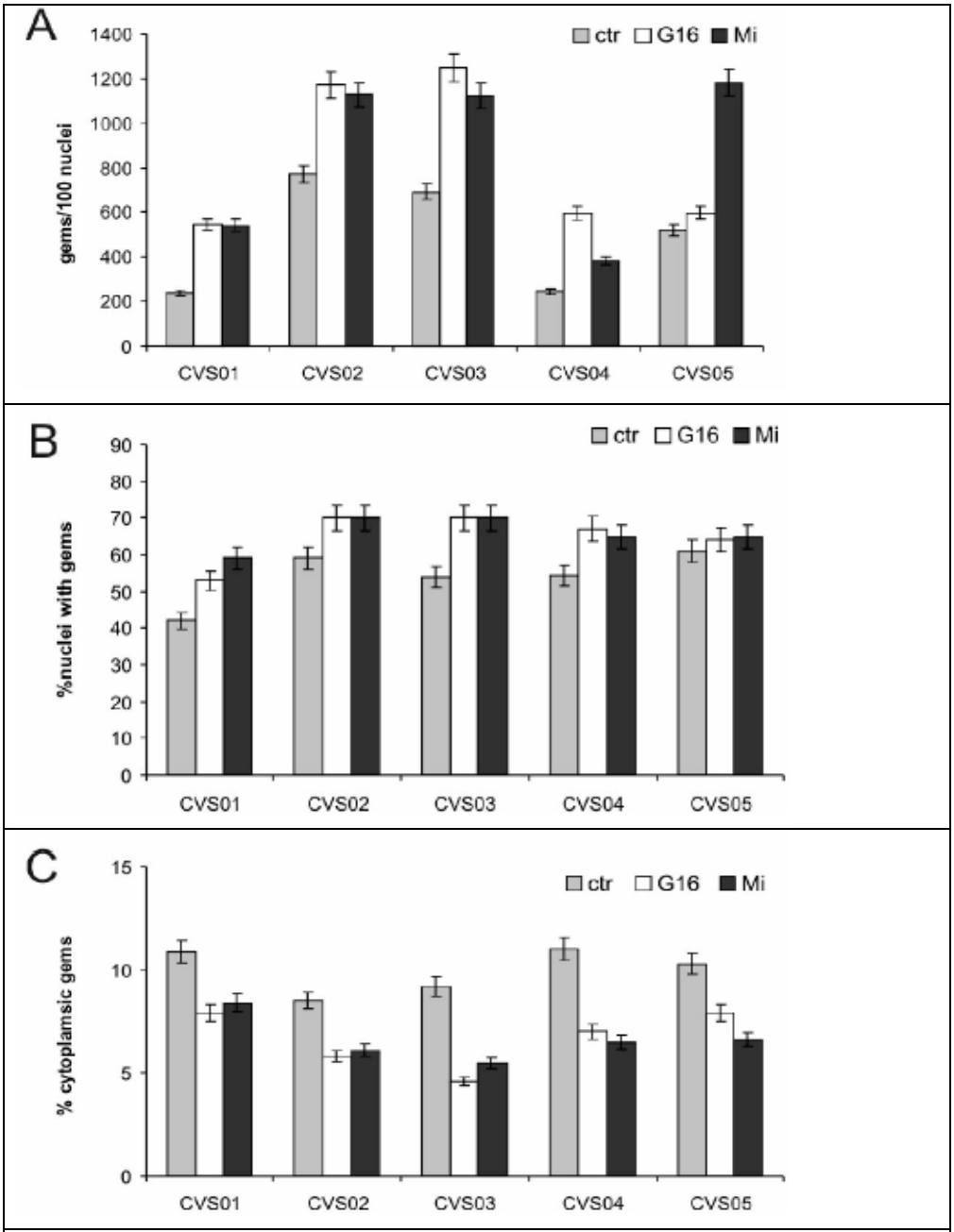

FIG. 4. Evaluation of SMN protein expression in transfected and untransfected cells by gem counts: Gems per 100 nuclei value: Gems per 100 nuclei value (**A**), percentage of nuclei with gems (**B**), and percentage of cytoplasm with gems (**C**) in untreated and treated cells observed by immunolocalization.. Shaded columns correspond to control untransfected samples (ctr), solid columns to G-16-electroporated cells (G-16), and open columns to microinjected cells (Mi). Error bars indicate the SD. Samples were always compared at the same number of cell doublings. Gem counts were performed on three different coverslips and two operators evaluated each sample. Mean values obtained from treated cells (G-16 and Mi) were significantly different from those obtained from untreated cells. $p < 0.05$ was considered statistically significant.

A statistically significant 2-fold increase in the number of gems per 100 nuclei was estimated in all transfected samples, both microinjected and electroporated (Figs. 3 and 4A, and Table 2). Moreover, the increase in nuclear gems was associated with a decrease in cytoplasmic gems in both electroporated and microinjected cells (Fig. 4B and C). These data support the hypothesis that the SFHR-mediated transition leads to an increase in full-length SMN mRNA, which restores appropriate import of the SMN protein into the nucleus. According to the mRNA analyses, no changes in gem numbers or localization were observed in cells electroporated according to other protocols or in water-injected control cells (data not shown).

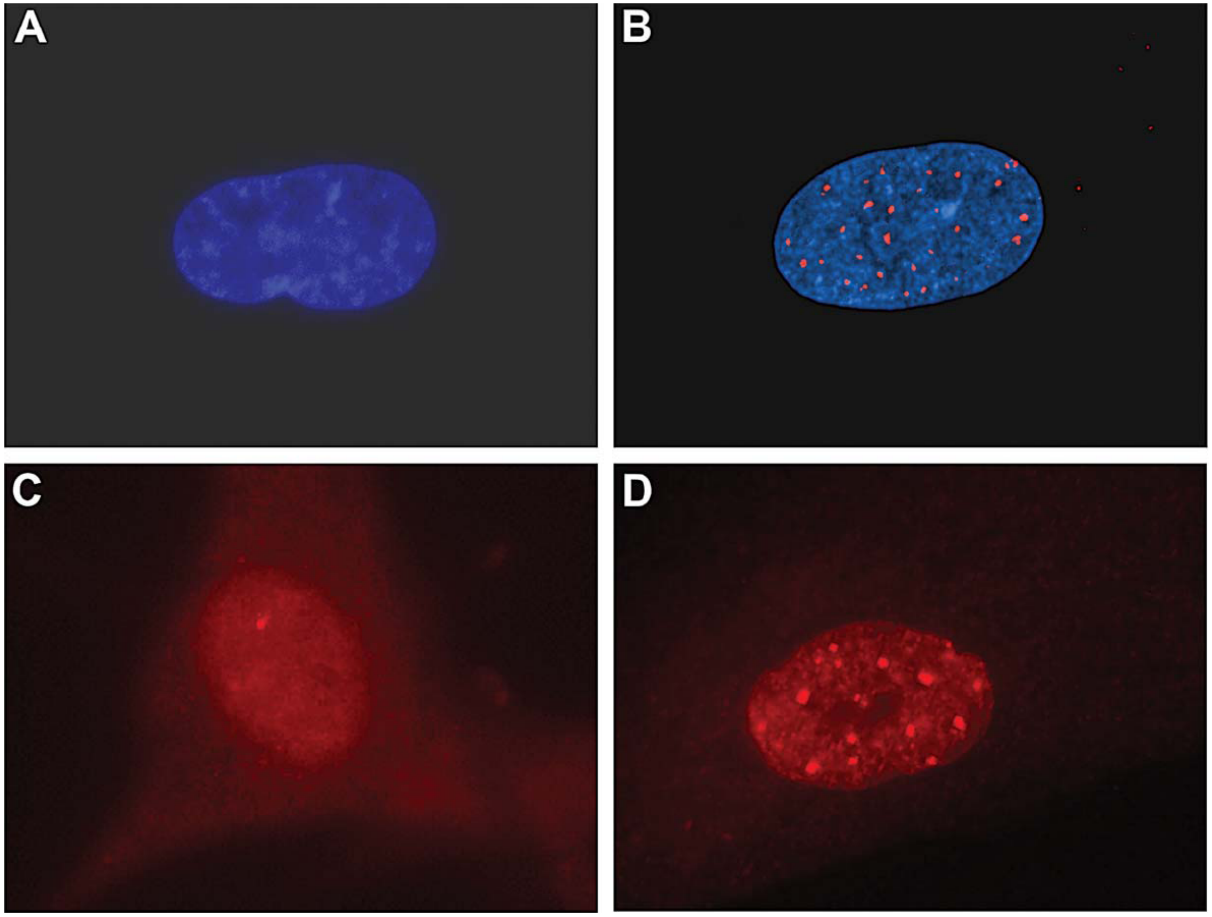

FIG. 3. Immunocytochemistry for SMN protein in untransfected (**A** and **C**), SDF-microinjected (**B**), and electroporated (**D**) SMA cells. In (**A**) and (**B**), nuclei were counterstained with Hoechst.

Western blot analysis

To confirm the increased synthesis of *SMN1* protein, Western blot analysis was performed (Fig. 5A). Because the commercially available antibody was unable to discriminate between SMN isoforms, a polyclonal antiserum was produced against *SMN* exon 7-encoded peptide in order to quantify changes in full-length protein after cell transfection (Hsieh-Li *et al*., 2000). Densitometric analysis of Western blot results (Fig. 5B) showed an SMN1:actin ratio consistent with results obtained in the immunocytochemical analyses. In particular, a 2.3- and 1.7-fold increase in SMN protein level was observed when microinjected (Mi) and electroporated (G-16) cells were compared with their respective untransfected controls (CTR Mi and CTR G-16). Wild-type and SMA samples were also loaded as controls.

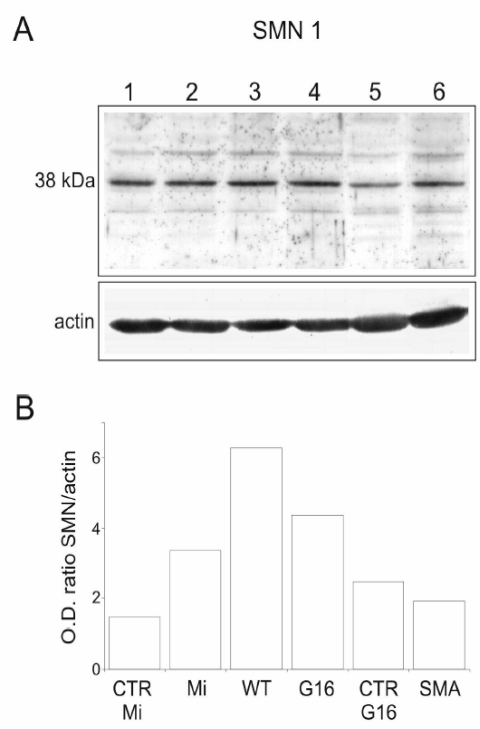

FIG. 5. Western blot analysis of SMN protein in untransfected and transfected cells. (**A**) Expression of SMN was detected with a polyclonal antibody, produced against an *SMN* exon 7-encoded peptide, that is able to recognize only the full-length SMN1 isoform. Lanes 1 and 2, water-microinjected (CTR Mi) and SDF-microinjected (Mi) samples, respectively; lane 3, wild-type (WT) cells; lanes 4 and 5, SDF-electroporated (G-16) and bufferelectroporated (CTR G-16) control, respectively; lane 6, untreated SMA cells (SMA). (**B**) Densitometric analysis was performed, normalizing the SMN value to actin (used as a loading control).

Stability of the genomic modification

To monitor the stability of SFHR-induced genomic modification over time, transfected CVS01 cell cultures were passaged *in vitro* for more than 2 months, equivalent to 45 doublings, and again analyzed both by molecular and biochemical assays. Cells transfected by either electroporation or microinjection maintained higher levels of full-length transcript and protein expression, evaluated by gem count, compared with untransfected controls (data not shown). The persistence of SFHR-induced genomic change was monitored in these cells by *Bsm*I restriction analysis (ARMS-PCR; Fig. 6).

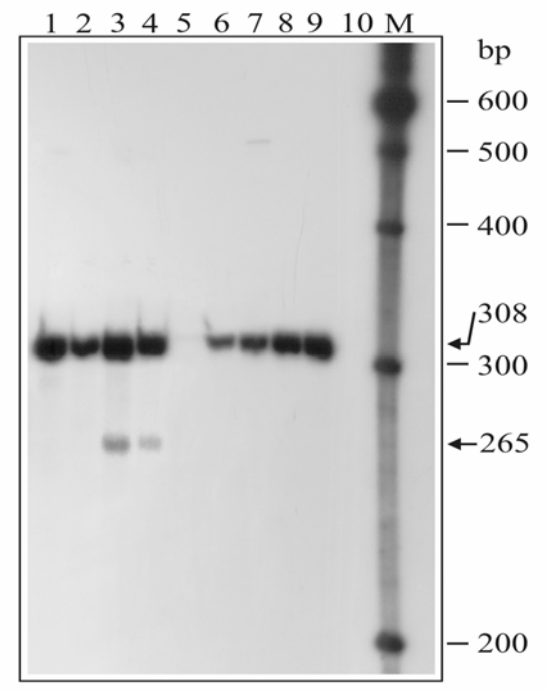

FIG. 6. Allele specific PCR using hSMN7WT/hSMN7Comm primers from genomic DNA of CVS01 transfected cells after 45 cell doublings (two months).
Lane 1 and 2 are amplicon from genomic DNA.
Lane 3 and 4 are the same amplicon of lane 1 and 2, digested by BsmI restriction enzyme.
Lane 5 is the negative control (no DNA).
M: 100 bp ladder.

Moreover, the isogeneic nature of cells cultured *in vitro* for 2 months was detected by DNA fingerprinting analysis, using an AmpF*l*STR Identifiler PCR amplification kit (Applied Biosystems) and genotyping data on an ABI PRISM 310 genetic analyzer (Applied Biosystems). The fingerprint patterns of the transfected cell lines cultivated for 2 months were identical to the starting ones, suggesting that they are isogeneic and derived from untransfected cells (data not shown). We further characterized cells grown in culture for the abovedescribed immunochemistry analysis. To do this, we double stained cultured cells with anti-vimentin and anti-pancytokeratin antibodies to identify mesenchymal-derived and epithelial cells, respectively. Results showed that whereas in primary cultures after 10 days both cytokeratin-positive cells (20–30%) and vimentin-positive cells (60–70%) were present (data not shown), in cells obtained after about 30

passages nearly all of the cells were vimentin positive. Thus, from a heterogeneous primary culture, containing many cell types present in the original tissue, a more homogeneous cell line has been selected in favor of vimentin-positive mesenchymal cells.

DISCUSSION

In humans, two copies of the *SMN* gene, which is the SMAdetermining gene, are present, called *SMN1* and *SMN2*. It is known that the mutated *SMN2* gene is unable to produce sufficient amounts of SMN protein for survival of motor neurons because of a single nucleotide difference in exon 7, resulting in defective splicing of the pre-mRNA and in a protein that is rapidly degraded and therefore unable to form gems efficiently (Le *et al*., 2000; Lorson and Androphy, 2000). Correcting the *SMN2* splicing defect in motor neurons should provide a therapeutic benefit to patients with SMA. In line with this notion, the introduction of different copies of the human *SMN2* gene in knockout mice caused the rescue of the SMA phenotype (Monani *et al*., 2000). Moreover, the possibility of modulating the *SMN* splicing process was demonstrated by generating minigenes containing, or not containing, the splicing enhancer element and expressing those constructs in cell culture or in transgenic mice. Only in the presence of the splicing enhancer element were high levels of SMN detected (Di Donato *et al*., 2001). Three unrelated asymptomatic individuals, with family histories of SMA, were found to have a homozygous deletion of the *SMN1* gene (Prior *et al*., 2004). Quantitative studies indicated that these individuals had increased *SMN2* copy numbers compared with the general population, specifically up to five, supporting the role of *SMN2* in modifying the SMA phenotype. Various experimental approaches have been taken to modulate *SMN2* splicing, such as the use of small molecule modulators and antisense oligonucleotides directed to a splicing silencer in intron 7 or to the intron 7–exon 8 junction (Lim and Hertel, 2001; Miyajima *et al*., 2002). In this paper,

we demonstrate that the splicing pattern of the *SMN2* gene can be modified in embryonic cells, and that the increased incorporation of exon 7 into the transcript can restore protein levels and gem numbers to nearly normal levels. We documented the *in vitro* conversion of the *SMN2* gene into the *SMN1* gene in cells obtained from the fetal villi of SMA embryos, using the SFHR technique. When a 498-bp SDF containing the *SMN1* exon 7 sequence was transfected into these cells, using two different techniques (electroporation and microinjection),

a high increase in full-length transcript was obtained (from 7 to 53%). In terms of protein, this resulted in a 2-fold increase. The relatively high percentage of cells producing high levels of SMN1 protein is likely due to both the recombinogenicity of the *SMN* locus and/or to optimization of the transfection protocols. However, it is also possible that fetal cells are more "receptive" to SFHR than are mature and differentiated cells (Kunzelmann *et al*., 1996). Critical considerations on gene repair strategies, on their occurrence and potency, have emerged, highlighting the necessity to better understand basic mechanisms. The SFHR technique has been the subject of criticism; for example, De Semir and Aran (2003) described how PCR artifacts due to SDF contamination could give false positive results. With this in mind, the methodology used in this study was tailored to validate the protocol used. In fact, we adopted an accurate procedure aimed at avoiding artifacts because the production of SDFs to the analysis of gene conversion. The specificity of the SFHR-mediated conversion was demonstrated by genomic DNA analysis testing the presence of the *Bsm*I restriction site. PCR primers utilized for analysis were outside the region of homology defined by the SDF in order to exclude any artifact from fragment contamination. We estimated that at the time of cell harvest, there was less than the equivalent

of 312,500 fragments per cell, a figure compatible with the absence of PCR artifacts (Kunzelmann *et al*., 1996; Goncz *et al*., 1998, 2001; Gruenert *et al*., 2004). In addition, total RNA was DNase treated before analysis, which was performed with primers located in different exons to avoid any contamination from SDF and from genomic DNA.

However, our study offers a functional evidence of induced genomic change. In fact, a significant increase in SMN functional protein was documented in transfected cells as revealed by both immunocytochemistry and immunoblot analyses. In this respect, the increase in the number of nuclear gems observed in transfected cells provides direct and formal proof of a genetic modification of transfected cells, because only full-length SMN protein is assembled into such structures (Liu and Dreyfuss, 1996; Lefebvre *et al*., 1997). This was corroborated by the use in Western blot analysis of a selective antibody, specifically directed against exon 7, that was able to detect only the wild-type SMN protein. Notably, this is evidence of the stability of the SDF-induced correction of the *SMN* locus and of the maintenance of a wild-type cellular phenotype. This aspect is crucial for future human applications. The principal finding of this paper is that an appropriately designed SFHR protocol can induce a functional and stable genetic modification in fetal SMA cells. This approach offers several advantages, such as stable and long-term expression of the recombinant gene, in comparison with gene complementation strategies based on viral transfection.

Two papers have reported SMN correction using adenovirus-mediated gene delivery (Di Donato *et al*., 2003) or, more recently, lentiviral vectors (Azzouz *et al*., 2004). Although the restoration of cellular phenotype seems to be better than that achieved by SFHR, there are several drawbacks to the

use of viral systems, such as the lack of long-term expression, the position effects on gene expression, and, more important, the presence of viral promoters driving gene expression. The present study performed for the first time on fetal cells highlights the possibility of rescuing an SMA fetus by delivering SDF *in utero* for an early treatment of SMA as proposed (Azzouz *et al*., 2004). We suggest that SDFs, delivered by microinjection, might cure SMA in humans by an *ex vivo* approach.

Abbreviaions:

SMA: Spinal Muscular Atrophy; *SMN1-2*: survival motor neuron gene 1-2; SFHR: small fragment homologous replacement; HR: homologous recombination; SDFs: Small DNA Fragments; CFTR: cystic fibrosis transmembrane conductance regulator, CF: cystic fibrosis, SDF: small DNA fragment, ES cells: embryonic stem cells.

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ORAL CONTRIBUTIONS

Filareto A., Sarra M., Malgieri A. , Bruscia E. , Fina D., Spitalieri P., Monteleone G., Sanchez M., Novelli G., Sangiuolo F. *SFHR GENE TARGETING STRATEGY OPTIMIZATION: useful tools and increased efficiency of repair*. American Society of Gene Therapy 10° Annual Meeting; Seattle, WA - May 30−June 3, 2007

the common bile duct (Type IC). Type II cysts are true diverticula of the common bile duct. Type III cysts, or choledochoceles, are cystic dilations involving only the intraduodenal portion of the common bile duct. Type IV cysts consist of multiple intra- and extrahepatic cysts (Type IVA) or multiple extrahepatic cysts only (Type IVB). Type V cysts comprise single or multiple cystic dilation of the intrahepatic bile ducts.

Prenatal investigation of choledochal cysts using MRI has rarely been described. MacKenzie *et al.* (2001) first reported the use of MRI by demonstrating a fetal choledochal cyst at 16 weeks' gestation. In their report, the second-trimester MRI image of the choledochal cyst was presented as a simple intraabdominal cyst that was not different from the image obtained by prenatal ultrasound. Chen *et al.* (2004) presented the MRI imaging scan of a type IA choledochal cyst at 30 weeks' gestation and provided evidence that MRI is able to present more precise information than ultrasound in the early third trimester. The present case further shows that MRI in the late third trimester is able to demonstrate clearer images of both the extrahepatic biliary duct and the intrahepatic duct and to delineate a more distinctive spatial anatomical relationship of the choledochal cyst with other internal organs such as liver, gallbladder, stomach, intestines, kidney and urinary bladder. The information acquired is very useful for prenatal identification of the type of choledochal cyst and differential diagnosis of other causes of intraabdominal cysts.

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Prenatal diagnosis of spinal muscular atrophy with respiratory distress (SMARD1) in a twin pregnancy

Autosomal recessive spinal muscular atrophy with respiratory distress type 1 (SMARD1; OMIM 604320) is the second anterior horn cell disease in infants in which the genetic defect has been defined. SMARD1 results from mutations in the gene encoding the immunoglobulin μ-binding protein 2 (*IGHMBP2; OMIM 600502*), located on chromosome 11q13 (Grohmann *et al.*, 2001). The disease is characterised by degeneration of α-motoneurons in the anterior horns of the spinal cord, leading to neurogenic muscular atrophy with subsequent symmetrical muscle weakness of the trunk and limbs in infancy (Mellins *et al.*, 1974; Rudnik-Schöneborn *et al.*, 1996; McWilliam *et al.*, 1985; Murphy *et al.*, 1985; Bove and Iannaccone, 1988; Bertini *et al.*, 1989; Grohmann *et al.*, 1999; Fenichel, 2001). Patients show severe respiratory distress due to bilateral diaphragmatic paralysis, and require respiratory assistance in the first weeks of life (Bove and Iannaccone, 1988; Bertini *et al.*, 1989; Novelli *et al.*, 1995). Despite a substantial overlap in clinical features, the phenotypes of SMARD1 versus spinal muscular atrophy (SMA1) infants can be distinguished. In contrast to SMA, distal muscles are more severely affected in SMARD, and life-threatening respiratory distress with clinical and radiological evidence of unilateral or bilateral paralysis of the diaphragm is the most prominent presenting symptom (Mellins *et al.*, 1974; Bertini *et al.*, 1989; Rudnik-Schöneborn *et al.*, 1996; Grohmann *et al.*, 1999; Zerres and Davies, 1999).

As in SMA1, the prognosis of SMARD1 is poor because of acute life-threatening respiratory distress.

Here, we describe a case of prenatal diagnosis of SMARD1 in a pair of twins at 11 weeks of gestation. The parents had one previous child who died at one month of age. He was previously characterised as homozygous for a 1540G→A transition in exon 11, causing a glutamic acid to lysine substitution (E514K) in the *IGHMBP2* gene (Grohmann *et al.*, 2001). The couple at risk for SMARD1 was seen by our genetic service preconceptually, and the mutation was confirmed in our laboratory. They were counselled regarding the potential outcomes of the pregnancy. Because of a

dichorionic twin gestation, two chorionic villi samplings (CVS) were performed.

Molecular analysis for SMARD1 was performed on genomic DNA extracted from CVS using intronic primers designed from the published sequence of the *IGHMBP2* gene (Grohmann *et al.*, 2001): exon 11 forward 5′-ATAGACAGAAACGTGCCCGA-3 and exon 11 reverse 5′-AGCTGCTCTGTAGAGGGACAAA-3′, which amplified exon 11 containing codon 514. PCR was performed in a total volume of 25 µL (200 mM each of four dNTPs, 2.5 mM $MgCl_2$, 0.125 U Taq polymerase, 20 pmol of each primer) and 200 ng genomic DNA. After an initial denaturation of 95 °C for 5 min, 30 cycles were performed of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with a final extension step of 72 °C for 7 min. The amplicon was purified by Seq Prep Kit (Beckmann Coulter, USA) and sequenced using CEQ (Beckmann Coulter, USA) DTCS Quick Start on a CEQ 2000 DNA Analysis System-Automated sequencer (Beckman Coulter, USA), according to the manufacturer's instructions (Figure 1). Sequence analysis revealed the presence of the E514K mutation in heterozygous state in one foetus, while in the other one it showed a homozygote wild-type genotype. To confirm the mutation, a PCR protocol was developed using primers specific for the mutation. In brief, we synthesized the following oligonucleotides: exon 11 reverse primer (11R/5′-AGCTGCTCTGTAGAGGGACAAA-3′) and the altered forward primer (11F/5′-CCTGGATGTGCAAACTGACGAGGCGGACGT-3′; the primer mismatch is underlined). A touch-down PCR programme was performed: 10 cycles (94 °C for 20 s, 10 s at 68 °C to 60 °C, 65 °C for 25 s); 30 cycles (94 °C for 20 s, 58 °C for 10 s, 65 °C for 25 s); 65 °C for 7 min. The reaction was in a total volume of 50 µL (200 mM each of four dNTPs, 2.5 mM $MgCl_2$, 15 pmol of each primer, and 200-ng genomic DNA, using 2.5U HotMaster Taq Polymerase (Eppendorf). The nucleotide variant inserted by the primer, together with the E514K substitution removes an AatII restriction site, otherwise present on wild-type chromosomes. The digestion product was analysed on a 3% nusieve agarose gel. Figure 2 shows the restriction analysis pattern obtained, which confirmed the sequence results. One foetus was confirmed as being heterozygous for E514K with only one chromosome AatII digested and the other one not (Lane 2), while the other foetal DNA had been completely digested confirming a homozygous wild-type genotype (Lane 3). This pattern was identical to wild-type control loaded in Lane 5. No AatII site was present in the SMARD1-affected child (Lane 4). Mutation analysis was confirmed by segregation analysis using a set of 11q microsatellite markers, proximal and distal to the *IGHMBP2* gene, *(D11S1314, D11S4590, RH52011 and RH47911)*. FAM-labelled PCR products were run by the ABI PRISM 310 system (Applied Biosystems, USA), in the presence of ROX fluorescent size markers (Perkin-Elmer Applied Biosystems, USA). GENESCAN 3.1 and GENOTYPER 2 softwares (Perkin-Elmer Applied Biosystems, USA) were used for data collection and allele sizing. The 1211 haplotype linked to the mutation was shown to be inherited from the father by the female foetus, predicted to be a heterozygote, and not by the male foetus, previously characterised as homozygous wild-type (data not

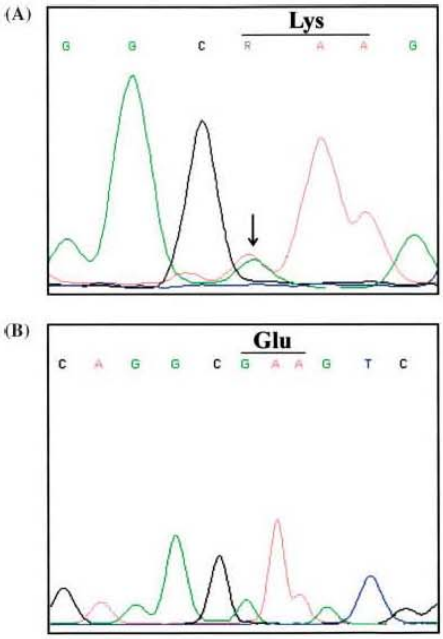

Figure 1—Exon 11 sequence analysis for the E514K mutation of the *IGHMBP2* gene: (A) heterozygous CVS, (B) homozygous wild-type CVS. The G→A transition is indicated by an arrow in the heterozygous foetus

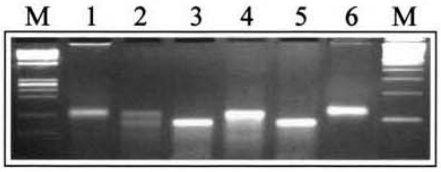

Figure 2—AatII digestion for the E514K mutation on the *IGHMBP2* gene. The E514K mutation removes the AatII recognition site, otherwise present on the wild-type chromosome. M: marker ΦX174/Hae III, Lane 1: undigested PCR product (124 bp), Lanes 2 CVS heterozygous for the E514K mutation with one chromosome digested (two bands: 98 bp and 26 bp) and the chromosome carrying the mutation undigested (124 bp), Lanes 3 CVS with both chromosome wild-type (98- and 26-bp bands), Lane 4 E514K homozygous affected brother (124-bp bands), Lane 5: digested healthy control, Lane 6, undigested PCR products: 100-bp ladder. 26-bp band is not clearly detectable on agarose gel

shown). In addition, CVS DNA samples were used in a multiplex PCR amplification using AmpFISTR Identifier PCR reaction Kit (Applied Biosystems, USA). Genotyping was carried out on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA) using the ABI PRISM 310 data collection software and Genotyper 3.7 analysis software (Applied Biosystems, USA). No maternal contamination was evident since the maternal alleles were not over-represented in the CVS sample.

Karyotype analyses performed on CVS revealed that dichorionic twins were different in sex (46,XX and 46,XY). In the female foetus, we were also able to detect the presence of an extra chromosome marker in a mosaicism condition at the 20% level. Amniocentesis was performed at 17 weeks of gestation and chromosome analysis showed a normal karyotype in both foetuses (46,XX and 46,XY), demonstrating the presence of a confined placental mosaicism evidenced only on cultured CVS cells.

Molecular analysis was again performed on cultured amniocytes confirming the genotypes of both foetuses that showed in the CVS. Two healthy babies were delivered by elective Caesarean section at 38 weeks of gestation, a male homozygous wild-type and a female heterozygous for IGHMBP2 mutation, as confirmed by further molecular tests performed on peripheral blood samples.

This is the first case of molecular genetic prenatal diagnosis of SMARD1 reported to date. SMARD1 diagnosis is of particular importance as it allows accurate genetic counselling and assists in the decision-making process for the initiation of mechanical ventilation of an affected infant.

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Prenatally diagnosed trisomy 6 mosaicism

INTRODUCTION

Chromosomal analysis from chorionic villi or amniotic fluid is being routinely used to determine the chromosomal complement of the fetus. A diagnosis of mosaicism from these cells always poses the problem of predicting the correct fetal karyotype and hence the fetal outcome. The pathological cell lines are present in at least two different culture vessels in level III mosaicism that excludes a cell-culture artifact. The pathological cells might be confined to extraembryonic tissue or might be due to true fetal mosaicism.

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Oligonucleotide-based gene targeting technologies

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The major impact of the human genome sequence is the understanding of disease aetiology with deduced therapy. The completion of this project has shifted the interest from the sequencing and identification of genes to the exploration of gene function, signalling the beginning of the postgenomic era. The goal of all gene therapy protocols is to repair the precise genetic defect without additional modification of the genome. It should involve a lasting and tissue specific expression of the functional gene. Besides, in gene therapy preferably any technology used should fulfil several requirements including safety, simplicity of use, cost effectiveness and amenability to industrial scale. The oligonucleotide-base gene targeting strategies now in use are based on the naturally occurred mechanism of homologous recombination (HR). Simply stated, the process involves targeting the mutation *in situ* for gene correction and for restoration of a normal gene function. HR in eukaryotes is less efficient than in prokaryotic system. Anyway recent advances in gene targeting and novel strategies have led to the suggestion that gene correction based on HR might be used as clinical therapy for genetic disease and used in the cellular genetic therapy approach. Cellular genetic therapy is the ultimate frontier for those pathologies that are the consequent to a specific non-functional cellular type, by the replacement of sick cells with healthy ones, obtained from the same patient or a different donor and previously cured with gene therapy techniques. The purpose of this paper is to review the oligonucleotides based gene targeting technologies: the small fragment homologous recombination (SFHR), the chimeric RNA/DNA oligonucleotides (RDO), triplex forming oligonucleotide (TFO) and the homologous recombination dependent gene targeting (hrdGT). With reference to the naked DNA approach, the recent availability of better delivery methods (*e.g.* electroporation and microinjection) has made the non-viral gene transfer an increasingly more important and viable method for gene therapy.

KEY WORDS: Oligonucleotides - Targeting technologies - Gene transfer.

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The genome-based approaches are aimed to an *in situ* permanent correction of defective endogenous gene (gene targeting approach). These procedures are clearly distinct from the cDNA-based strategies that utilize viral cDNA-based methods by relying on non-viral DNA delivery vehicles. Furthermore, they are preferable to the delivery of such exogenous non-integrating vectors, transiently expressing wild type versions of non functional genes or their cDNA (gene augmentation approach).

Gene therapy has very straight-forward goals to effectively correct the pathogenic phenotype resulting from genetic mutation(s) and to insure that the therapeutic strategy is safe for the patient.

The gene therapy approaches can be broadly divided into viral and non-viral gene transfer technology. Viral vectors take the advantage on the facile integration of the gene of interest into the host and high probability of its long-term expression but are plagued by safety concerns vehicles. The main risk of retrovirus-

mediated gene transfer is insertional mutagenesis resulting from random retroviral integration. In fact, random integration has been reported in viral gene delivery to increase the possibility of gene disruption, including disruption of genes involved in cell cycle or tumour suppression,[1, 2] and as a result gene targeting strategies have being received increasing attention.

Recently the observation that retroviral gene transfer induced T cell leukaemia in 2 children of 10 patients treated for typical X-linked severe combined immunodeficiency (SCID-X1) has raised significant safety concerns for traditional gene strategies.[3-5] Hacein-Bey-Abina *et al.* showed retrovirus vector integration in proximity to the LMO2 proto-oncogene promoter, leading to aberrant transcription and expression of LMO2, suggesting that retrovirus vector insertion can trigger deregulated premalignant cell proliferation with unexpected frequency, most likely driven by retrovirus enhancer activity on the LMO2 gene promoter. Accordingly, the American Food and Drug Administration (FDA) placed on clinical hold all active gene therapy trials using retroviral vectors and suspended the enrolment of new patients in clinical trials that involve the use of retroviruses. Furthermore, in the 2005, another clinical trial reports the failure of retroviral gene therapy in 2 such patients affected by SCID-X1, c-deficiency, despite effective gene transfer to bone marrow CD34+ cells, suggesting that there are intrinsic host-dependent restrictions to efficacy.[6]

Non-viral vectors (naked DNA fragments and plasmid DNA), although less efficient at introducing and maintaining foreign gene expression, have the profound advantage of being non-pathogenic and non-immunogenic.

Above all, gene targeting strategies retain the integrity of the target gene in terms of relationship between the protein coding sequences and gene-expression. By preserving the integrity of the targeted gene, the relationship between the coding sequences and regulatory elements remains intact and the corrected gene would still undergo the cell-specific regulatory elements. Therefore, targeted gene would be expressed at physiological levels in the appropriate cell type. Consequently, cell-specific expression is not altered.

It is, therefore, mandatory to develop vectors with an improved safety profile and/or transfect cells in *ex vivo*, followed by checking them carefully by genetic analysis before infusing back into the patient.

At the present time, the recent availability of better delivery methods (*e.g.* electroporation and microinjection) has made the non-viral gene transfer an increasingly more important and viable method for gene therapy. Therefore, if further developed, gene targeting strategies will gain a higher capacity of correction and will lead to fewer mutagenic side effects than do methods that randomly insert genes into the genome.[7]

Small fragment homologous replacement

Small fragment homologous replacement (SFHR) is a gene repair strategy that involves the introduction of small DNA fragments (SDFs) (up to 1 kb) into cells. These SDFs involve homologous exchange between their sequences and the endogenous (genomic or episomal) ones, resulting in phenotypic changes (Figure1). The technique has been used to modify endogenous genomic DNA in both human and mouse cells. After entering the cells, the fragment pairs with its genomic homologue and replaces the endogenous sequence with the exogenous fragment through an, as yet, undefined mechanism,[8-10] that probably involves similar pathways to those of homologous recombination (HR) and/or uncharacterized pathways of DNA repair.[11] It is likely that SFHR functions by targeting and replacement, involving the necessary proofreading and annealing to homologous target regions, with subsequent strand invasion, and exchange of genetic material.

Typically, small DNA fragments are utilized double stranded although in 2004 Kamiya's group reported a very significant enhancement of the SFHR-mediated gene correction efficiency using a sense ssDNA fragment. In this model, an inactivated Hyg-EGFP fusion gene was utilized in a gene correction assay, obtaining a twelve-fold enhancement in gene correction.[12]

Genotype and phenotype analyses have shown specific modification of disease causing genetic loci and suggested that it has potential as a therapeutic modality for the treatment of inherited disease, if not with a single treatment, with repeated applications. Different kinds of genomic mutations have been altered by using this approach, suggesting a broad range of utility in terms of target genes and cell types able to support SFHR. Moreover, the SFHR technique appears to be effective both *in vivo* and *in vitro*. In fact, genetic modification of several kinds of cells was reported, including mouse embryonic stem cells.[8, 13-15] The recent application of this strategy to stem cell and demonstration that these cells can be efficiently targeted would mean that a single treatment could

correct a genetic defect within a given organ for the lifetime of the patient, using an *ex vivo* strategy.[14, 16, 17] By targeting gene repair to stem cell populations, it is possible that long-term correction might be achieved through clonal expansion.

Different efficiency values of SFHR-mediated modification were reported. This variability can depend from purity of the synthetized olionucleotides and transfection efficiency, specific for each cell line.[18] In this way any improvement in gene delivery will be fundamental to the success of gene repair, especially for somatic gene repair *in vivo* where transfection efficiencies are considerably lower than *in vitro*. The efficiency of transfection could be increased using a different way of delivery of SDF into cell nuclei. Recent studies have reported the successful use of microinjection techniques in progenitor stem hematopoietic cells-HPCs, human lung sarcoma-HT1080, and immortalized lung epithelial-16HBE14o- and primary human fibroblasts and human trophoblast affected by spinal muscular atrophy (SMA).[13, 14, 19] This technique, together with an innovative electroporation protocol, might overcome the inefficient nuclear delivery of DNA that was observed using chemical delivery vehicles. Another factor surely influencing SFHR efficiency is represented by the absence of a selection mechanism to accurately define the genetic changes induced by this strategy. For this purpose a subcloning of the transfected cells by using the limited dilution technique could be attempted,[20] demonstrating the stability of the modification over multiple generations. The SFHR technique has been successfully applied to modify mouse genomic loci, as the cystic fibrosis transmembrane conductance regulator (Cftr) gene, and the dystrophin gene, both *in vitro* and *in vivo*. Phenotypic and genotypic changes have been demonstrated for the Cftr gene, while only genotypic modifications have been shown in mdx mice.[15] A recent study by Goncz *et al.*,[14] has demonstrated the importance of the delivery during the application of SFHR-mediated protocols. The authors used the SFHR technique to introduce in the genome of a wild-type mouse a 3bp deletion within the cftr gene. SDFs were transferred in normal mouse lung via intratracheal instillation, after complexation with 4 different transfecting agents. Detectable levels of sequence alteration were observed, but the data suggests a gradient of these DNA vehicles in terms of reproducibility and specifically, the artificial viral envelope (AVE) gave better results than LipofectAMINE, dimethyl-dioctadecyl-ammonium-bromide (DDAB) and SuperFect. This study underscored the difficulties in extrapolating results from the *in vitro* to *in vivo* settings. A similar approach was used in our laboratory to introduce the same modification in mouse embryonic stem cells (D3) using cationic liposome as the SDFs transfection vehicle (Sangiuolo *et al.*, Cftr gene targeting in mouse embryonic stem cells mediated by small fragment homologous recombination [SFHR]Hum Gene Ther 2005;6. In press). Confocal microscopy

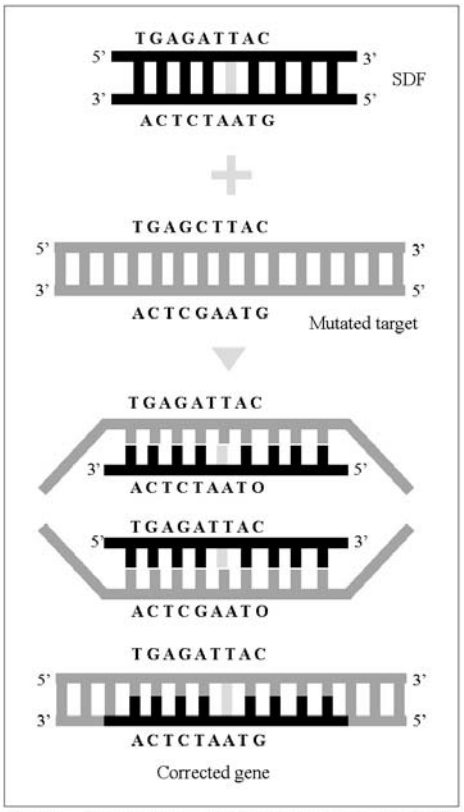

Figure 1.—Small fragments homologous recombination (SFHR). Scheme of the homologous exchange between the SDF sequences introduced into the cell and endogenous target locus.

was carried out to track the entry of Cy-5 labelled SDFs (red) into the nuclei (green). The 3 bp deletion (TTT) was detected at the DNA and mRNA level, after amplicon cloning, to avoid polymerase chain reaction (PCR) artefacts. Modification efficiency of cftr locus was quantified at the mRNA level by using real-time PCR, resulting in more than 5% of transfected cells. In addition, we demonstrated that a mutated gene product was obtained using this technique. This high frequency of selective change in ES cells using SFHR is encouraging for the future development of therapeutic protocols based on cell therapy.

The Mdx mouse model of Duchenne muscular dystrophy (DMD),[15, 21] was also repaired by using SFHR. A nonsense mutation in the dystrophin locus was targeted in both primary myoblast cultures and by direct injection of affected muscle (tibilias anterior). *in vitro* and *in vivo* application of a wild-type SDF (603bp) was used to mediate a T to C conversion in exon 23 of the dystrophin gene. Different conditions in the lipofectamine complex enhanced the efficiency of SFHR-mediated modification *in vitro*. Conversion was observed at both the DNA and RNA levels. The conversion of mdx to wild-type sequence *in vitro* was about 15% by PCR analysis, although there was no detection of normal dystrophin protein. *in vivo* the correction efficiency was up to 0.1% in the tibialis anterior of male mdx mice, but again there was no evidence of gene expression at either the transcript or protein level. It was suggested that the disparity between the genomic repair and protein expression was possibly due to toxicity of the transfected agent on myoblasts, or a delay in protein expression. The correction in myoblasts from mdx mice persisted at least 28 days in culture and up to 3 weeks *in vivo*. These genomic conversion frequencies were lower than those reported for chimeraplasty, which did result in protein expression,[22, 23] after direct injection into muscles of mdx mice. Two weeks after single injections into tibialis anterior muscles, the maximum number of dystrophin-positive fibers in any muscle represented 1-2% of the total number of fibers in that muscle. The expression appears to be stable until 10 weeks after single injections.

Recently, in our laboratory SFHR were used in order to obtain an *in vitro* restoration of SMN protein in human trophoblasts affected by SMA. SMN2-correcting SDFs were transfected *in vitro* into cells obtained from 5 human foetal chorionic villi of embryos, homozygous for SMN1 deletion, by either electroporation or microinjection. Transfected SMA cells showed an increase of up to 53% in full-length SMN mRNA compared to untransfected controls, as detected by real-time PCR. Also immunocytochemistry an immunoblotting analyses revealed a significant 2 times fold increase in SMN protein [Sangiuolo *et al.*, Cftr gene targeting in mouse embryonic stem cells mediated by small fragment homologous recombination (SFHR) Hum Gene Ther 2005;6. In press].

Triplex forming oligonuclotides

Triplex forming oligonucleotides (TFOs) research was largely the concern of DNA structural and physical chemists [24] until automated oligonucleotide synthesis became routine some 30 years after the first description of the structure. Two groups showed that synthetic oligonucleotides could form stable triplex [25, 26] opening the field to a broader group of investigation, including those with biological interest.

Later on it was suggested that TFO could be developed as sequence specific gene targeting reagent in living cells and the same vein as gene specific antisense oligonucleotides.[27-30]

This approach utilizes the ability of single-strand nucleic acid to bind major groove of double helix DNA by Hoogsteen hydrogen bonds in sequence-specific manner and form triple-helix structure (TFOs).[31-33] TFOs, typically from 15 bp to 25 bp long, bind specifically to duplex DNA and provide a strategy for site-directed modification of genomic DNA by forming stable and specific triple helical structures with homopurine/homopyrimidine -rich areas of the genome. The changes of target sequences can be permanent, and, therefore, this strategy has potential as a tool for gene knock-out and/or correction. DNA damaging agents have been successfully coupled to TFOs to induce site-directed DNA damage (Figure 2).[34-36]

The third strand consists of either purines or pyrimidines (depending on the target sequence) and the complex is stabilized by hydrogen bonds between third strand bases and the bases in the strand of the duplex.[37] The purine-motif oligonucleotides can be used for G:C rich targets, but, in most of the cases, pyrimidine-motif oligoucleotides for A:T rich targets are required. In fact, genomic homopurine-rich areas (15-30 bp) occur about every kilobase of genome, and so it is possible that the majority of genes will contain TFO targets.[30, 38, 39] As a consequence, stable triplexes, formed on polypurine:polypyrimidine tracts, are over represented in the human genome, being par-

ticularly common within promoters and introns.[40-42]

To overcome this potential limitation, phosphodiester bonds were replaced with positively charged N,N-diethylethylenediamine linkages, in order to enhance TFOs intracellular activity and allow targeting of relatively short polypurine sites, thereby substantially expanding the number of potential triplex target sites in the genome. This study performed in mouse cells demonstrated that chemically modified TFOs were able to target a shorter (10 bp) site in a chromosomal locus inducing site-specific mutations.[30]

TFOs have successfully produced specific mutations in a variety of somatic cells.[43]

Also, TFOs have been used in combination with mutagenic adjuncts, such as psoralen that induces site specific chromosomal breaks within the genome [44] and, therefore, increasing the HR frequency. An interesting study demonstrated that stable Hprt-deficient clones of CHO cells were created by using psoralen-modified TFOs. In fact, 85% of the 282 analyzed clones contained deletions or insertions within the triplex binding site, indicating a TFO-specific induction of mutagenesis.[45]

In the 2000, Glazer's group demonstrated that TFOs facilitate HR, since TFO binding itself may be mutagenic and recognized as abnormal by repair systems [46] LTK(-) mouse cell line carrying 2 mutant copies of the herpes simplex virus thymidine kinase (TK) gene as direct repeats in a single chromosomal locus were used. The spontaneous frequency of recombination between these repeats (4×10^{-6} under standard culture conditions) can produce a functional TK gene.[44] Nevertheless, the cells microinjected with 30 bp polypyrimidine TFOs specific for a site situated between the 2 TK genes, recombined with a frequency in the range of 1% (2 500-fold above the background). Also, TFOs transfected with cationic lipids also induced recombinants in a highly sequence-specific manner but were less effective, reporting recombination frequencies of six- to seven-fold over background.

In the same year Vasquez *et al.* demonstrated that TFOs can be exploited to induce mutations at specific genomic sites in somatic cells of adult mice. Mutation frequency in supF gene was about five-fold greater than mice treated with a scrambled sequence control oligomer, demonstrating the site-specific genome modification by TFOs in mouse.[47] Alterna-tively, the replacement of deoxyribose rings characteristic of DNA with a morpholine ring (MOR) in the TFOs was demonstrated to induce the formation of pyrimidine motif triplex at neutral pH, with an increase of 60 times compared with unmodified TFOs. Therefore, MOR backbone modification of TFO could be a key modification and may eventually lead to progress in therapeutic applications of the antigene strategy *in vivo*.[48]

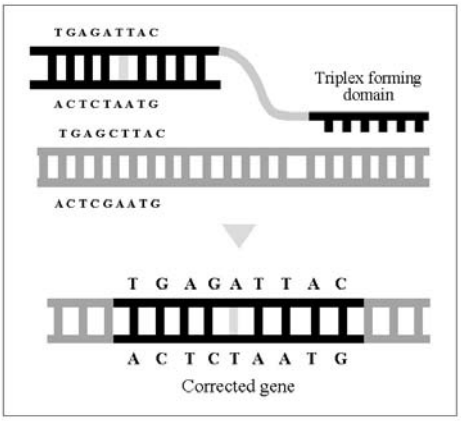

Figure 2.—TFO model. Schematic representation of the triple-helix region formation between the exogenous sequence and the complementary genomic locus.

Chimeric RNA/DNA oligonucleotides

Chimeraplasts or chimeric RNA/DNA oligonucleotides (RDO) are designed to target a homologous genomic sequence and induce a site-specific base change.[49-51] The chimera is a single-stranded molecule, usually 70-80 bases in length. These 68-mers contain a mutator region of 5 DNA nucleotides complementary to the target sequence, except for the targeted point mutation flanked by 2 blocks of 2'-O-methyl RNA residues of 10 nucleotides each, also complementary to the target locus.

The 2'-O-methylation serves as protection from nucleases present in the cell nuclei. A nick is embedded in the duplex by extending the 3' end of the oligonucleotide to form a 5-bp-long GC clamp bridged by 4 T residues (a T-loop) at the end. The nick is provided as a swivel to permit topological intertwining of the RDO into the target double stranded DNA. It is recessed within the duplex to enhance thermal stability and nuclease resistance (Figure 3). The successful repair

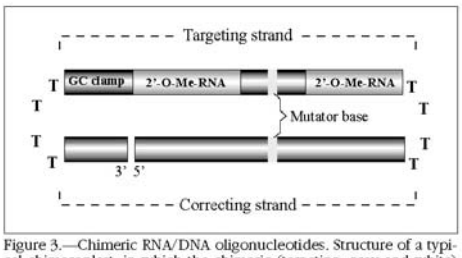

Figure 3.—Chimeric RNA/DNA oligonucleotides. Structure of a typical chimeroplast, in which the chimeric (targeting, grey and white) strand is entirely base-paired to the all-DNA (correcting) strand (white).

of the chimeraplast may depend entirely on mismatch affinity [52] and protein recognition, and so the secondary structure, the quality of synthesis and subsequent purification, the surrounding genomic sequence, and the transfection efficiency all influence the conversion rates.

In an elegant study, melanocytes from albino mice were isolated and transfected with chimeraplasts to repair the genetic defect, a point mutation in the tyrosinase gene that is an essential enzyme in melanin synthesis.[52]

Genes have been targeted in various tissues including liver, muscle, and skin. Receptor-mediated transfer of RDOs into mice liver has been used to introduce a specific mutation in the factor IX gene in approximately 40% of the liver cells,[54] and for the correction of a mutation in the Gunn rat model of Crigler-Najjar syndrome type 1.[55]

In both cases the nucleotide conversion was confirmed at the protein level. The ApoE2 allele gene has been changed to ApoE3 in approximately 25% of liver cells by intraperitoneal injection of RDOs complexed with polyethylenimine.[7, 56] In mouse and canine models of Duchenne's muscular dystrophy injections of RDOs into muscle tissue led to the repair of a dystrophin point mutation with a frequency of 1-2% and 10%, respectively.[22, 23] Skin has been targeted by topical application and intradermal injection of RDOs in albino mice. By this approach a point mutation in the tyrosinase gene was corrected leading to restored pigmentation.[57] Finally, a mutation associated with carbonic anhydrase II-deficiency has been corrected in the kidney.[58, 59]

RDO-mediated gene correction has been achieved in plants as well. Introduction of point mutations in specific plant genes leading to herbicide resistance has been accomplished *in vivo* in both tobacco [60] and maize plants.[61, 62] Surprisingly, mixed base sequences other than the predicted nucleotide conversion have been observed, suggesting a plant-specific repair mechanism different from the high-fidelity repair observed in the mammalian system. This unspecificity has later been confirmed in an *in vitro* study using a tobacco cell-free extract.[63]

Although successful chimeraplasty was demonstrated in a variety of tissues, curiously little data are available for stem cells.

A major criticism *versus* this technique is that the high conversion frequencies observed in some experiments, are consequence of PCR artefacts and/or cell to cell contamination.[64, 65] Many scientists believed that the results were most likely art factual, predicting that no one would be able to use it. The question of reproducibility has plagued these protocols, especially when the issue of the high frequencies of gene repair was discussed. In fact the main problem is centred on the variability among the data of different research groups, varying from 0.5% to 20% even within the same laboratory.[66-68] However, it may be of general concern that a broad application of this technique is still to be awaited, despite the number and the extent of positive reports.

The synthesis and purification of the RDOs is complex and costly. In attempts to improve and analyze the gene correction activity, recent studies have focused on the use of oligonucleotides with modifications to the original RDO design (Figure 1B-D). An RDO with a mismatched base only in the lower all-DNA strand [69] as well as ssODNs with varying length (25-61 nucleotides) and polarity [70-72] have been utilized in gene correction experiments. The ssODNs utilized either contain 4 terminal 2'-O-methyl uracil or terminal phosphorothioate linkages for nuclease protection.

Delivery systems

Certainly one of the deepest effects on the targeting ratio is the method of delivery of the vector DNA. Aware of that, scientists attempted different gene-transfection methods during the past years and parallel studies, meant to increase the efficiency of the most promising ones, were carried on. At the present time the 3 most common techniques still are liposome-mediated delivery, microinjection and electroporation, therefore

now most of the efforts have shifted to improving their efficacy both for *in vitro* and *in vivo* applications.

Cationic-liposome delivery

The cationic-liposome based gene delivery, first reported by Felgner in 1987,[73] allows transfer of genetic material trough the cell-membrane and its subsequent release into the cytoplasm. Once inside, the DNA is thought to pass into the nucleus, maybe by physical association with the chromatin during mitosis or by crossing the nuclear membrane. Initially the negatively charged DNA is electro-statically bound to the liposome in a lipocomplex that is able to associate with the cellular membrane (negatively-charged) and is then up-taken by the cell. Although both the uptake and the release mechanisms have not been completely clarified yet, it has been claim that, maybe, endocytosis and/or lipocomplex fusion with the membrane permit the liposome entrance into the cell, and that once inside the DNA could be released by mixing of the lipid membranes with the liposomes and the consequent disruption of the latter.[74]

Cationic liposomes (CL) are wildly used as delivery system in mammals,[75] although they show a general high toxicity to cells. In fact, CLs are membrane active elements, that is to that they can hamper the membrane functions and equilibrium.[76]

This was recently confirmed by transfection experiments on salmonid cell lines of hepatocyte and macrophage origin.[77] Those observations lead to the conclusion that differences in the transfection efficiency and in CL toxicity-levels between the cell lines had to be mainly addressed to their different membrane compositions, although in the above mentioned cell lines the CL cytotoxicity increased with the increasing lipid concentration.

Furthermore, in general, different results suggest that the DNA/liposome mixing ratio significantly affects the intracellular trafficking of plasmid DNA complexed with the cationic liposomes.[78] Nevertheless liposome transfer has several advantages: they can be used both for *in vitro* and *in vivo* transfections, are non –immunogenic, can transfer any type of nucleic acids and their use and preparation is relatively simple.

Microinjection

Another way of physically introducing DNA into a cell is through microinjection that consists in directly injecting foreign DNA into cells. A cell positioned under a microscope, is manipulated to a blunt capillary and the DNA or RNA is inserted into the cytoplasm or nucleus.

Microinjection has been successfully used with large frog eggs, cultured mammalian cells, mammalian embryos, and plant protoplasts and tissues.

Examples of its application to transfer in the cells molecules capable of either transitorily compensate mutated gene or directly repair mutations on the chromosomal DNA, is reviewed by Davis *et al.* In particular, the work focuses on glass needle-mediated microinjection as a method for the delivery of genetic material into blood stem cells.[79]

An illustration of the use of the microinjection technique was reported in a recent study where the efficiency of correction of 2 differently designed chimeraplasts were compared.[13] These constructs were evaluated for their ability to correct a point mutation in the gene encoding recombinant enhanced green fluorescent protein (eGFP) that rendered the protein non fluorescent. To realize this, a plasmid encoding this mutant eGFP gene and a chimeraplast were co-introduced directly into the nuclei of primary fibroblasts by microinjection.[80]

Electroporation

Electroporation is an efficient method that can be used both *in vitro* and *in vivo* to transfer physiologically the gene into the cell without complicated preparations.

This technique is based on the application of external electric fields that results in transient, reversible breakdown of the cell membrane. During the process, the membrane becomes extremely conductive and as a consequence of the current passage inside the cell, is achieved formation of pores, trough which small molecules are transferred into the cytosol.

Developments over the past decades have led to sophistication of equipment and optimization of protocols; among all the different successful innovation, the most interesting one is the electroporation of small molecules delivered directly inside the nucleus, using the new Amaxa nucleofector technology that is able to introduce the genetic material directly into the nucleus. This reduces assay time to a couple of hours *versus* 24 to 48 h for standard transfection methods that use liposome mediated delivery. Ideally the system is matched for selected primary cell populations (includ-

ing hematopoetic cells, chondrocytes, cardiomyocytes, endothelial cells, keratinocytes, stem cells, neurons, and dendritic cells [DC]). Furthermore it is not restricted to DNA only but it can also be used for transferring siRNA (small interference RNA) into cells, especially primary cells. The only drawbacks to the machine are that not optimized cell systems require a protocol optimization and in general it must be used trial and error on each of the programs provided. Very recently the nucleofector method was exploited to transfect different cell lines. It showed a transfection frequency higher than 50% used in nature killer (NK) cells,[81, 82] 60% in DC cells.[83] Transfection of primary cells and stem cells, a problem in the laboratory routine since most methods working effectively for cell lines in culture fail to transfect them, has been achieved as well by Hamm *et al.*[84]

Gene targeting by homologous recombination

The current gene therapy approaches are aimed to an *in situ* modification at the chromosomal loci of point mutations, nucleotide deletions and/or additions.

Overall, such repair systems are all based on the pairing of an extra-chromosomal DNA fragment with a chromosomal DNA molecule, and from different observations it seems that recombination event and then a mismatch correction of the newly formed DNA heteroduplexes.

HR is a natural mechanism found in all the organisms, from a molecular point of view, it is a multi step process during which 2 homologous DNA sequences pair and perform an exchange of genetic material.

Most likely the process is similar in lower and higher organisms but its complexity gradually increases going from eubacteria to mammalian cells, as well as the genetic control of the HR process. Furthermore, it seems that both in eubacteria and eukaryotes HR is sensible to the length of homology shared between the donor and the receiver DNA and to the perfection of homology.[85, 86]

Studies made on *E. coli* disclosed most of the information about the HR mechanism,[87] which can be exemplified dividing it in 6 successive steps.

The process seems to be promoted particularly when double strand breaks (DBSs) are indistinctively produced on the donor or the recipient DNA. This HR enhancement caused by existing DBSs has been confirmed also in higher eukaryotes.[88, 89] For instance, double strand breaks provoked in the tobacco genome by transient expression of the *E. coli* restriction enzyme I-SceI resulted in a HR-directed gene targeting frequency of up to 10^{-2} in this site.[90]

Therefore, it is probably correct to assess the DBSs formation as a preliminary step for HR events (step 1).

In the cell, starting from the DBSs encountered, a specific set of enzymes (a rare 5'-exonuclease maybe together with a helicase) produces 3'-terminal single-stranded DNA overhangs (ssDNA), (step 2). At this point an active nucleoprotein filament can be established by ssDNA strands and a RecA-type protein coupled together. This enzymatic complex produces strand exchange and heteroduplex DNA formation (step 3) by searching for homology within different DNA filaments and switching over combinations between the donor and the recipient DNA. Successively, other intermediate formations, known as Holiday structures, are induced by the gap repairing activity of different cellular enzymes (step 4). In step 5 branch migration proteins can move around and also extend the Holiday structures which, in the successive step are finally cleaved by a DNA resolvase enzyme (step 6).

The cleavage can proceed in 2 different manners therefore giving different recombinant double stranded DNA (dsDNA). Although these dsDNA contain region of heteroduplex DNA, not always it is possible to find areas on imperfect homology within them. Anyway, in the latter case the consequent repair system take action, playing a positive or negative selection. Since it corrects 1 of the 2 mismatching nucleo-tides, either it will revert the newly introduced nucleotide to the original chromosome sequence (negative selection) or insert a point mutation by correcting the chromosomal original nucleotide (positive selection).

For the gene targeting in eukaryotes such as plants and mammals, HR does not seem to be of simple application. A series of studies have been carried out on homologous recombination dependent gene targeting (hrdGT) in plants.[91, 92] Among several, Miao *et al.* successfully disrupted a non-selectable gene in Arabidopsis thaliana by creating a mutant of the TGA3 locus via targeted insertion of a gene conferring kanamycin (Km) resistance.[93] In this case the hrdGT frequency obtained was in a range of 3.8×10^{-4} events that, together with the frequency of almost 1×10^{-3} reported yet again in Arabidopsis by Kempin *et al.*,[94] represent the 2 highest hrdGT mediated gene-correction frequencies in plants. The average number of

targeted events in plants overall is, in fact, very low, yielding 1×10^{-5}, 10^{-6} or even fewer transformants.

It is generally believed that the HR-mediated gene targeting application is rather hampered by the high frequency of non-homologous end-joint events (NHEJ), more than by the low frequency of HR. In fact, NHEJ takes place in about one cell per 10^2 to 10^4 treated cells, with a frequency 1 000-fold higher than the HR frequency. As a result, targeted recombinants are often covered by random integrants.[39]

Therefore, scientists have attempted different strategies to enrich the number of targeted events, whose effect was however only quite limited. Neither the extension of the length of homology up to 23 kb or the use of a positive-negative selection brought to a significant improvement.[95, 96] An exceptional is the predominantly haploid moss Physcomitrella patents, in which a conversion efficiency of more than 90% was successfully obtained.[97-99]

In mammalian cells HR-mediated gene targeting has been exploited for more than 10 years, allowing the creation of transgenic mice as models for human diseases and helping understanding gene function.

Pioneering experiments in vertebrate cells were independently realized by Smithies and Capecchi. It was firstly established that desired modifications could be obtained in mammalian cells by HR; in fact, transfection rate of 10^{-3} was achieved when a plasmid carrying the globulin gene sequences integrated into the globin locus.[99]

In embryo-derived stem (ES) cells, site-directed mutagenesis by gene targeting was proved by Thomas and Capecchi to mutate the endogenous hypoxanthine phosphoribosyl transferase (HPRT) with an estimated ration of 1:1000.[100] Also, this work demonstrated that targeting efficiency of the recombinant vectors was strongly dependent upon the length of the homology shared between exogenous and chromosomal DNA. This date was also confirmed by recent *in vitro* knock-out experiments in human stem cells.[101]

From these data it is clear that the efficiency of gene targeting is not a fixed quantity but it varies in relation with different genetic environments, utilization of different vectors, various delivery methods and finally currently investigated alternative technologies.

The repair of point mutations directed by modified single-stranded DNA oligonucleotides is dependent on the activity of proteins involved in HR. As a consequence, factors that stimulate HR, such as double strand breaks, can impact the frequency with which repair occurs. Recently, another set of experiments aims to increase the cellular HR event frequency either by overexpressing the proteins involved in the HR process or transfecting the cells with molecules known to modify the HR mechanism and improve HR against NHEJ.

In mammalian cells, HR induced by altered replication activity, is dependent on Rad51, a member of the Rad52 epistasis group.[102]

Also, in both yeast and mammalian cells, the mechanism of gene repair likely involves many of the members of the RAD52 epistasis group. In fact, they appear critical for the successful completion of this reaction.[103, 104] Haber's group also demonstrated that repair of a double strand break by HR requires binding of the strand exchange protein Rad51p to ssDNA, followed by synapsis with a homologous donor. The Rad51p filament is required to search the genome for homologous sequences. Rad51p binding to ssDNA requires Rad52p protein, and the absence of Rad55p delays Rad51p binding to ssDNA and prevents its strand invasion and localization. Lack of Rad54p does not significantly impair Rad51p recruitment and its initial association, however Rad54p is required at or before the initiation of DNA synthesis after synapsis has occurred at the 3' end of the invading strand.[103]

It seems logical therefore that increasing the cellular concentrations of these proteins would elevate the frequency of gene repair. On the other hand, probably the over expression of only a few of these proteins may not lead to a significant stimulation in gene repair because the activity of this pathway requires that the proteins be present and functional in exacting stechiometries. Thus, a global stimulation of the HR pathway may be necessary to achieve high correction levels.

Data presented in 2 very recent papers now suggest that this could be achieved by lengthening the time in which the cells transit through S phase or by reducing the rate of replication fork movement.

Wu *et al.* systematically investigated the single strand oligonucleotides (SSO)-mediated gene repair at various phases of the cell cycle in a mammalian cell line and found that the efficiency of SSO-mediated gene repair was elevated by '10-fold in thymidine-treated S-phase cells. The increase in repair frequency correlated positively with the duration of SSO_thymidine coincubation with host cells after transfection. The paper supply evidence suggesting that these increased repair frequencies arise from a thymidine-induced slowdown of replication fork progression. Furthermore the study provides fresh insight into the mechanism of SSO-mediated gene repair in mammalian cells and

demonstrate how its efficiency may be reliably and substantially increased.[105]

Brachman *et al.* demonstrated that the stalling of replication forks can also activate the gene repair pathway and lead to an enhanced level of nucleotide exchange. The mammalian cell line, DLD-1, containing an integrated mutant eGFP gene, was used as an assay system to explore how replication fork activity affects the overall repair reaction. The addition of 2, 3-dideoxycytidine (ddC), a nucleoside analog that retards the rate of elongation and effectively stalls the replication fork, results in a lengthened S phase and an increased number of gene repair events. Overall, their results indicate that an expansion of S phase and a transient stalling of replication forks can increase the frequency of targeted gene repair.[106]

Finally, an alternative solution against the low HR recombination efficiency in gene therapy was reported by Holmes'group. Two fundamental processes, DNA recognition by C_2H_2 zinc-finger proteins and homology-directed repair of DNA double-strand breaks, were used combined together to correct an X-linked SCID mutation in the IL2Rγ. Remarkably, the *in vitro* use of this new technique in K562 cell line yielded more than 18% of gene-modified human cells without selection, and about 7% of the cells acquired the desiderate genetic modification on both the X chromosomes.[107]

Conclusions

The potential applicability of targeted gene conversion is immense. It is imperative that gene repair becomes an efficient and reproducible strategy before applying it to clinical medicine. The technique holds promise not only as a potential gene therapy approach towards genetic diseases caused by point mutations but also as a tool for functional genomics in defining gene function. Furthermore, it is particularly intriguing the possibility of the stem cells gene-targeting, that is a main objective in both therapeutic application and functional genomics. However, the obstacles associated with this technique are still many and, although many studies have focused on unravelling the mechanism of action, numerous steps in the gene correction process are still unknown. In fact, the precise model for heteroduplex formation, the exact process of repair pathway activation, further identification of involved key proteins, and mismatch type dependency and sequence context effects need further attention. In particular, the limitations of the technique have, to a large extent, been ascribed to poor delivery system efficiencies. Therefore, understanding the mechanisms underlying efficiency, efficacy, and specificity of gene correction will be paramount to the wider application of these molecules in functional genomics and ultimately as therapeutic agents.

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In Vitro Restoration of Functional SMN Protein in Human Trophoblast Cells Affected by Spinal Muscular Atrophy by Small Fragment Homologous Replacement

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ABSTRACT

The majority of patients affected by spinal muscular atrophy (SMA) have deletion of the *survival of motor neuron 1* (*SMN1*) gene, but they retain a "nonfunctional" copy of the duplicate gene (*SMN2*) in their genome. *SMN2* produces defective SMN protein because of a C → T transition in exon 7, which causes the skipping of exon 7 during SMN mRNA maturation. Many attempts have been made to correct altered *SMN* gene expression and to increase the level of normal SMN protein, but to date an effective treatment for this disease has not been established. Small Fragment Homologous Replacement (SFHR) is a site-specific gene modification approach that has the potential to maintain the genomic organization necessary for expression. The target modification in the genome is mediated by small DNA fragments (SDFs) 400–800 bp in length. In this study we used SFHR to induce a T → C transition at codon 280 in exon 7 of the *SMN2* gene in order to produce an increase in functional SMN protein. SDFs were transfected *in vitro* into cells obtained from five human fetal chorionic villi of embryos, homozygous for the *SMN1* deletion, by either electroporation or microinjection. Transfected SMA cells showed an increase of up to 53% in full-length SMN mRNA compared with untransfected controls, as detected by real-time polymerase chain reaction. Consistent with the RNA data, immunocytochemistry and immunoblotting revealed a significant 2-fold increase in wild-type SMN protein. Furthermore, genotype and phenotype of transfected cells remained stable after several *in vitro* passages, demonstrating the stability of the correction over time.

OVERVIEW SUMMARY

Spinal muscular atrophy (SMA), a severe neuromuscular disease characterized by degeneration of α motor neurons in the spinal cord, has an estimated incidence of 1 in 6000–10,000 live births. In the present paper, we investigated whether Small Fragment Homologous Replacement (SFHR) can restore the SMA phenotype in human fetal cells. After transfection of a 498-bp DNA fragment homologous to the *survival of motor neuron 1* (*SMN1*) sequence, significantly increased levels of both SMN full-length mRNA and protein were detected. Genomic modification remained stable after 45-fold cell population doublings. These results demonstrate for the first time that expression of functional SMN can be restored in human fetal cells by target modification of the genomic DNA and suggest a potential use for such methods in rescuing the SMA phenotype.

INTRODUCTION

SPINAL MUSCULAR ATROPHY (SMA; OMIM 601627) is a common recessive autosomal disorder that results in destruction of motor neurons in the anterior horn of the spinal cord

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(Dubowitz, 1995). Motor neuron degeneration affects voluntary muscles and causes proximal symmetrical weakness and atrophy of the limbs and trunk. SMA is the second most common fatal autosomal recessive disorder after cystic fibrosis, with a carrier frequency of 1 in 40 (Pearn, 1980), and is clinically subdivided into three groups on the basis of age of onset and clinical course (Munsat and Davies, 1992). SMA I is the most severe, with an onset before 3 months and death from respiratory failure occurring within 2 years. SMA II and SMA III represent milder forms. Adult-onset SMA, variably referred to as type IIIb or type IV, although less frequent, has also been reported. However, the large majority of SMA patients have onset of symptoms in infancy or early childhood.

The *survival of motor neuron* (*SMN*) gene has been shown to be the SMA-determining gene (Lefebvre *et al.*, 1995). Two SMN genes are present on chromosome 5q13: the telomeric or *SMN1* gene and the centromeric or *SMN2* gene (Melki *et al.*, 1994; Lefebvre *et al.*, 1995; Burglen *et al.*, 1996). All forms of SMA are caused by homozygous loss of the *SMN1* gene as a result of gene deletion, conversion, or mutation. In about 95% of affected patients exon 7 of the *SMN1* gene is absent, and other, more subtle mutations have been identified in the remaining affected patients (Melki *et al.*, 1994).

SMN2 copy number influences the severity of the disease (Campbell *et al.*, 1997; McAndrew *et al.*, 1997; Wirth *et al.*, 1999; Feldkotter *et al.*, 2002; Mailman *et al.*, 2002). Patients with a milder form of SMA, type II or III, have been shown to have more copies of *SMN2* than do type I patients. Eleven nucleotide differences exist between *SMN1* and *SMN2* transcripts, but none of these differences leads to changes in the amino acid sequence (Monani *et al.*, 1999). The critical difference between *SMN1* and *SMN2* is a C-to-T transition in the *SMN2* gene, which affects the activity of an exonic splicing enhancer (ESE), reducing the recognition of *SMN2* exon 7 by the spliceosome (Cartegni and Krainer, 2002). This results in the transcription of two isoforms, the full-length functional SMN protein and more predominant transcripts lacking exon 7 (Monani *et al.*, 1999; Lorson and Androphy, 2000; Lorson *et al.*, 1999).

The full-length *SMN* transcript produces a protein of 38 kDa that is present in both the cytoplasm and the nucleus with variable expression levels among the different tissues examined: greatest in the brain, spinal cord, and muscle and lowest in lymphocytes and fibroblasts (Coovert *et al.*, 1997; Lefebvre *et al.*, 1997). Nuclear SMN is concentrated in aggregates called Gemini of coiled bodies (gems; Liu and Dreyfuss, 1996). The number of gems per cell is drastically decreased in SMA patients, and can be used as a prognostic indicator of disease severity.

The SMN protein acts within macromolecular complexes together with the SMN-interacting proteins, gemin 2, 3, and 4. The complex in the nucleus is involved in pre-mRNA splicing, ribosome production, and transcription (Fischer *et al.*, 1997; Liu *et al.*, 1997; Charroux *et al.*, 1999, 2000), whereas that in the cytoplasm is involved in biogenesis of the spliceosomal UsnRNP (uridine-rich small nuclear ribonucleoprotein).

To date, no effective therapy exists to rescue the motor neuron degeneration of SMA. However, the discovery of the SMA -determining gene, and of the differences between *SMN1* and its highly homologous copy *SMN2* in the human genome, has led to the development of novel therapeutic strategies for SMA. In this regard, *SMN2* provides a natural target for therapeutic intervention in almost all SMA patients and many attempts have been made to devise strategies to enhance the incorporation of exon 7 during mRNA splicing, or to increase the activity of the *SMN2* promoter (Hofmann *et al.*, 2000; Zhang *et al.*, 2001; Young *et al.*, 2002; Brichta *et al.*, 2003; Skordis *et al.*, 2003; Andreassi *et al.*, 2004).

Several approaches are being explored to develop potential therapies for SMA, but no gene-targeting strategies have been successfully attempted. Gene targeting induces a site-specific chromosomal modification that leads to long-term and genetically inheritable expression of the correct gene, regardless of its size. Moreover, direct conversion of genomic sequences preserves the integrity of the gene in such a way that the coding sequences and regulatory elements remain intact. The availability of better delivery methods, such as electroporation and microinjection, has made nonviral transfer an increasingly safer and more viable method for gene therapy. One of these oligonucleotide-based gene-targeting strategies, Small Fragment Homologous Replacement (SFHR), has been successfully used to correct or modify gene sequences responsible for some inherited disease *in vitro* and *in vivo* (Kunzelmann *et al.*, 1996; Goncz and Gruenert, 1998, 2000, 2001; Goncz *et al.*, 1998, 2001, 2002; Kapsa *et al.*, 2001; Bruscia *et al.*, 2002; Sangiuolo *et al.*, 2002; Thorpe *et al.*, 2002; Gruenert *et al.*, 2003).

In this study, we investigated the efficacy of SFHR to induce a T → C transition in exon 7 of the *SMN2* gene in order to rescue physiological level of *SMN* transcript and protein, after transfection by either electroporation or microinjection in human fetal cells obtained from embryos affected by SMA.

MATERIALS AND METHODS

Small DNA fragment design and synthesis

Fragment was generated by polymerase chain reaction (PCR) of human genomic DNA wild type at the *SMN1* locus and lacking the *SMN2* gene. Primers used to generate a small DNA fragment (SDF) homologous to the human *SMN1* sequence were located within introns 6 and 7, respectively (Fig. 1A). The sense primer sequence was SMN1F (5′-AGTTGTGGGATTGTAGGCATG-3′) and the antisense primer sequence was SMN1R-BsmI (5′-GCATTCTAGTAGGGATGTAGA-3′). The reverse oligonucleotide was designed to incorporate a base change at base pair position 497 of the amplified fragment. The point modification together with the *SMN1* sequence allowed the incorporation of a unique *Bsm*I restriction site that can be used as a marker to verify SFHR-mediated specific correction (Goncz and Gruenert, 1998; Goncz *et al.*, 1998). PCR was performed (annealing temperature, 55°C) in a total volume of 50 μl, using 1.5 U of *Pfu* DNA polymerase, 20 pmol of each primer, and 300 ng of genomic DNA. The SDF was cloned into a plasmid vector for large-scale production and, before use, always gel and ethanol purified (DNA gel extraction kit; Millipore, Bedford, MA).

Cell culture

Primary cell cultures from human placental villi were established from five normal and five SMA embryos after transabdominal chorionic villus sampling (CVS) at week XII of preg-

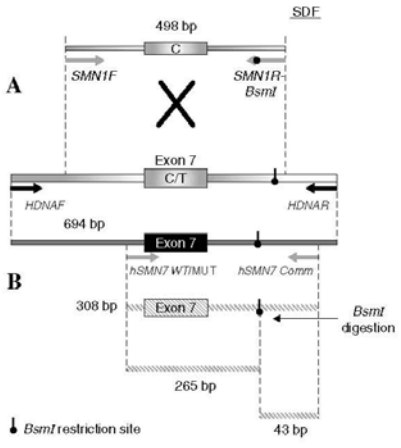

FIG. 1. (**A**) SDF design and synthesis using SMN1F and SMN1R-BsmI primers. The latter contains a mutation that inserts a *Bsm*I site. (**B**) Analysis of genomic DNA consists of two successive amplification rounds. The first was done with two primers (HDNAF and HDNAR) located outside of SDF and gave a 694-bp fragment. The second was performed with the first product as template after gel purification and was developed with allele-specific primers ($hSMN_7WT/hSMN_7Comm$ and $hSMN_7MUT/hSMN_7Comm$). The specificity of the 308-bp fragment was checked by *Bsm*I enzymatic restriction, releasing two bads of 265 and 43 bp, respectively.

nancy (Zahed *et al.*, 1988) from normal women and women at 25% risk of SMA who were undergoing prenatal molecular diagnosis. Informed consent from patients was obtained. Cells were grown in Chang medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin–streptomycin. After about 10 days of culture cells grown from the explants of the villi were detached from the dish, using a standard EDTA–trypsin solution (Sigma-Aldrich, St. Louis, MO) and subcultured in fresh medium for an additional 2–4 days as indicated, before transfection. In some experiments, after transfection, cells were propagated several times (>40 passages).

Cell transfection by microinjection

Injection was performed into cells cultured for at least 48 hr from the first subculture and at about 30% confluency. In each experiment, about 1000 cells were microinjected (Table 1) under a Nikon Eclipse TE 300 inverted microscope, equipped with a Narishige MO-188NE micromanipulator (Narishige International, East Meadow, NY) and a FemtoJet microinjector (Eppendorf, Hamburg, Germany). Microinjection needles had a 1.0 ± 0.2 μm outer tip and an inner tip diameter of 0.5 μm (Femtotips; Eppendorf).

In preliminary experiments, to establish the efficiency of the injection process in delivering molecules into the nucleus and cell viability after injection, SDFs were injected together with rhodamine B isothiocyanate–dextran 10S (RITC–dextran, MW 10,200; Sigma-Aldrich). Observation under a fluorescence microscope revealed that up to 85% of cells remained intact after microinjection and between 77 and 90% of these showed nuclear fluorescence (Table 1).

In each experiment up to 1000 cells were microinjected with 0.7 μl of SDF (~24 ng), corresponding to $\sim 4 \times 10^7$ molecules of SDF per cell. SDF concentration has been exactly calculated with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Cells were then grown and expanded for various periods as indicated, harvested, and stored for analysis. Untransfected cells harvested after the same amounts of time in culture were used as controls.

Cell transfection by electroporation

Nucleofection (i.e., transfection of DNA directly into the nucleus) of human CVS cells in culture was performed according to the optimized protocol for adult human dermal fibroblasts (NHDF-Adult) developed by Amaxa Biosystems (Cologne, Germany). Human CVS cells were grown in flasks (25 cm^2; Corning, Corning, NY) until they reached 80% confluence, corresponding to approximately 5.6×10^5 cells per flask. At this time cells were detached with EDTA–trypsin and resuspended in 100 μl of Nucleofector solution (Amaxa Biosystems), to which 3 μg of pEGFP or 3 μg of SDF-SMN1 ($\sim 1.0 \times 10^7$ molecules of SDF per cell) was added. Samples were nucleofected with the Amaxa apparatus. To assess the best nucleofection conditions (in terms of transfection efficiency and cell survival), we tested various Amaxa programs (U-23, P-22, G-16, and A-24), using pEGFP DNA. After electroporation, cells were immediately plated, expanded for various periods of time, harvested, and stored for analysis.

Analysis of DNA from transfected cells

Genomic DNA from transfected cells was amplified with two oligonucleotide primers (HDNAF and HDNAR) located outside the SDF region. This amplicon (694 bp) was gel purified (DNA gel extraction kit; Millipore) and used as a template for an amplification-refractory mutation system (ARMS)-PCR using $hSMN_7WT/hSMN_7Comm$ and $hSMN_7MUT/hSMN_7Comm$

TABLE 1. MICROINJECTION CONDITIONS FOR HUMAN FETAL TROPHOBLASTS[a]

Sample	*Cells plated*	*Cells injected*	*Viable cells injected*
CVS01-Mi 01	700	531	389 (73%)
CVS01-Mi 03	1000	976	800 (82%)
CVS01-Mi H_2O	700	515	400 (77%)
CVS02	750	630	478 (75%)
CVS03	900	751	600 (80%)
CVS04	850	670	570 (85%)
CVS05	910	790	630 (79%)

Abbreviation: Mi, microinjection.

[a]The growth rate of rhodamine B isothiocyanate–dextran 10S (RITC–dextran)-injected, SDF-injected, and water-injected controls was similar: 1 population doubling every 24 hr.

primers, specific for *SMN2* and *SMN1* sequences, respectively (Fig. 1B). The presence of the *Bsm*I restriction site has been demonstrated to be a marker of the SDF-induced recombination event. The reaction was run on a 6% polyacrylamide gel.

Quantitative expression analysis of SMN *transcripts*

mRNA was reverse transcribed to cDNA according to protocols provided with the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Fifty microliters of 2× RT master mix (2× RT buffer, 2× dNTP mixture, 2× random primers, 5 U of MultiScribe reverse transcriptase) was added into each tube containing 50 μl of RNA sample (about 1500 ng). Incubation conditions included 10 min at 25°C and 2 hr at 37°C. Real-time RT-PCR was performed on a TaqMAN ABI PRISM 7000 sequence detection system (Applied Biosystems) as previously reported with further modification (Andreassi *et al.*, 2004). The relative level of expression was calculated from the ratio of *SMN1* cycle threshold (C_t) to *SMN2* C_t values, both of which were normalized to the control transcript β_2-microglobulin C_t value using the formula $2 \times [\Delta C_t\ (SMN1) - \Delta C_t\ (SMN2)]$, in which ΔC_t represents the differ-

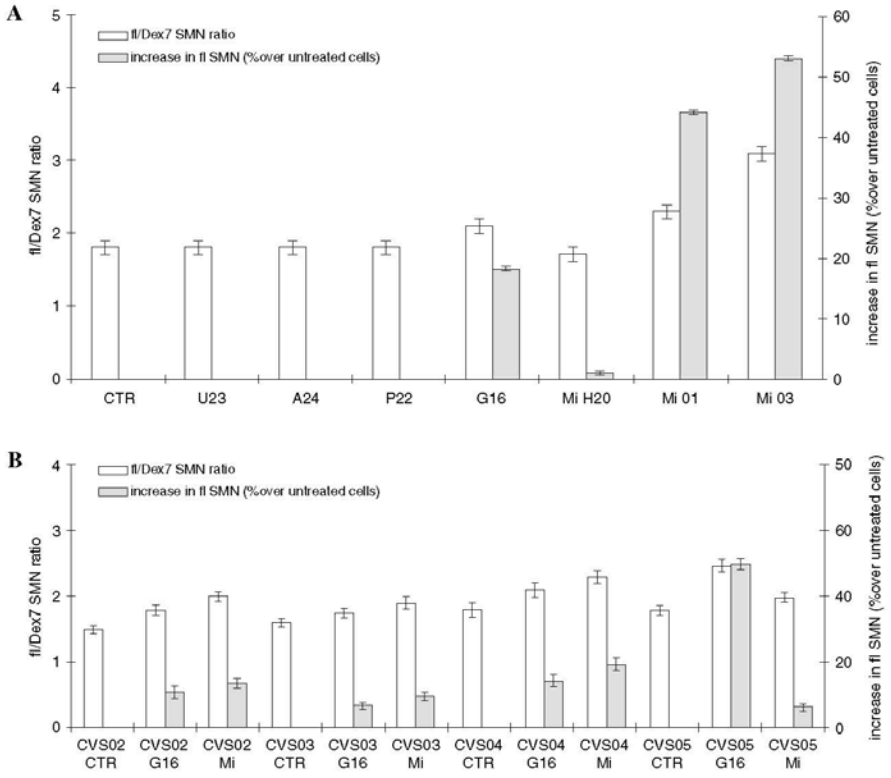

FIG. 2. Quantitative analysis of full-length and Δ7 SMN mRNAs in CVS01 cells transfected by several modes of electroporation or by microinjection (**A**); all other cultured cells (CVS02, CVS03, CVS04, CVS05) were transfected by the G-16 electroporation program or by microinjection (Mi) (**B**). Open columns represent the full-length:Δ7 SMN ratio for each transfected sample (*left axis*). Shaded columns represent the increase in SMN full-length amount in transfected cells (*right axis*). CTR, untransfected sample; P-22, U-23, G-16, and A-24 indicate different electroporation protocols. Mi01 and Mi03 are two independent microinjected samples. MiH20 corresponds to a sample microinjected with water. All values obtained from treated cells represent the mean of at least three independent experiments performed in triplicate. Values were significantly different from those obtained with untreated cells. Error bars indicate the SD. Samples were always compared at the same number of cell doublings. $p < 0.05$ was considered statistically significant.

ence in C_t values between the *SMN1* (wild-type) transcript and the *SMN2* (Δ7) transcript. All PCRs were performed in triplicate.

Western blot analysis

Electroporated or microinjected cells were grown to confluency, rinsed in phosphate-buffered saline (PBS), and scraped from the culture flask in ice-cold lysis buffer (50 m*M* Tris-HCl [pH 7.4], 150 m*M* NaCl, 1% Triton X-100, 1% sodium deoxycholate, 100 m*M* NaF, 0.1% sodium dodecyl sulfate [SDS]) containing freshly added protease inhibitor cocktail (Sigma-Aldrich). Samples were analyzed for protein concentration by Bradford protein assay (Bio-Rad, Hercules, CA). Thirty micrograms of protein resuspended in Laemmli buffer was electrophoresed on a 10% SDS–polyacrylamide gel and subsequently electrotransferred to a nitrocellulose membrane, in accordance with standard procedures. Membranes were incubated in blocking solution (PBS, 3% bovine serum albumin [BSA], 0.1% Tween 20) overnight at 4°C and then incubated with anti-SMN polyclonal antibody, diluted 1:250 in blocking solution, for 1 hr at room temperature. The antibody was produced in rabbit, using 250 μg of purified peptide (Inbios, Pozzuoli, Italy), the sequence of which corresponds to exon 7 of *SMN*, as reported (Hsieh-Li *et al.*, 2000). The membranes were washed in PBS containing 0.1% Tween 20 and incubated with the secondary antibody (anti-rabbit IgG conjugated to horseradish peroxidase) for 1 hr at room temperature. After extensive washes the immune complexes were revealed by autoradiography, using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences/GE Healthcare, Piscataway, NJ). Blots were reprobed with a mouse polyclonal anti-actin antibody, diluted 1:1000 (Sigma-Aldrich). Western blot quantification was performed by scanning the autoradiographs, using a VersaDoc imaging system (Bio-Rad).

Immunofluorescence analysis

Cells grown on glass coverslips were rinsed in PBS and fixed in methanol for 5 min at −20°C. Samples were then rinsed for 30 min at room temperature in PBS containing 3% BSA and 0.1% Triton. Primary antibodies against SMN protein (H195; Santa Cruz Biotechnology, Santa Cruz, CA), pancytokeratins (C-2562; Sigma, St. Louis, MO), and vimentin (V6630; Sigma) were added (diluted 1:150) and applied to the coverslips overnight at 4°C. After extensive washes in PBS, cells were incubated for 1 hr in the dark in the presence of the appropriate secondary antibodies (Molecular Probes, Eugene, OR), diluted 1:800. Last, samples were treated with Hoechst (diluted 1:2000 in PBS) for 5 min to identify nuclei and mounted with a solution of 50% glycerol in PBS. Fluorescence was observed under a Zeiss Axioplan microscope, equipped with a ×100 objective. Negative controls included cells incubated with IgG of

Table 2. Gem Counts of Fetal Trophoblasts from Human Placental Villi

Individual	*Phenotype*	*Total no. of cells*	*Nuclei with gems (%)*	*Gems/100 nuclei*	*Cells with cytoplasmic signal (%)*
Normal					
CVS-1	Normal	309	70	1947	0.09
CVS-2	Normal	301	70	1651	1.04
CVS-3	Normal	212	70	1483	1.05
CVS-4	Normal	291	69	1820	0.35
CVS-5	Normal	200	68	2044	0.35
Untransfected					
CVS-01	SMAI	235	42	240	10.09
CVS-02	SMAI	213	59	773	8.05
CVS-03	SMAI	326	53.9	693	9.02
CVS-04	SMAI	234	54.4	247	11.00
CVS-05	SMAI	270	61	521	10.03
	Phenotype–treatment				
Transfected					
CVS-01	SMA-Mi 01	357	48	363	9.04
	SMA-Mi 03	342	53	546	7.09
	SMA-G-16	330	59	543	8.04
CVS-02	SMA-Mi	215	70	1172	5.08
	SMA-G-16	193	70	1127	6.01
CVS-03	SMA-Mi	331	70	1251	4.06
	SMA-G-16	330	70	1126	5.05
CVS-04	SMA-Mi	310	67	597	7.00
	SMA-G-16	300	65	385	6.05
CVS-05	SMA-Mi	299	64	599	7.09
	SMA-G-16	305	65	1181	6.06

Gem counts were performed on three different coverslips and two operators evaluated each sample. $p < 0.05$ was considered statistically significant.

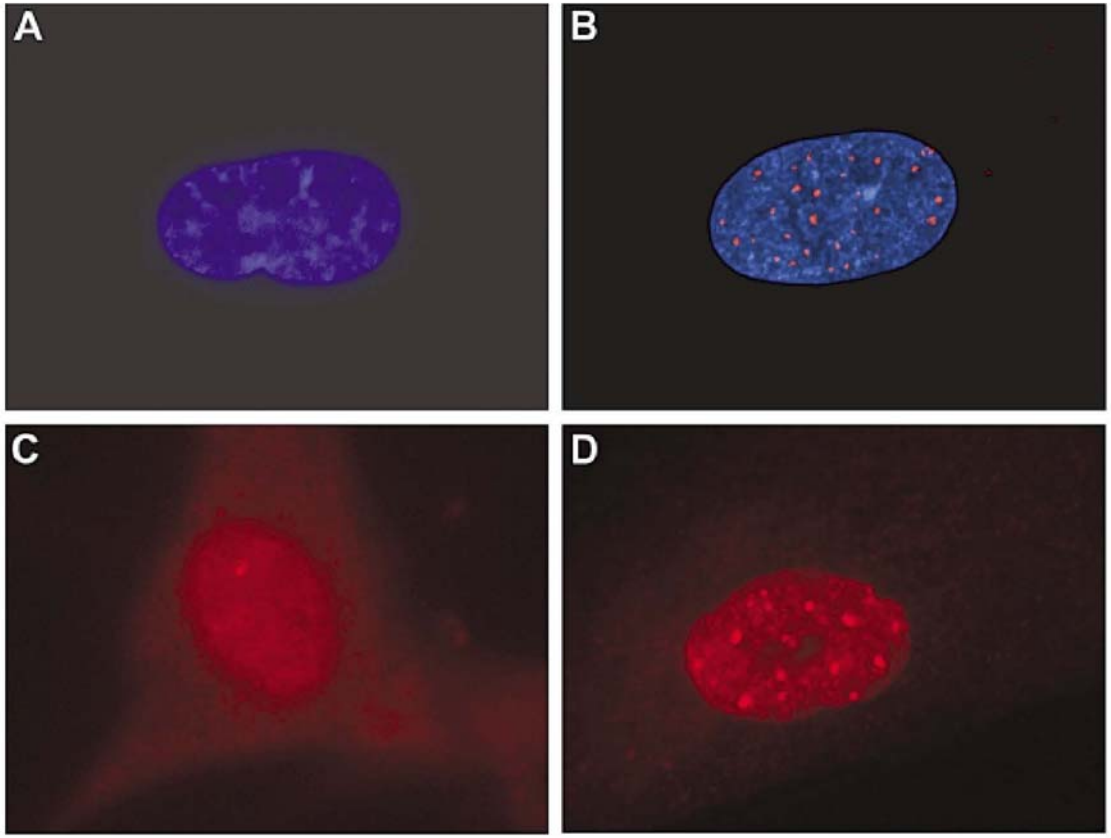

FIG. 3. Immunocytochemistry for SMN protein in untransfected (**A** and **C**), SDF-microinjected (**B**), and electroporated (**D**) SMA cells. In (**A**) and (**B**), nuclei were counterstained with Hoechst.

the same species as the primary antibodies instead of the primary specific antibodies.

Statistical analysis

Real-time PCR data were analyzed by analysis of variance (ANOVA) (SPSS version 10.0; SPSS, Chicago, IL). For gem number studies, variance between the means was analyzed by paired *t* test analysis (SPSS version 10.0). A p value less than 0.05 was considered statistically significant.

RESULTS

Molecular analyses of transfected cells for SMN transcripts

Genotypes of the cultured cells obtained from normal and SMA fetal villi were confirmed by DNA diagnostic tests. The latter showed deletion of the *SMN1* locus and retention of the *SMN2* locus. SMA cells were then transfected by either electroporation or microinjection and analyzed in comparison with untransfected cells harvested after the same number of cell doublings.

Figure 2A shows the setting of the optimal transfection protocol on *CVS01* samples, using electroporation or microinjection. Only one of the four electroporation programs tested, specifically G-16, led to high transfection efficiency (95%) associated with high viability of cells (75%).

In fact, G-16-electroporated cells revealed an increase of about 18% in the amount of full-length *SMN* and a 2.1-fold increase in the ratio of full-length *SMN1* to *SMN2* (Δ7) isoforms compared with untreated cells. Conversely, microinjection experiments showed that the amount of full-length *SMN* mRNA increased up to 53% compared with untransfected cells, whereas the full-length:Δ7 isoform ratio increased up to 3.11-fold (Fig. 2A). No modification in transcript levels was observed in cells electroporated according to the U-23, A-24, and P-22 programs and in water-microinjected cells.

These results were confirmed successively in four independent samples of villi transfected under the optimal conditions selected (Fig. 2B). With the exception of *CVS05* (see below), microinjection always gave better results than electroporation, underlining the importance of the route of delivery of SDFs into cells. After electroporation, the full-length SMN transcript increased up to 17% and the full-length:Δ7 ratio reached a value of 2 in *CVS04*. Microinjected *CVS04* cells also gave the maximum value of 20% full-length SMN transcript increase and a 2.29 *SMN1*:Δ7 ratio (Fig. 2B). In *CVS05* cells the situation seemed to be reversed; in fact, the electroporated sample showed an increase in full-length mRNA up to seven times than seen in the microinjected sample.

All samples (*CVS01* to *CVS05*) were previously tested for DNA genomic modifications and were positive by *BamH* analysis, except for samples electroporated according to U-23, A-24, and P-22 (data not shown).

SMN protein expression in transfected cells

We first analyzed cells from wild-type villi and untransfected SMA cells by immunocytochemistry (Table 2). As previously described, the SMN protein was found both in the cytoplasm and in the nucleus of cells as gems (Liu and Dreyfuss, 1996). Unlike wild-type cells, however, in which many gems are localized to the nucleus, in SMA cells few gems were present in the nucleus. In such cells most of the gems were localized in the cytoplasm, likely because of inefficient import of the Δ7 SMN isoform into the nucleus (Liu *et al.*, 1997; Patrizi *et al.*, 1999). We predicted that corrected SMA cells should produce more full-length SMN protein and, therefore, should have an

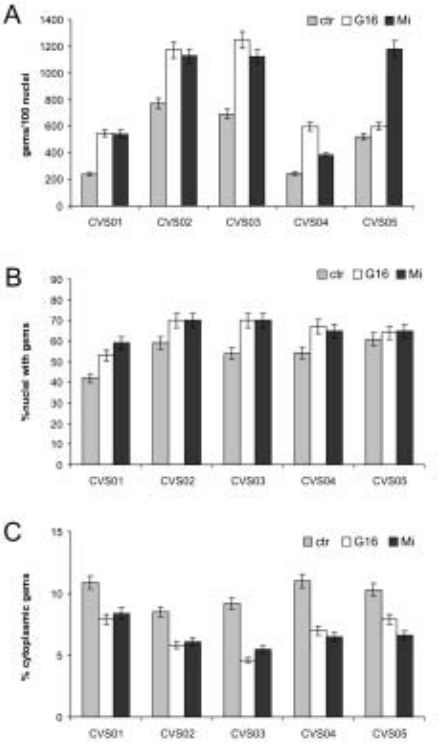

FIG. 4. Evaluation of SMN protein expression in transfected and untransfected cells by gem counts: Gems per 100 nuclei value (A), percentage of nuclei with gems (B), and percentage of cytoplasm with gems (C) in untreated and treated cells observed by immunolocalization. Shaded columns correspond to control untransfected samples (ctr), solid columns to G-16-electroporated cells (G-16), and open columns to microinjected cells (Mi). Error bars indicate the SD. Samples were always compared at the same number of cell doublings. Gem counts were performed on three different coverslips and two operators evaluated each sample. Mean values obtained from treated cells (G-16 and Mi) were significantly different from those obtained from untreated cells. $p < 0.05$ was considered statistically significant.

increased number of gems in the nuclei (Di Domato *et al.*, 2003). After transfection, cytoplasmic and nuclear gems were counted and compared between untransfected and transfected SMA cells from all samples of cultured villi (Table 2). A statistically significant 2-fold increase in the number of gems per 100 nuclei was estimated in all transfected samples, both microinjected and electroporated (Figs. 3 and 4A, and Table 2). Moreover, the increase in nuclear gems was associated with a decrease in cytoplasmic gems in both electroporated and microinjected cells (Fig. 4B and C). These data support the hypothesis that the SFHR-mediated transition leads to an increase in full-length *SMN* mRNA, which restores appropriate import of the SMN protein into the nucleus. According to the mRNA analyses, no changes in gem numbers or localization were observed in cells electroporated according to other protocols or in water-injected control cells (data not shown).

Western blot analysis

To confirm the increased synthesis of *SMN1* protein, Western blot analysis was performed (Fig. 5A). Because the commercially available antibody was unable to discriminate between SMN isoforms, a polyclonal antiserum was produced against *SMN* exon 7-encoded peptide in order to quantify changes in full-length protein after cell transfection (Hsieh-Li *et al.*, 2000). Densitometric analysis of Western blot results (Fig. 5B) showed an SMN1:actin ratio consistent with results obtained in the immunocytochemical analyses. In particular, a 2.3- and 1.7-fold increase in SMN protein level was observed when microinjected (Mi) and electroporated (G-16) cells were compared with their respective untransfected controls (CTR Mi and CTR G-16). Wild-type and SMA samples were also loaded as controls.

Stability of the genomic modification

To monitor the stability of SFHR-induced genomic modification over time, transfected GM501 cell cultures were passaged *in vitro* for more than 2 months, equivalent to 45 doublings, and again analyzed both by molecular and biochemical assays. Cells transfected by either electroporation or microinjection maintained higher levels of full-length transcript and protein

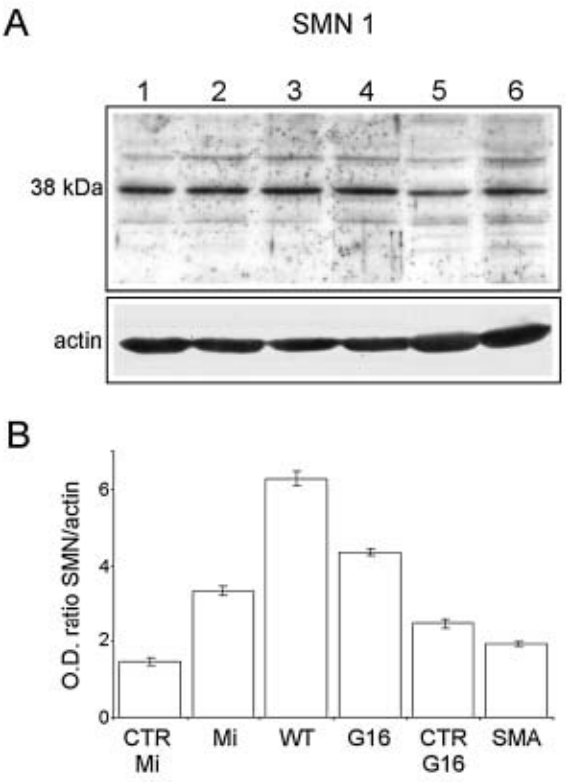

FIG. 5. Western blot analysis of SMN protein in untransfected and transfected cells. (A) Expression of SMN was detected with a polyclonal antibody, produced against an *SMN* exon 7-encoded peptide, that is able to recognize only the full-length SMN1 isoform. Lanes 1 and 2, water-microinjected (CTR Mi) and SDF-microinjected (Mi) samples, respectively; lane 3, wild-type (WT) cells; lanes 4 and 5, SDF-electroporated (G-16) and buffer-electroporated (CTR G-16) control, respectively; lane 6, untreated SMA cells (SMA). (B) Densitometric analysis was performed, normalizing the SMN value to actin (used as a loading control).

expression, evaluated by gem count, compared with untransfected controls (data not shown). The persistence of SFHR-induced genomic change was monitored in these cells by *Bsm*I restriction analysis (ARMS-PCR; Fig. 6). Moreover, the isogeneic nature of cells cultured *in vitro* for 2 months was detected by DNA fingerprinting analysis, using an AmpF*l*STR Identifiler PCR amplification kit (Applied Biosystems) and genotyping data on an ABI PRISM 310 genetic analyzer (Applied Biosystems). The fingerprint patterns of the transfected cell lines cultivated for 2 months were identical to the starting ones, suggesting that they are isogeneic and derived from untransfected cells (data not shown).

We further characterized cells grown in culture for the above-described immunochemistry analysis. To do this, we double stained cultured cells with anti-vimentin and anti-pancytokeratin antibodies to identify mesenchymal-derived and epithelial cells, respectively. Results showed that whereas in primary cultures after 10 days both cytokeratin-positive cells (20–30%) and vimentin-positive cells (60–70%) were present (data not shown), in cells obtained after about 30 passages nearly all of the cells were vimentin positive. Thus, from a heterogeneous primary culture, containing many cell types present in the original tissue, a more homogeneous cell line has been selected in favor of vimentin-positive mesenchymal cells.

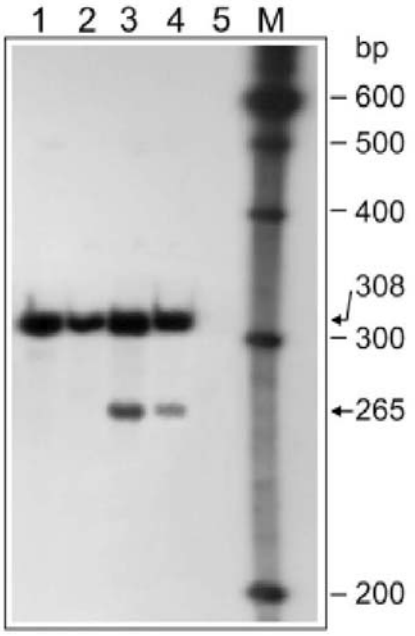

FIG. 6. Allele-specific PCR using $hSMN_7WT/hSMN_7Comm$ primers from genomic DNA of CVS01-transfected cells after 45 cell doublings (2 months). Lanes 1 and 2, amplicon from genomic DNA; lanes 3 and 4, the same amplicon as in lanes 1 and 2, digested with *Bsm*I restriction enzyme; lane 5, negative control (no DNA); lane M, 100-bp ladder.

DISCUSSION

In humans, two copies of the *SMN* gene, which is the SMA-determining gene, are present, called *SMN1* and *SMN2*. It is known that the mutated *SMN2* gene is unable to produce sufficient amounts of SMN protein for survival of motor neurons because of a single nucleotide difference in exon 7, resulting in defective splicing of the pre-mRNA and in a protein that is rapidly degraded and therefore unable to form gems efficiently (Le *et al.*, 2000; Lorson and Androphy, 2000). Correcting the *SMN2* splicing defect in motor neurons should provide a therapeutic benefit to patients with SMA.

In line with this notion, the introduction of different copies of the human *SMN2* gene in knockout mice caused the rescue of the SMA phenotype (Monani *et al.*, 2000). Moreover, the possibility of modulating the *SMN* splicing process was demonstrated by generating minigenes containing, or not containing, the splicing enhancer element and expressing those constructs in cell culture or in transgenic mice. Only in the presence of the splicing enhancer element were high levels of SMN detected (Di Donato *et al.*, 2001).

Three unrelated asymptomatic individuals, with family histories of SMA, were found to have a homozygous deletion of the *SMN1* gene (Prior *et al.*, 2004). Quantitative studies indicated that these individuals had increased *SMN2* copy numbers compared with the general population, specifically up to five, supporting the role of *SMN2* in modifying the SMA phenotype.

Various experimental approaches have been taken to modulate *SMN2* splicing, such as the use of small molecule modulators and antisense oligonucleotides directed to a splicing silencer in intron 7 or to the intron 7–exon 8 junction (Lim and Hertel, 2001; Miyajima *et al.*, 2002).

In this paper, we demonstrate that the splicing pattern of the *SMN2* gene can be modified in embryonic cells, and that the increased incorporation of exon 7 into the transcript can restore protein levels and gem numbers to nearly normal levels. We documented the *in vitro* conversion of the *SMN2* gene into the *SMN1* gene in cells obtained from the fetal villi of SMA embryos, using the SFHR technique. When a 498-bp SDF containing the *SMN1* exon 7 sequence was transfected into these cells, using two different techniques (electroporation and microinjection), a high increase in full-length transcript was obtained (from 7 to 53%). In terms of protein, this resulted in a 2-fold increase.

The relatively high percentage of cells producing high levels of SMN1 protein is likely due to both the recombinogenicity of the *SMN* locus and/or to optimization of the transfection protocols. However, it is also possible that fetal cells are more "receptive" to SFHR than are mature and differentiated cells (Kunzelmann *et al.*, 1996).

Critical considerations on gene repair strategies, on their occurrence and potency, have emerged, highlighting the necessity to better understand basic mechanisms. The SFHR technique has been the subject of criticism; for example, De Semir and Aran (2003) described how PCR artifacts due to SDF contam-

ination could give false positive results. With this in mind, the methodology used in this study was tailored to validate the protocol used. In fact, we adopted an accurate procedure aimed at avoiding artifacts because the production of SDFs to the analysis of gene conversion. The specificity of the SFHR-mediated conversion was demonstrated by genomic DNA analysis testing the presence of the *Bsm*I restriction site. PCR primers utilized for analysis were outside the region of homology defined by the SDF in order to exclude any artifact from fragment contamination. We estimated that at the time of cell harvest, there was less than the equivalent of 312,500 fragments per cell, a figure compatible with the absence of PCR artifacts (Kunzelmann *et al.*, 1996; Goncz *et al.*, 1998, 2001; Gruenert *et al.*, 2004).

In addition, total RNA was DNase treated before analysis, which was performed with primers located in different exons to avoid any contamination from SDF and from genomic DNA. However, our study offers a functional evidence of induced genomic change. In fact, a significant increase in SMN functional protein was documented in transfected cells as revealed by both immunocytochemistry and immunoblot analyses. In this respect, the increase in the number of nuclear gems observed in transfected cells provides direct and formal proof of a genetic modification of transfected cells, because only full-length SMN protein is assembled into such structures (Liu and Dreyfuss, 1996; Lefebvre *et al.*, 1997). This was corroborated by the use in Western blot analysis of a selective antibody, specifically directed against exon 7, that was able to detect only the wild-type SMN protein. Notably, this is evidence of the stability of the SDF-induced correction of the *SMN* locus and of the maintenance of a wild-type cellular phenotype. This aspect is crucial for future human applications.

The principal finding of this paper is that an appropriately designed SFHR protocol can induce a functional and stable genetic modification in fetal SMA cells. This approach offers several advantages, such as stable and long-term expression of the recombinant gene, in comparison with gene complementation strategies based on viral transfection.

Two papers have reported SMN correction using adenovirus-mediated gene delivery (Di Donato *et al.*, 2003) or, more recently, lentiviral vectors (Azzouz *et al.*, 2004). Although the restoration of cellular phenotype seems to be better than that achieved by SFHR, there are several drawbacks to the use of viral systems, such as the lack of long-term expression, the position effects on gene expression, and, more important, the presence of viral promoters driving gene expression. The present study performed for the first time on fetal cells highlights the possibility of rescuing an SMA fetus by delivering SDF *in utero* for an early treatment of SMA as proposed (Azzouz *et al.*, 2004). We suggest that SDFs, delivered by microinjection, might cure SMA in humans by an *ex vivo* approach.

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In Vivo and In Vitro Studies Support That a New Splicing Isoform of *OLR1* Gene Is Protective Against Acute Myocardial Infarction

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Abstract—Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), encoded by the *OLR1* gene, is a scavenger receptor that plays a fundamental role in the pathogenesis of atherosclerosis. LOX-1 activation is associated with apoptosis of endothelial cells, smooth muscle cells (SMCs), and macrophages. This process is an important underlying mechanism that contributes to plaque instability and subsequent development of acute coronary syndromes. Independent association genetic studies have implicated *OLR1* gene variants in myocardial infarction (MI) susceptibility. Because single nucleotide polymorphisms (SNPs) linked to MI are located in intronic sequences of the gene, it remains unclear as to how they determine their biological effects. Using quantitative real-time PCR and minigene approach, we show that intronic SNPs, linked to MI, regulate the expression of a new functional splicing isoform of the *OLR1* gene, LOXIN, which lacks exon 5. Macrophages from subjects carrying the "non-risk" disease haplotype at *OLR1* gene have an increased expression of LOXIN at mRNA and protein level, which results in a significant reduction of apoptosis in response to oxLDL. Expression of LOXIN in different cell types results in loss of surface staining, indicating that truncation of the C-terminal portion of the protein has a profound effect on its cellular trafficking. Furthermore, the proapoptotic effect of LOX-1 receptor in cell culture is specifically rescued by the coexpression of LOXIN in a dose-dependent manner. The demonstration that increasing levels of LOXIN protect cells from LOX-1 induced apoptosis sets a groundwork for developing therapeutic approaches for prevention of plaque instability. (***Circ Res.*** **2005;97:152-158.)**

Key Words: OLR1 ■ myocardial infarction ■ LOX-1 ■ oxLDL ■ apoptosis

Recent studies have shown that atherogenesis is no longer an inevitable consequence of aging but instead is a complex disease with multi-factorial etiology.[1,2] Traditional risk factors associated with atherogenesis include hypercholesterolemia, smoking, male gender, hypertension, diabetes, and age. However, newly defined nontraditional risk factors are emerging as being equally important.[2] Among these are the elevated plasma and tissue levels of oxidized low-density lipoprotein (OxLDL).[3] OxLDL is now considered to play a fundamental role in the entire process of atherogenesis. OxLDL elicits endothelial dysfunction, a key step in the initiation of atherosclerosis, favoring generation of reactive oxygen species, inhibition of nitric oxide synthesis, and enhancement of monocyte adhesion to activated endothelial cells.[4,5] In addition, oxLDL is involved in inducing smooth muscle cell migration and proliferation, and are avidly ingested by macrophages, resulting in foam cells formation.[5] Increased levels of oxLDL relate to plaque instability in human coronary atherosclerotic lesions. Thus, oxLDL levels show a significant positive correlation with the severity of acute coronary syndromes such as myocardial infarction (MI) and unstable angina, and the more severe lesions contain a significantly higher percentage of oxLDL–positive macrophages.[6] According to this scenario, it has been shown that oxLDL is cytotoxic to cultured cells and induces apoptosis and necrosis of vascular endothelial cells, smooth muscle cells (SMCs), and macrophages.[7–10] These processes have been proposed to lead to plaque vulnerability and potential rupture, which is ultimately responsible for acute atherothrombotic vascular occlusion and tissue infarction.

Most of these effects are mediated by the interaction of oxLDL with its major receptor, named LOX-1, a type-II

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membrane protein belonging to the C-type lectin family. LOX-1 consists of 4 domains: a short N-terminal cytoplasmic, a transmembrane, a connecting neck, and a lectin-like domain at the C terminus which binds oxLDL.[11] Importantly, the integrity of the lectin-like domain is critically required for its binding activity and is highly conserved among species.[12] LOX-1 is expressed in endothelial cells, macrophages, SMCs, and platelets. Furthermore, LOX-1 is present in atheroma-derived cells and is overexpressed in humans and animal atherosclerotic lesions in vivo.[13] An association of polymorphisms in the human *OLR1* gene and MI susceptibility has been recently reported.[14,15] In particular, we have identified 7 different single nucleotide polymorphisms (SNPs), 6 of them located within introns 4, 5, and 3′ UTR (untranslated region), comprised in a linkage disequilibrium (LD) block strongly associated with the elevated risk to develop MI.[14] Because the SNPs related to an increased risk of MI did not affect the coding sequence of the gene, we decided to explore whether the SNPs could give rise to a functional product by examining the existence of messenger RNA (mRNA) isoforms as a consequence of alternative splicing. Using a minigene approach, we show that SNPs located in the LD block regulate the level of the new fully functional transcript by modulating the retention of exon 5 of the *OLR1* gene. The identification and characterization of the new splice variant of the *OLR1* gene suggest that this variant may have a functional role on plaque instability and therefore in the pathogenesis of myocardial infarction.

Materials and Methods

RT-PCR Experiments

Total RNA was purified from monocyte-derived macrophages and COS-7 cells using RNeasy Mini Purification kit (Qiagen). Poly A^+ RNA were purified using the Oligotex mRNA Mini Kit (Qiagen). Reverse transcription was performed with a High-Capacity cDNA Archive Kit (Applied Biosystems). RT products were amplified using forward OLR1-FW primer (5′-TGTTGAAGTTCGTGACTGCTT-3′), reverse OLR1-RW primer (5′-TTCTGCAGCCAGCTAAATGA-3′), and then subcloned using a TA cloning kit (Invitrogen).

DNA Constructs

To amplify the genomic sequence surrounding the alternatively spliced exon 5 of *OLR1* gene, we used the primer pair *OLR1-Xho*IF (5′-ACA GTC *CTC GAG* GTG AGT GTT CAT GGA TAT TTG-3′) and OLRI-*Eco*RIR (5′-TGT GTG *GAT ATC* CTG CAG GTA GGA AAA ACA AAA-3′). We used human genomic DNA homozygous with respect to "risk" or "non-risk" haplotype at LD block of *OLR1* gene as the PCR template. The PCR products were cloned into pSPL3 vector (Invitrogen) and sequenced using the primer *OLR1*SEQF (5′-GTT TCC TAT TCT TTG CTG AAC-3′) and *OLR1*SEQR (5′-GTG GGG AGT AAT GTT TCT GAG-3′).

To generate LOX-1-GFP and LOXIN-GFP constructs, the coding sequences of *OLR1* gene and the splicing variant LOXIN were PCR-amplified from cDNA, which was derived from the human heart poly A^+ RNA (Clontech) using selected oligos. For the amplification of *OLR1*, primers F1 (5′ CCG*CTCGAG*ATGACTTT-TGATGACCTAAAG 3′) and F2 (5′ CGC*GGATCC*T GTGCTCT-TAGGTTTGC 3′) were used. For the amplification of LOXIN, primers F1 and F3 (5′*CGCGGATCC*ATCA GATCAGCTGTGC-TATT 3′) were used. The PCR products were cloned into the XhoI/BamHI-digested pEGFP-N1 vector (Clontech) and sequenced using CEQ2000 (Beckman–Coultard).

Quantitative Real-Time PCR

Real-time RT-PCR was performed on a TaqMAN ABI 7000 Sequence Detection System (Applied Biosystems). By using the Primer Express 2.0 software (Applied Biosystems), we designed primers and MGB probes for the discrimination analysis of the 2 alternatively spliced isoforms (primers and MGB probes sequences are available on request, patent pending).

Commercially available predeveloped TaqMan endogenous reference GAPDH gene (Applied Biosystems) was used to normalize the amount of cDNA added per sample. A comparative C_T method was used to determine relative quantification of RNA expression. All PCR reactions were performed in triplicate.

Cell Culture and Transfection

Human monocytes were isolated from peripheral blood mononuclear cells (PBMCs) of subjects homozygous for the "risk" and "non-risk" haplotype. We promoted their transition to macrophages in vitro as previously described.[11] Differentiation was determined by flow cytometry using anti-CD36 FITC monoclonal antibody (cell purity >95%). Simian COS-7 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS). Transient transfection of COS-7 was performed using the Nucleofector kit (Amaxa Biosytems). 1 μg of plasmid DNA was added to 5×10^5 COS-7 suspended in 100 μL of human dermal fibroblast Nucleofector solution. The program A-24 was selected for a high density of transfection. All subjects gave informed consent, and the study protocol was approved by the Tor Vergata University Ethics Committee.

Evaluation of Apoptosis

Differentiated macrophages were cultured without serum for 15 hours and then incubated with oxLDL (100 μg/mL; Intracel) for 8 hours before detection of apoptosis. Apoptosis was evaluated by multiparameter flow cytometry using a method that distinguishes nuclei from apoptotic, necrotic, or viable lymphoid cells.[16] Isolated nuclei were analyzed by fluorescence and by forward- and side-angle scatter multiparameter analysis using a FACScan Flowcytometer (Becton Dikinson). A minimum of 5000 events was collected for each sample.

Apoptotic fibroblasts were visualized by staining with the blue fluorescent dye Hoechst 33342 (Sigma) and phosphatidylserine assay as described previously.[17] Annexin V and Hoechst 33342 positive cells were counted from cells transfected with GFP, LOX-1-GFP, and LOXIN-GFP recombinant proteins.

Immunofluorescence Staining

Immunofluorescence was performed as described.[18] Affinity purified anti-rabbit LOX-1 (Santa Cruz), goat anti-calnexin (Santa Cruz), and mouse anti-Golgi 58K (Sigma) protein were used as primary antibodies. Texas Red goat anti-rabbit IgG (Calbiochem), Texas Red goat anti-mouse IgG (Calbiochem), and Rhodamine Red–X-conjugated affinipure rabbit anti-goat IgG (Jackson Immunoreasearch) were used as secondary antibodies. Hoechst 33342 dye was used at 1 μg/mL. Samples were examined with a DMRA Leica fluorescence microscope equipped with CCD camera. Acquired images were deconvolved using Leica Q-fluoro software and processed using Adobe Photoshop.

Western Blot Analysis and Enzymatic Digestion

PBMCs and COS-7 were lysed for 30 minutes in ice-cold cell extraction buffer (EB) (100 mmol/L NaCl, 10 mmol/L EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mmol/L Tris-HCl, pH 7.4, 1 mmol/L PMSF, 10 μg/mL pepstatin A, 10 μg/mL leupeptin and 0.3 μmol/L aprotinin). Nuclei and large debris were removed by centrifugation at 290g for 10 minutes at 4°C. The supernatants were then precipitated with 5 volumes of MeOH at −20°C for 2 hours. After centrifugation (16 000g, 10 minutes, 4°C), protein pellets were dissolved in 4× sample buffer (500 mmol/L Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 40 mmol/L DTT and 0.02% bromophenol blue) and heated at 95°C for 5 minutes.

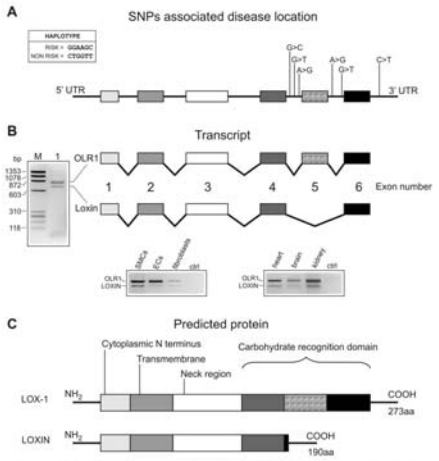

Figure 1. *OLR1* from gene to protein. A, Genomic organization of the *OLR1* gene. The exon-intron structure of *OLR1* gene is shown along with SNPs position, comprised in a LD block. The top box represents the "risk" and "non-risk" haplotype at the LD block. B, Identification of alternatively spliced forms of *OLR1* gene and expression profile in different cell types and tissues as indicated. C, Predicted protein schematic representation.

Blots were probed with rabbit anti-LOX-1 antibody (Santa Cruz). Immunoreactive bands were detected with sheep anti-rabbit IgG horseradish peroxidase (Amersham) and visualized by ECL (Sigma). Enzymatic deglycosylation was performed as previously described.[18]

Statistical Analysis

All statistical analyses were performed using SPSS version13 software. Data are presented as means +1 SD. Normal distribution of continuous variables has been verified by Kolmogorov–Smirnov with Lillefors correction. Differences in means of continuous variables were analyzed by impaired *t* test or ANOVA as needed. Bonferroni correction was used for multiple comparison.

Results

Identification of an Alternatively Spliced Forms of *OLR1* Gene

We started from total or poly (A)$^+$ RNA of human monocyte-derived macrophages and subjected it to reverse transcription to test the potential effect of the SNPs located in the LD block on RNA splicing of the human *OLR1* gene. We performed PCR amplification using primers located in the 5′UTR and 3′UTR of the *OLR1* gene and sequenced the amplified fragments. This analysis identified a reproducible pattern of alternative splicing around exon 5. In particular we identified 2 *OLR1* transcripts in both RNA fractions. One of these products corresponded to the full-length transcript (*OLR1*), while the other lacked exon 5; we named it LOXIN (Figure 1B). The newly spliced mRNA has a stop codon in the open reading frame that leads to a premature termination of the translation product and generates a predicted protein that lacks 2/3 of the lectin-like domain (Figure 1C). Both isoforms were detected in several cell types (endothelial cells, fibroblasts, and smooth muscle cells), and tissues (heart, kidney, and brain), suggesting that they reflect a physiological pattern of expression of the *OLR1* gene (Figure 1B).

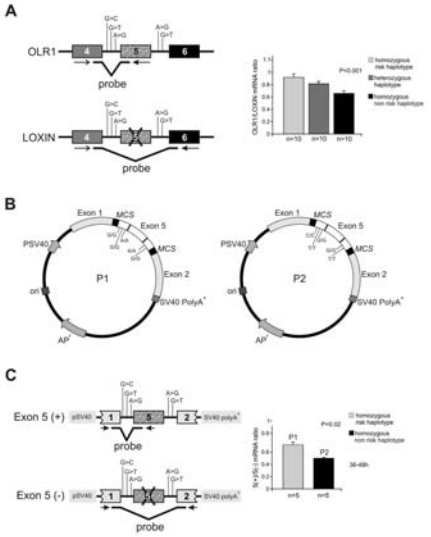

Figure 2. SNPs located in the LD block modulate the levels of the *OLR1* mRNA isoforms. A, Quantification of the *OLR1* and LOXIN transcripts using real-time quantitative PCR. Total RNA was isolated from monocyte-derived macrophages of 10 subjects homozygous for the "risk" haplotype and 10 for the "non-risk" haplotype and 10 heterozygotes. The 2 isoforms were quantified using isoform specific primers and probes. Bar graphs show the relative amount of the 2 isoforms expressed as a ratio of *OLR1* isoform (containing exon 5) to the LOXIN isoform (lacking exon 5). B, Map of the 2 minigene plasmids P1 and P2. The plasmids P1 and P2 contain the genomic sequence homozygous for the "risk" haplotype and the "non-risk" haplotype, respectively. C, Quantification of the P1 and P2 minigenes expression. Total RNA was isolated from COS-7 cells at 36 and 48 hours after transfection with P1 or P2 plasmids. The corresponding cDNA were quantified using an isoform-specific real-time PCR. Bar graphs show the ratio of the transcript containing exon 5 to the transcript lacking exon 5.

SNPs Located in the LD Block Modulate the Levels of the mRNA Isoforms

To find in vivo evidence that SNPs located in the LD block modulate the level of the two mRNA isoforms, we performed an isoform-specific real-time PCR starting from total RNA of human monocyte-derived macrophages of selected patients carrying the "risk" and "non-risk" haplotype at *OLR1* gene. By this analysis we noted a marked difference in the mRNA ratio (*OLRI*/LOXIN) according to the haplotype. In particular, the *OLR1*/LOXIN mRNA ratio was 33% higher in human monocyte-derived macrophages of subjects homozygous for the "risk" haplotype compared with homozygous for the "non-risk" haplotype (Figure 2A). The finding that the relative amount of the LOXIN transcript is significantly

greater in subjects carrying the "non-risk" haplotype strongly suggests a negative link between levels of LOXIN mRNA and the incidence of MI in humans.

To further confirm the regulatory role of the intronic polymorphism, we extended these studies by constructing minigenes carrying the "risk" and "non-risk" haplotypes with genomic sequences containing intron 4, exon 5, and intron 5 (Figure 2B). These constructs were transfected in COS-7 fibroblasts and the ratio of unspliced (exon 5+) to spliced (exon 5−) transcript was analyzed by real time isoform-specific PCR. As can be seen in Figure 2C, the ratio was 27% higher in RNA extracted from cells transfected with minigenes carrying the "risk" haplotype. These in vitro experiments not only suggest that the relative abundance of the 2 isoforms is modulated by the intronic SNPs mapping within the LD block, but also confirm the previously described in vivo results (Figure 2A).

Subcellular and Membrane Distribution of LOX-1 and the LOXIN Splice Variant

To investigate the cellular localization of the full-length and the splice variant, we constructed two plasmids that allowed the efficient expression of LOX-1 and LOXIN in mammalian cells fused, C-terminally, to the green fluorescent protein (GFP). Immunofluorescence analysis of transfected COS-7 fibroblasts revealed that the intracellular expression of the full-length LOX-1–GFP causes a cell-lethal phenotype. Many transfected cells are roundly shaped and tend to detach from the dish. On the contrary, LOXIN-GFP isoform is efficiently expressed, and its expression does not result in cytotoxicity. Notwithstanding the toxic effect, many LOX-1–GFP transfected cells retain normal morphology, and this has allowed us to study its intracellular distribution. As shown in Figure 3A, (panels A through C), LOX-1 distributed in the ER and in the Golgi apparatus. At 24 hours after transfection, LOX-1 colocalized almost exclusively with the Golgi 58K protein, indicating that the protein initially traffics along the secretory pathway. In contrast, LOXIN-GFP was not detectable in the Golgi patches of permeabilized COS-7 cells. In these cells we observed a more widespread staining, characteristic of typical ER distribution. In 40% to 50% of transfected cells, an accumulation of fluorescence in the perinuclear area was also detected (Figure 3A, panels D through F).

The 2 GFP-tagged isoforms were also labeled for surface receptors in live cells (Figure 3B and 4). At 24 hours after transfection, over 90% of cells expressing LOX-1–GFP showed a typical punctuate plasma membrane–associated fluorescence (Figure 3B, panel C). Remarkably, <10% of COS-7 cells transfected with LOXIN-GFP showed surface staining (Figure 3B, panel F). The very low expression of LOXIN at the plasma membrane demonstrates that truncation of the C-terminal portion in LOXIN protein leads to a profound effect on cellular trafficking of the protein to the plasma membrane.

The expression level of the 2 isoforms in transfected fibroblasts was also confirmed in Western blot. A single band corresponding to LOX-1–GFP and LOXIN-GFP fusion proteins were detected (Figure 4A, lanes 1 and 2). The molecular weight of the bands corresponded to the predicted molecular

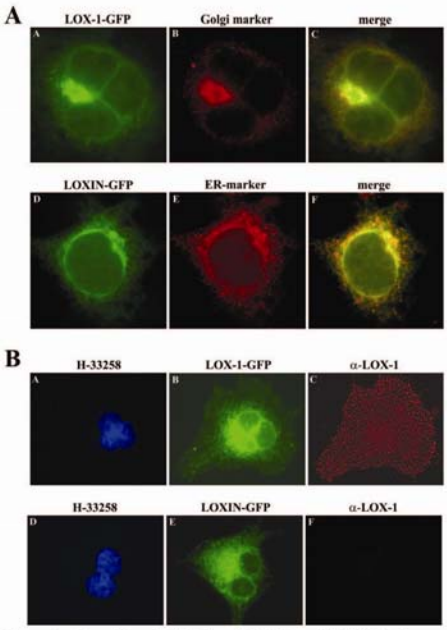

Figure 3. Subcellular distribution of LOX-1 and LOXIN isoforms. A, COS-7 cells were transfected with LOX-1–GFP (panel A) and LOXIN-GFP (panel D) and costained with antibodies against the Golgi 58K protein (panel B) and calnexin (panel E). Panels C and F represent the merged images. B, Unpermeabilised transfected COS-7 cells were immunostained with anti–LOX-1 antibodies.

weight of nonglycosylated proteins. The LOX-1–GFP band was, however, much fainter, probably because of the previously mentioned cytotoxic effect of this construct (Figure 4A, lane 2).

In Vivo Expression of LOX-1 and LOXIN Isoforms

To verify whether LOXIN transcript is indeed translated in vivo and to analyze the level of its expression, we used Western blot to examine the relative amounts of the 2 isoforms in PBMCs derived from selected subjects with different haplotypes. As shown in Figure 4B, immunoreaction of cell lysates showed 2 major bands of 34 and 22 kDa, corresponding to the predicted molecular weight of the 2 isoforms. Interestingly, we noticed a relative increase in the amount of LOXIN in cells derived from subjects homozygous for the "non-risk" haplotype (Figure 4B). Removal of N-linked glycans by PNGase digestion (Figure 4B) resulted in the disappearance of few faint bands, indicating that the two bands, 34 and 22 kDa, correspond to the unglycosylated LOX-1 and LOXIN proteins. In many gels, including the one shown in Figure 4B, a third band of 26 kDa was also observed. This band may represent a degradation product of the LOX-1 protein that we are currently studying.

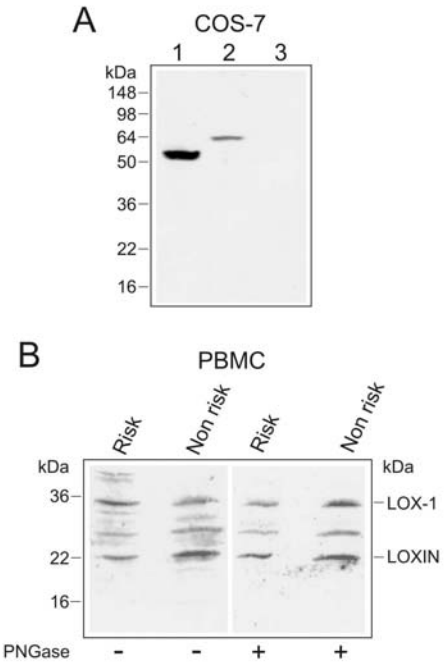

Figure 4. Intracellular expression of LOX-1 and LOXIN isoforms. A, Western immunoblot analysis of cellular lysates derived from mock-transfected (lane 3) or COS-7 cells transiently transfected with DNA encoding for LOXIN-GFP (lane 1) and LOX-1–GFP (lane 2). The 2 isoforms were detected with rabbit anti–LOX-1 antibodies. B, Western immunoblot analysis of cellular lysates of PBMCs derived from subjects carrying the "risk" (lanes 1 and 3) and "non-risk" (lanes 2 and 4) haplotypes incubated for 3 hours with PNGase F (1000 U), as indicated.

In Vivo Proapoptotic Effect of LOX-1 and Rescue By LOXIN

We considered that an altered balance between the 2 isoforms could be related to the increased susceptibility to apoptosis. To test this notion, we analyzed oxLDL-induced apoptosis in a monocytes/macrophages in vivo assay.[19] Human monocytes were isolated from buffy coats of healthy donors carrying the "risk" and "non-risk" haplotype at LD block of *OLR1* gene. After Ficoll gradient centrifugation, monocytes were separated to induce differentiation in macrophages. 100 μg/mL of oxLDL was added to these cells to induce apoptosis. Remarkably, flow cytometric analysis of macrophages derived from subjects homozygous for the "non-risk" haplotype showed a 24% reduction in apoptotic cell number compared with the homozygous for the "risk" haplotype when treated with the oxLDL (Figure 5A). Our data suggest that increased levels of LOXIN in macrophages may relate to reduced level of apoptosis.

Because the 2 isoforms are physiologically coexpressed in PBMCs with a different balance related to the haplotype, and

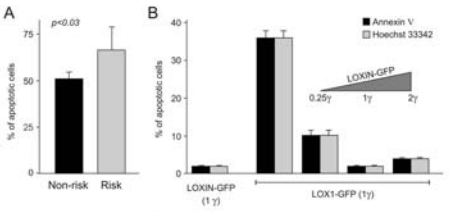

Figure 5. Protective effect of LOXIN expression. A, Monocytes were isolated from PBMCs of 10 subjects homozygous for the "risk" haplotype and 10 for the "non-risk" haplotype. Their transition to macrophages was promoted in vitro. After 8 hours of incubation with 100 μg/mL of oxLDL, the number of apoptotic cells was counted by multiparameter flow cytometry. B, Simian COS-7 fibroblasts were transiently transfected with DNA encoding for LOXIN-GFP and LOX-1–GFP alone or cotransfected with a fixed amount of LOX-1 and increasing concentration of LOXIN-GFP plasmids (as indicated). At least 100 positively transfected cells for each plasmid were counted and checked for their positive reaction to phosphatidylserine and Hoechst 33342. The average of the percentage of apoptotic cells in cells transfected with GFP alone (6%) was subtracted to all experimental points. The results shown are the average of 3 different experiments.

increased LOXIN levels relate to the "non-risk" haplotype, we explored the possibility that LOXIN isoform may have a protective effect versus apoptosis. To test this hypothesis, we transfected COS-7 fibroblasts with DNA encoding for LOXIN-GFP and LOX-1–GFP alone or cotransfected with a fixed amount of LOX-1 and increasing concentration of LOXIN-GFP plasmids (as indicated in Figure 5B). The phenotype of transfected cells was analyzed using 2 markers of apoptosis. First, we used Annexin V, which detects alteration at the level of the plasma membrane. Next, we examined the uptake of blue-fluorescent Hoechst 33342 dye, which stains the condensed chromatin of apoptotic cells more brightly than the chromatin of nonapoptotic cells. On the basis of the combined staining patterns of these dyes, we were able to distinguish between normal, apoptotic, and dead cells. As mentioned above, cells expressing the LOX-1–GFP fusion protein did not thrive, with many of the cells exhibiting cell shrinkage, which is a feature of apoptosis. Thus, LOX-1–GFP alone resulted in 36% of cells developing apoptosis. In contrast, LOXIN expression was not toxic and the number of apoptotic cells was comparable to the mock-transfected cells. Interestingly, the coexpression of LOXIN-GFP resulted in a complete dose-dependent rescue of the LOX-1-GFP–induced phenotype. It is worth noting that a very low dose of LOXIN, corresponding to a ratio of 1:4 with LOX-1, resulted in a 72% reduction in the number of apoptotic cells, and a 1:1 ratio of the 2 plasmids used for transfection completely prevented the phenotype. This finding suggests that small differences in LOX-1/LOXIN balance and, especially, a small increase in LOXIN expression, may have a very profound effect on the cytotoxic phenotype also in vivo.

Discussion

During genetic screening, it is a common experience to end up with a large fraction of orphan variants with an unclear

pathogenetic role.[20] It is therefore essential to find a plausible biological basis for each such association to strengthen the genetic significance of SNPs in prediction of complex diseases. This article reports, for the first time, the identification and characterization of a new functional isoform of the *ORL1* gene and provides a functional explanation of the genetic association between SNPs within *ORL1* gene and myocardial infarction.

We show that SNPs located in the LD block modulate the relative abundance of the 2 transcripts. Both in vivo and in vitro experiments indicate that the new splice variant LOXIN is expressed at a similar level as the full-length receptor LOX-1. Interestingly, macrophages from subjects carrying the "non-risk" haplotype express more LOXIN and result in fewer cells undergoing apoptosis on oxLDL induction. Macrophages play an important role in all phases of atherosclerosis, from the development of the fatty steak to the process that ultimately contribute to plaque rupture and myocardial infarction.[21–23] Indeed, evidence from pathological studies link apoptosis of plaque resident macrophages with rupture and thrombosis of atherosclerotic lesions and subsequent development of acute vascular complication. In particular, it has been proposed that extensive apoptosis of macrophages occurs only at sites of plaque rupture and possibly contributes to the process of rupture and thrombosis.[24] Our finding that increased levels of LOXIN in macrophages relate to reduced levels of apoptosis suggests that this in turn could result in plaque stabilization by influencing the nature of the vulnerable plaque.

We report that C-terminal splice variants of LOX-1 receptor differentially traffic from the ER to the plasma membrane. In cell lines we show that LOXIN isoform displays no surface expression in 90% of transfected cells and lower expression in 1/10 of the cells. This is attributable to retention of LOXIN in the ER and accumulation of the protein in the perinuclear region. Regulation of the trafficking of LOX-1 receptors to the plasma membrane may provide an essential mechanism for the control of its function in vivo. In this context, different splice variant isoforms in the C-terminal domain have been reported for other receptors, such as glutamate receptors. In this case, the domain has been shown to be the site of interactions of proteins involved in the trafficking and stabilization of glutamate receptors in the synaptic membrane and, in turn, to play a role in synaptic strength and plasticity.[25,26] Whether LOXIN forms heteromeric receptors with LOX-1 and regulates its intracellular traffic in vivo is under study.

LOX-1 receptor, when ectopically expressed in fibroblasts, is toxic and results in a high percentage of cells undergoing apoptosis. We demonstrate here that LOXIN molecules, when coexpressed with LOX-1 receptors, have a protective role and are able to rescue the apoptotic phenotype. The mechanism by which LOXIN exerts its protective role is not known but we propose different hypotheses. First, LOXIN may act on LOX-1 receptors by forming inactive heterodimers. Because the LOXIN splice variant lacks exon 5, it misses the binding region of ox-LDL. By increasing the intracellular LOXIN relative amount, the number of functional receptors able to bind oxLDL may be reduced. Because oxLDL induces apoptosis in macrophages, thereby reducing oxLDL binding sites, fewer cells will go through apoptosis. Secondly, LOXIN may exert its action on the transport of LOX-1 receptors toward the cellular membrane, blocking them into the ER and downregulating their membrane expression. Thirdly, the LOXIN itself may even have an antiapoptotic activity. These different hypotheses warrant further studies.

It is important to note that the expression level of both isoforms in vivo is similar. Subjects homozygous for "non-risk" haplotype show a small increase in LOXIN expression compared with homozygous for "risk" haplotype. In vitro experiments confirmed this fine balance between the 2 isoforms. A small amount of LOXIN is sufficient to rescue the lethal phenotype.

We conclude that SNPs located in the LD block of *OLR1* gene, with their functional role, may represent an important genetic risk factor for prediction of susceptibility to myocardial infarction. Moreover LOXIN could represent a new target for prevention of progression and destabilization of atherosclerotic plaque. Therefore, new research or therapeutic strategies favoring increasing expression of LOXIN could be effective for preventing plaque instability.

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ELSEVIER

Cellular Genetic Therapy

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ABSTRACT

Cellular genetic therapy is the ultimate frontier for those pathologies that are consequent to a specific nonfunctional cellular type. A viable cure for there kinds of diseases is the replacement of sick cells with healthy ones, which can be obtained from the same patient or a different donor. In fact, structures can be corrected and strengthened with the introduction of undifferentiated cells within specific target tissues, where they will specialize into the desired cellular types. Furthermore, consequent to the recent results obtained with the transdifferentiation experiments, a process that allows the in vitro differentiation of embryonic and adult stem cells, it has also became clear that many advantages may be obtained from the use of stem cells to produce drugs, vaccines, and therapeutic molecules. Since stem cells can sustain lineage potentials, the capacity for differentiation, and better tolerance for the introduction of exogenous genes, they are also considered as feasible therapeutic vehicles for gene therapy. In fact, it is strongly believed that the combination of cellular genetic and gene therapy approaches will definitely allow the development of new therapeutic strategies as well as the production of totipotent cell lines to be used as experimental models for the cure of genetic disorders.

CELLULAR GENETIC THERAPY is the ultimate frontier for those pathologies that are consequent to a specific nonfunctional cellular type. A possible cure for these kinds of diseases is the replacement of sick cells with healthy ones, obtained from the same patient or a different donor.

Using available cellular therapy strategies, altered structures can be corrected and strengthened with the introduction of undifferentiated cells within the specific target organs or tissues, where they specialize into various cellular types. In particular, the introduction of those undifferentiated cells was successfully utilized as part of the curative treatment of tumor tissues previously treated with chemotherapy and/or radiation therapy, in leukemia children and in immunodeficiency diseases.

Furthermore, cellular therapy has gained more interest after the results obtained with the transdifferentiation experiments of the last 5 years. This process allows the in vitro differentiation of embryonic and adult stem cells.[1–3] In fact, it has become immediately clear that many advantages could be obtained from the use of the stem cells to produce drugs, vaccines, and therapeutic molecules. As well, they may be exploited to set up new diagnostic technologies for prevention of hereditary diseases and to generate cellular clones to be used in transplantation and tissue regeneration.[4]

EMBRYONIC AND ADULT STEM CELLS

Stem cells can be obtained from embryo, adult, or fetal differentiated tissues. Embryonic cells are "totipotent," which means that they are able to produce all possible cellular types and therefore all the tissues of an adult body. The stem cell definition is shifting constantly. At first it referred to blood and germinal line cells only and later was also extended to cells belonging to all the tissues believed to be able to display autoregeneration. In the latest years, the definition of stem cell has changed again, consequent to the recently discovered functions, properties, and localizations.

In the 19th century authors such as Regaud,[5] who studied spermatogenesis, hypothesized the existence of tissues able to regenerate in the course of an organism's life cycle. At the same time, the hematologists Weidendreich and Maximow theorized the possibility that blood cells arose from a common ancestoer cell type, and therefore were indispensable for their constant regeneration.[6] These considerations

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set the initial bases for the standardization of several clinical protocols such as the one for the bone marrow transplant.[7] Up to now, nervous, bone, and muscle tissues were demonstrated to contain stem cells, although it is not completely clear if they were able to display in vivo regeneration.

Often a cell is defined as a stem cell by its biological abilities only demonstrated experimentally, since, after being isolated and amplified in vitro, they generated unlimited quantities of the native tissue. As well, stem cells may have an ability to both independently proliferate and produce cellular clones only ex vivo.

Among the characteristics of a stem cell is a capacity for asymmetric generation of two novel cells: one identical to the parental one and the other able to differentiate into all cell types derived from the three embryonic layers—mesoderm, ectoderm, and ectoderm. The overall biological proprieties previously cited allowed us to recognize the presence of stem cells also in some tissues that apparently did not show any autoregenerative ability.[8]

Different sources of stem cells have been identified: blastocysts obtained from frozen embryos at different developmental stages using the technique of in vitro fertilization; extra embryonic fetal tissues (amniotic liquid, fetal blood) acquired from postimplant fetal trophoblast (from premature spontaneous abortion); and cell lines commercially available after in vitro inception, starting from clones initially isolated in laboratories. Actually there are at least 72 different cell lines on the market.

To those "traditional" sources has been recently added a "revolutionary" technique that allows in vitro production of stem cells directly from the cells of a sick patient (autologous) by reprogramming the nucleus of the mature somatic cell. The nucleus is introduced into an enucleated oocyte, consequently generating the formation of totipotent "embryoid spheres," able to proliferate and differentiate into numerous cell types.[9] Such a method opens the route for the "induced transdifferentation" that will hopefully bring production of stem cells directly from a somatic cell. This will be possible when the cytoplasmic factors of the oocyte have been identified. The cells so obtained possess the same nuclear genome as the somatic cell donor; therefore they are immunologically compatible for an self transplant.

There are five different stem cell types:

- *Heterologous stem cells:* Derived from the internal area of the embryo, before its implantation into the uterine wall. They reproduce themselves easily and can differentiate into all the different cell types present in an adult organism. They can be isolated from the embryo during its early developmental stages and then cultivated in the laboratory.
- *Autologous stem cells:* Isolated after the transfer of a nucleus from a somatic cell into an enucleated oocyte. These types of stem cells have the same genetic heritage as the donor and can be transplanted without any risk of rejection.
- *Fetal stem cells:* Pluripotent cells derived from abortions. From the information available it is not possible to clarify their ability to differentiate for the production of different tissues.
- *Stem cells from umbilical cord:* They would allow the creation of a personal cell bank for each newborn baby, a natural resource for therapy through the years. Their value has been recently demonstrated in vivo[10] in mice affected by an autosomal hereditary recessive disease, the amyotrophic lateral sclerosis.
- *Adult stem cells:* Those cells provide the maintenance and damage repair of tissues. Sources of stem cells are also some differentiated tissues, such as bone marrow, liver, skeletal muscle, umbilical cord, and adipose tissue. Recently stem cells have been localized even in the nervous central system.[11–13]

Most of the information regarding particular stem cells has been derived from the bone marrow and its ability to generate different cell types. Recent publications demonstrated that they can differentiate into muscle, cerebral tissue, cardiac cells, liver, kidney, and epithelial cells.[14–17]

Nevertheless, recently, a lot of incestious exist regarding adult stem cell "plasticity."[18] In fact, some of those "plasticity" experiments have not been reproduced; for example, while the Margaret Goodell group failed to find in vivo transdifferentiation of bone marrow cells into neural cells, in the same year another paper demonstrated a direct differentiation of embryonic cells in to motor neuronal cells.[19] Two different studies showed that in some cases there is no transdifferentation but a cellular fusion that brings chromosomal aberrations and therefore cannot be used for clinical applications.[20,21]

Also, it is possible that some of the regeneration potentials, which have been described for tissues such as muscule and bone, are due to the hematopoietic stem cells already circulating, as reported in a recent study on stem cells derived from muscle tissue.[22] Therefore, in order to avoid similar artifacts, it is a priority to characterize novel specific markers that identify stem cells of different origin. As well, it is necessary that genetic markers be expressed by the transdifferentiated cells not only under in vitro but also in vivo conditions.

Anyway, the regeneration potential is inferior to adult stem cells where compared to embryonic stem cells. First, adult stem cells are more difficult to isolate and propagate in vitro than embryonic ones. Also, embryonic stem cells can be genetically manipulated by homologous recombination techniques,[23] whereas for adult stem cells only transgenic retroviruses have been introduced up to now. Such products are overexpressed at variable levels once introduced into the host cell, but unfortunately it has also been shown that, due to their random insertion into the genome, they can cause mutagenic phenomena that induce cancer in some cases.[24] Finally embryonic stem cells can be differentiated to originate all cellular types desired by utilizing the

appropriate in vitro culture conditions, whereas this cannot be obtained yet with adult stem cells.

Another limit to stem cell therapeutic use is the possible immunological incompatibilities once introduced into a receipient organism; for example, a major technical limit in a transplant aimed to repair damaged tissues is the rejection of the transplanted stem cells.

STEM CELL–MEDIATED GENE THERAPY

Somatic gene therapy is a genetic engineering technique aiming to correct a genetic defect through the insertion into the target cell of exogenous genetic material, which is driven in by specific transfer vectors. In this way the cell receives the exact genetic information, so in the mutated cells the correct structure of the corresponding protein and its functions are restored.

Up to now, different attempts have been performed in vitro and in vivo, and different strategies, have been tested involving both viral and nonviral vectors.[6,25–27]

The idea of the gene therapy is to use the DNA as a drug. Anyway, most of the time, it means supplementing DNA able to produce a protein with a therapeutic function. For example, this means the production of a protein that is lacking or deficient in the patient, as for the protein in Duchenne disease or the CTFR protein in cystic fibrosis disease, two serious inherited pathologies. Otherwise a protein, such as erythropoietin, a hormone that regulates hematic cell production, in some cases of anemia and talassemia can restore to normal the low efficiency of red corpuscles, although it does not substitute for the defective hemoglobin in such patients. Another application regards the cure of cancers; in fact, although tumors are not caused by the lack of a single protein in the body, it is believed possible to boost the immune system of the sick organism by inducing, with the use of the gene therapy, an overproduction a cytokine that regulate the immune system or of other proteins that make the carcinogenic cells more easily recognizable to the immune system.

Many difficulties are encountered in the attempt of applying gene therapies protocols, which are different in relation to the target organ to be cured. All the current gene therapy techniques imply the use of carrier vectors, able to transport the genetic material inside the cell nucleus. At the beginning of the 1990s, in particular the retrovirus vectors brought a lot of hope and optimism, since they were easy to use and efficient. Unfortunately, later it was observe that undesired immune responses resulted from the use of these retrovirus vectors and that it was not possible to obtain prolonged expression of the introduced genes.

The safest way to transport exogenous genetic material into target organisms is to introduce these directly as naked DNA into the cells and tissues. The main limit to the application of such a procedure is the huge degradation that the introduced naked DNA encounters before reaching the nucleus. Anyway, recent new promising techniques of "microinjection" seem to allow DNA introduction directly into the nucleus of a single cell by the use of extremely small needles. At present all efforts are concentrated in improving techniques for the transfer of genetic material into the cell/tissue. In the latest years different vectors have been used. Some of them provide the possibility of expressing an exogenous gene only in a specific cellular type or ensure prolonged expression of a gene, therefore providing few or even only a unique treatment option for the patient.

Up to now, the vectors tested may be distinguished as viral and nonviral. The viral system comprises the retrovirus, able to convert their RNA into DNA so as to infect the cell. They insert themselves into the genome and may interfere with other gene activities. In fact recently after the application of gene therapy protocols with the use of retrovirus vectors to two children with X-linked severe combined immunodeficiency, they manifested the onset of leukemia.[28]

Among the other types of viral system utilized are the adenoviruses, the adeno-associated virus, and lentiviruses such as HIV, which allow the infection of mature cells and cells in which adenoviruses do not work as vectors, and the herpes viruses, particularly studied since they are highly specific for the nervous system. Nonviral vector systems comprise liposomes (lipid spheres where the DNA is enclosed) or positively charged cationic polymers, able to condense the DNA.

Liposomes are the vectors largely used both in vitro and in vivo. They transport DNA contained into the target cell, through an endocytosis process. In comparison with viruses liposomes are much more safe but less efficient and selective.

Different approaches have been used in gene therapy. The DNA can be transfer directly into the cells and tissues of the patient (in vivo gene therapy); otherwise, it is transferred into cells previously isolated from the patient that, once corrected, are reintroduced in the patient (ex vivo gene therapy). Other alternative gene therapy protocols aim to obtain the in situ correction of the defective gene by exploiting events of homologous recombination naturally occurring in the cells between two specific DNA sequences (gene targeting).[29] The correction event is obtained by introducing into the cell an exogenous DNA sequence able to specifically recognize and modify the target DNA sequence in the genome. It has been demonstrated that the exchange of genomic material happens through mechanisms of cellular homologous recombination. A main advantage of these techniques is that the gene correction obtained is permanent; therefore, there is no need for various DNA dosages to maintain the therapeutic effects in the patient. Also, by correcting the endogenous gene in situ, its integrity is preserved and, equally importantly, its expression is still naturally regulated by its own promoter.

The effectiveness of this technique has been proven in both immortalized or primary culture of human cells. The main drawback is that the homologous recombination events happen with a low frequency (10^{-5} to 10^{-7}). Many

attempts have been made to increase the recombination efficiency, but although improvements have been noticed for some time,[30] the system appears to be difficult to reproduce and also dependent on the target cell type. It is also believed that both cellular differentiation and chromatin condensation state influence the "targeting" efficiency. For this reason stem cells may be a better "target," since they have a great capacity for replication and also their chromatin is not very condensed. Totipotent stem cells are able to accept and easily tolerate exogenous genes. Once a stem cell is corrected, it can be differentiated into all the different cell types, allowed with the single introduction of an exogenous gene, namely, the correction of many cell types, such as blood cells, skin cells, liver cells, and even brain cells. The intrinsic corrective capacity of these cells is at least 10 times higher than of the already differentiated adult cells. Therefore they are definitely more suitable for the gene therapy approach.[31] An experiment has recently been described where embryonic stem cells were genetically modified through homologous recombination.[32] The engineered cells were then differentiated in vitro into neuronal cells, to demonstrate the stability of the genetic modification.

The work of Rideout et al[23] demonstrated the capacity of correction of the gene targeting approach. In this experiment nuclei from murine cells affected by severe immunodeficiency were transferred into enucleated oocytes. As a second step, embryonic stem cells were isolated from the blastocysts that had been cloned. Successively, the genetic defect present in the embryonic stem cells was corrected by homologous recombination. This approach combined two different techniques, opening new route for the cure of genetic diseases.

Another work demonstrated the possibility of obtaining in vitro oocytes from murine embryonic stem cells.[33] It is important to establish whether it is possible to obtain oocytes also from human embryonic stem cells. In this way it could be possible to generate autologous embryonic stem cell lines and ethical problems related to the use of oocytes would be avoided.

Finally, it has been reported that in Id knockout mouse embryos, which display multiple cardiac defects, the midgestation lethality was rescued by the injection of 15 wild-type embryonic stem cells into the mutant blastocysts. The myocardial markers altered in Id mutant cells were restored to normal throughout the chimeric myocardium. Also, intraperitoneal injection of embryonic stem cells into female mice before conception partially rescued the cardiac phenotype with no incorporation of embryonic stem cells. In addition, insulin-like growth factor I, a long-range secreted factor, in combination with WNT5a, a locally secreted factor, accounted for complete reversion of the cardiac phenotype. Thus, embryonic stem cells have the potential to reverse congenital defects through Id-dependent local and long-range effects in a mammalian embryo.[34]

In addition to all these considerations about the possible utilization of the embryonic stem cells, it is clear from these studies that they can also provide factors missing from mutant mammalian embryos and induce neomorphic effects that can compensate for the effects of the mutation. Such properties may imbue embryonic stem cells with a much greater therapeutic value than previously imagined.

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Therapeutic Strategies for the Treatment of Spinal Muscular Atrophy (SMA) Disease

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Abstract: Spinal Muscular Atrophy (SMA) is a progressive neurodegenerative disorder characterised by the loss of upper and/or lower motor neurons. SMA is the leading genetic cause of infant mortality with an incidence of 1 in 6000 live births and a carrier frequency of about 1 in 50. Different types of disease (from SMAI to SMAV) have been described based on clinical severity and age of onset. The SMA-determining gene, Survival of Motor Neurons (SMN), is part of a 500 kb-inverted duplication on chromosome 5q13. Within the duplicated genes SMN1 and SMN2 can be found. Most (95%) SMA patients have deletions or conversion events of SMN1. The SMN2 gene primarily produces a transcript which lacks exon 7 and of which only 10-20% of its protein is functional.

Although a variety of therapeutic trials are ongoing, only life-prolonging treatments are being developed. The knowledge gained regarding the pathogenesis of SMA remains limited, because the precise function of SMN is not yet known. Furthermore, it is not quite clear why motor neurons of the patients are the only cell type for which SMN expression level are unadequate for their normal activity, even if the affected genes have "housekeeping" functions.

Both pharmacological or genetic approaches have been conducted for the therapy of SMA. Moreover, stem cells provide a further aspect to be analysed. In fact, the genetic modification of a small number of stem cells could give rise to a dividing population of therapeutic cells.

These innovative approaches when united could be usefully adopted to replace lost cells and at the same time protect surviving motor neurons in SMA patients.

Key Words: Spinal Muscular Atrophy, gene therapy, gene targeting, pharmacological treatment, survival motor neuron gene.

INTRODUCTION

Spinal Muscular Atrophy (SMA) is a heterogeneous group of disorders characterised by the degeneration of alpha motor neurons in the anterior horns of the spinal cord and the lower brainstem. SMA occurs in childhood with muscle weakness and respiratory failure. Muscle weakness is always symmetrical and associated with a marked decrease in deep reflexes, muscle fasciculation and hypotonia. The disease represents the most frequent inherited cause of infant mortality [1].

Depending on the age of manifestation and achieved motor abilities, SMA has been classified into four different types. Type I patients (approximately 50% of all SMA cases) are never able to sit or stand unaided and usually die before the age of 2 years. Type II SMA patients are able to sit, but are never able to stand, whereas type III SMA patients are able to sit and stand, but mostly get wheelchair bound while muscle weakness is progressing [2]. The mildest form of SMA, type IV, is defined as a slowly progressing disease, which typically occur after the age of 30 [3]. Regardless of SMA type, disease progression and life expectancy is strongly correlated to age of onset. Moreover, patients with SMA tend to have the greatest rate of loss of muscle power at disease onset [3].

Mutations of the Survival of Motor Neuron (SMN) locus, on chromosome 5q13, are responsible for the disease. In humans, the SMN locus contains an inverted duplication consisting of the telomeric SMN1 gene and the centromeric, highly homologous SMN2 gene. More than 95% of SMA patients are homozygously deleted for SMN1 [4], and the remaining cases (5%) show missense, nonsense or splicing mutations within SMN1 gene [5]. All patients deleted for SMN1 retain at least one centromeric copy of the gene, *SMN2*, which is unable to complement SMN1 because of a crucial mutation in an exonic splicing enhancer [6], leading to an alternative splicing phenomenon. In fact the SMN2 gene mainly produces a protein isoform, which has exon 7 deleted (delta7 SMN) (about 80%), and reduced amounts of full-length (FL-SMN) mRNA (about 20%). Delta7 SMN encodes for an unstable protein and consequently the insufficient levels of SMN protein in motor neurons represents the cause of the disease phenotype. To support this hypothesis, there is evidence of a clear inverse correlation between the severity of the SMA phenotype and the SMN2 copy number. SMN2 is the major modifier of SMA phenotype and the critical parameter being the amount of total SMN (both full-length and delta7) it can produce [7]. In fact, the more SMN2 copies a patient has, the milder the phenotype [8-10]. Other evidence sustaining this supposition comes from *Smn*

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knockout mice. In fact introducing one or two copies of human SMN2 in this murine model makes it possible to improve the clinical phenotype, trasforming the embryonic lethal phenotype into a severe one [11, 12].

SMN is a protein of 38 kDa involved in a series of pathways, the most essential of which is the housekeeping function in snRNP biogenesis and spliceosome assembly [13,14] and the neuron-specific function in RNA transport along the axons [15-17]. The SMN gene does not have an exclusively neuronal expression or function, but it is ubiquitously expressed, with only few tissue and developmental differences [18,19]. Within a single cell, the protein is present in both the cytoplasm and the nucleus, where it is concentrated in two sub-structures that often overlap, Cajal bodies and Gems [16,17,20,21]. Gems contain high levels of factors involved in transcription and RNA processing. At least seven additional proteins (from Gemin2 to Gemin8) are stably associated with the SMN protein in large macromolecular complexes [22,23].

The number of gems inversely correlates with disease severity, with type I patients showing few or even no gems [24,25] while there is an increase in type II and III patients. For this reason the number of gems per cell drastically decreases in SMA patients, and represents a useful prognostic indicator of disease severity. Also the localisation of the gems correlates with the phenotype, whereas in affected cells the gems are localised more within the cytoplasm than in the nuclei compared to wild type cells [13,26].

However the precise function of SMN is not yet known. Furthermore, it is not quite clear why SMA motor neurons are the only cell type for which SMN expression levels are unadequate for their normal activity.

To date, no effective therapy exists which is able to rescue the motor neuron degeneration of SMA patients. However, the discovery of the SMA-determining gene, and of the differences between *SMN1* and its highly homologous copy *SMN2* in the human genome, has led to the development of novel therapeutic strategies for SMA.

To this aim, *SMN2* provides a natural target for therapeutic intervention in almost all SMA patients. The general hypothesis is that increasing full-length SMN protein levels should have a positive impact on the onset and progression of SMA disease. This goal can be reached by increasing the expression of SMN, or changing the splicing pattern of the SMN2 gene or even stabilizing the SMN protein.

The therapeutical approach could be pharmacological or genetic. Moreover the development of stem-cell therapies has great potentiality, since this recent developments in stem cell differentiation and transplantation.

PHARMACOLOGICAL THERAPY

Different mechanisms have been investigated for increasing the expression of a stable SMN protein. They include the increase of the full-length/delta7 (FL/Δ7) ratio of the SMN transcript, the suppression of exon 7 alternative splicing in the SMN2 derived transcript or the stabilization of SMN protein level.

Aclarubicine and sodium vanadate (a phosphatase inhibitor) act exclusively facilitating the inclusion of exon 7 into the SMN2 transcript. This effect can be detected in type I SMA fibroblasts in which both SMN protein and gems are restored to normal levels, suggesting how alteration of the splicing pattern in SMA disease could represent a promising therapeutic approach [27]. Additionally sodium vanadate markedly promotes the exon 7 inclusion within the *SMN2*-derived mRNAs *in vivo* [28]. Unfortunately, the toxicity of these drugs makes it impossible to use them its long-term basis in SMA patients.

Another approach consists of epigenetically regulating the SMN promoter activity. To do this different histone deacetylase (HDAC) inhibitors have been used, such as sodium butyrate [29], valproic acid [30,31], phenylbutyrate [32] and benzamide M344 [33]. It has been demonstrated that these compounds increase the full-length SMN2 transcript and protein levels by modulating the histone acetylation state of the SMN promoter sequence both *in vitro* and *in vivo*. The mechanism is clear: HDAC inhibits histone acetyltransferases so making the promoter chromatin more transcriptionally active. Moreover, some of these drugs are already FDA approved and used in therapeutic protocols of several diseases.

Of the HDAC inhibitors, sodium butyrate was the first to be reported as able to increase FL transcript levels in SMA I cells *in vitro*. Subsequently both phenylbutirrate and valproic acid have been used in clinical trials in SMA patients, who well tolerated them [34]. The two substances also favour the raising of SMN2 gene expression .

Valproic acid (VPA) has been shown to increase the total amount of functional SMN protein and also promote neurite outgrowth *in vitro* [35] through an independent mechanism. Another group reported the effect of VPA *in vitro* [30] and *in vivo* [36] both in SMA patients and carriers. This drug is able to increase SMN transcript and protein levels using tolerated doses, but the response is variable among different subjects[30]. VPA could prevent neuron death [37] and enhances axonal regeneration [38], so preserving motor function in SMA. A recent paper for the first time confirmed the therapeutic effects of VPA in a SMA model mice (type III) [39]. Authors reported an attenuation in motor neuron death with an increase of SMN protein level in the spinal cord, in blood cells and a partial normalization of motor function in the affected mice. Clinical trials are now ongoing at the University of Utah in collaboration with Families of Spinal Muscular Atrophy, and Sigma Tau Pharmaceuticals, Inc. Abbott.

Benzamide M344, a novel HDAC, has been described as an effective treatment for restoring both the correct splicing of SMN2 and consequently the total number of gems per nucleus as well as the number of nuclei containing gems *in vitro*. The therapeutic effect is the highest reported compared to other HDAC inhibitors [33].

Recently a histone deacetylase inhibitor of second-generation, SAHA (suberoylanilide hydroxamic acid), was tested as a potential candidate drug for the treatment of SMA. Used at low micromolar concentrations SAHA increases SMN levels in several neuroectodermal tissues, in-

cluding rat hippocampal brain slices and motoneurone-rich cell fractions, and also using a novel human brain slice culture assay. SAHA is well tolerated with no dose-limiting toxicity, as already experimented in clinical trials for cancer treatment. By epigenetic SMN2 gene activation, this compound slows down the progressive α-motor neuron degeneration caused by insufficient amounts of SMN protein [40].

Due to its capacity in increasing the full-length SMN transcript and the gem number in SMA lymphoblastoid cell lines [41], another compound known to be a cell cycle inhibitor, hydroxyurea, is now used in clinical trial in Taiwan and in the United States.

Also the read-through effect of aminoglycoside antibiotics has been tested on type I SMA patient fibroblasts. More specifically amikacin and tobramycin have been shown to be able to quantitatively increase gem numbers and protein levels, without being cytotoxic. The effect is due to the read-through of the SMNΔ7 stop codon in exon 8, conferring additional stability to the unstable SMNdelta7 product. Aminoglycosides act differently from the other compounds and for this reason can be considered as an integrating treatment for increasing SMN levels [42].

Novel aminoglycosides (structurally related to neomycin and kanamycin) were recently tested for determining their capacity to induce higher levels of SMN expression measured by gems and protein levels in SMA I fibroblasts. Six of these were found to be able to suppress accurate stop codon recognition , increasing the protein length in five amminoacides and so stimulating SMN protein levels up to wild-type patterns [43]. However authors can not assure the suitability of these compounds for SMA treatment.

Indoprofen is a non steroidal anti-inflammatory drug able to gain the translation efficiency of SMN2-derived transcripts. It has been tested *in vitro* on human type I SMA patient fibroblasts, demonstrating its ability to increase both nuclear gems and SMN protein level. Moreover, tested on SMA model pregnant mice ($Smn^{-/-}$; $SMN2^{+/+}$), indoprofen also raised the viability of SMA model mice [44], even though a poor central nervous penetration was reported.

Finally another approach for SMA therapy was conducted using riluzole, a glutamate inhibitor already tested in amyotrophic lateral sclerosis (ALS) patients. It was tested both on SMA mice [45] and in SMA I patients [46]. No adverse effect was reported, but the tested group was too small to draw any concrete conclusions regarding its real effect.

Several clinical trials are currently in progress, which have been carefully reviewed by Sumner *et al.* 2006 [47] and Hirtz *et al.* [48]. Of these, a private biopharmaceutical company (involved in developing drugs to prevent neuronal loss in patients with neurodegenerative disease) recently reported the successfull completion of phase 1 clinical trials for a novel compound named TRO19622 for treating neurodegenerative diseases (www.trophos.com). In preclinical studies, this compound has been demonstrated to promote the survival of a wide range of neurons *in vitro*, as well as in several *in vivo* models of neurodegenerative diseases. Due to a good patient tolerance and an excellent safety profile, this company will initiate a pivotal Phase II/III clinical trial for the treatment of SMA in the EU.

GENE THERAPY

A strategy that could theoretically lead to a cure for SMA involves the replacement/restoration of the *SMN1* gene or function using a gene therapy approach. Unfortunately, development of gene therapy in general has been limited because of the technical difficulties of efficient gene delivery to target tissues as well as problems related to the random insertion of the therapeutic gene into the host DNA. Di Donato *e coll.* first demonstrated the feasibility of a viral gene therapy approach which is adenovirus-mediated [49]. His paper reported the successful restoration of gene expression and consequently of gem number, after delivering SMN cDNA in primary fibroblast cell lines derived from SMA patients [49]. Moreover, the interaction of exogenous SMN protein with the other component of the gem complex brought about the restoration of gem function in SMA cells.

Another gene replacement approach was successfully conducted delivering the SMN gene both *in vitro* and *in vivo* (into SMA mice muscles) by using a lentiviral vector [50]. A beneficial effect was obtained, restoring SMN levels and increasing the life expectancy in type I SMA mice compared to the untreated animals.

Besides viral approaches of gene complementation, gene-targeting strategies have been successfully attempted. Gene targeting induces a site-specific chromosomal modification that leads to long-term and genetically inheritable expression of the correct gene, regardless of its size, preserving the integrity of the gene. The availability of better delivery methods has recently made nonviral transfer a safer and more viable method for gene therapy.

One of these oligonucleotide-based gene-targeting strategies, Small Fragment Homologous Replacement (SFHR), has been successfully used to correct or modify gene sequences responsible for some inherited diseases *in vitro* and *in vivo* [51], including SMA [52]. In this work, our group investigated the efficacy of SFHR to rescue the physiological level of *SMN* transcript and protein, after the transfection of a 498-bp DNA fragment containing the *SMN1* exon 7 sequence in human fetal cells, obtained from embryos affected by SMA. The *in vitro* conversion of the *SMN2* gene into the *SMN1* one and the modification of the splicing pattern of SMN was demonstrated. In particular a high increase in full-length transcript (from 7 to 53%) and consequently a significant 2-fold increase in wild-type SMN protein were obtained. Furthermore, genotype and phenotype of transfected cells remained stable after several *in vitro* passages, demonstrating the stability of the correction over time.

At the RNA maturation level, SNMN2 exon 7 is strictly regulated by complex interactions between RNA splicing factors and *cis*-acting elements located in both exonic and intronic sequences. A purine rich exonic splicing enhancer (ESE), located near the center of exon 7, is required for full-length SMN expression and is bound directly by the SR-like factor hTra2b1 [53,54]. Moreover, the critical C/T transition falls within an SF2/ASF binding site in SMN1 which is abrogated in the SMN2 pre-mRNA [55]. Additionally to this effect, the C/T transition creates a novel hnRNPA1 dependent splicing silencer (ESS) able to prevent efficient exon 7 splicing in SMN2 [56]. Other regulatory elements within

exon7 and the flanking introns have been described, including the Extended Inhibitory Context , at the 3' splice site (ss) of exon 7 and the ISS-N1 silencer, downstream of the 5' ss in intron 7 [57-60]. These discoveries highlight the complexity of SMN1/SMN2 pre-mRNAs splicing and provide unique targets for the development of new therapies aimed at compensating for the SMN1 genomic loss. Alteration of the SMN2 pre-mRNA splicing process to generate a higher fraction of full length SMN product, may therefore be a promising therapeutic approach to treat SMA. This strategy has significant advantages compared to gene replacement. In fact no site-specific integration in the genome is required and the endogenous gene expression pattern is maintained. Alteration of SMN2 gene splicing has been attempted by investigators who have used RNA-based gene therapy approaches with antisense oligonucleotides directed towards the 3' ss of SMN2 exon 8 [61]. These molecules reduce recognition of the 3' ss in exon 8 favouring the incorporation of exon 7, which contains the authentic translation stop, into the fully processed transcript. In *vitro* experiments have demonstrated a 5-fold increase in the ratio of SMN2 exon 7 inclusion over exon 7 skipping. An evolution of this system consists in the creation of synthetic chimeric effectors constituted by a minimal RS peptide domain, covalently linked to an antisense moiety that targets SMN2 gene sequences specifically [55]. To increase the efficacy and longevity of the antisense oligonucleotides, an expression system that incorporates the anti-SMN region into the U7 small-nuclear RNA has also been developed [62]. Similarly, a novel synthetic RNA class, called bifunctional RNAs, has been designed [63]. Bifunctional RNAs derive their name from the precence of two domains: an antisense RNA sequence specific to a target RNA and an untethered RNA portion that functions as a binding platform for splicing factors. In this way, bifunctional RNAs, although complementary to the target exon, do not block the splicing reaction at their binding site like conventional antisense RNA. SMN2-directed oligoribonucleotides contain an SMN2 exon 7 targeting domain and a non- complementary tail region that mimics exonic splicing enhancer motifs to provide trans-splicing activators. It is possible to express bifunctional RNAs that modulate SMN2 splicing from recombinant adeno-associated virus vectors (rAAV), which efficiently transduce both myocites and neurons and which result in a stable, long-term expression of the transgene [64]. In addition, rAAV virions can be retrogradely transported to neurons following intramuscular injection, which makes this approach particularly suitable for the development of a SMA molecular therapy.

CELL REPLACEMENT

Another strategy that might eventually play a role in SMA treatment is "cell replacement". Embryonic stem (ES) cells are pluripotent cells that can be differentiated into motor neurons, to replace the damaged ones, as in SMA patients. A major problem however is to get these cells to differentiate into the appropriate cell type and induce them to leave the spinal cord and make contact with muscle. ES cells could be also useful for providing trophic support and protecting surviving motor neurons [65,66]. Stem cells could be isolated and transplanted onto the affected spinal cord, after pre-differentiation and genetic modification in culture to form specific motor neuron cell types. Therefore, by combining both gene therapy and cellular therapies, it should be possible to reduce the risks associated with genetic (viral or not) and cellular therapies since the growth characteristics of the cells, the insertion site of the virus and the ability to regulate transgenes could be determined and checked prior to transplantation.

Recent studies have reported how to successfully differentiate *in vitro* ES cells into lower motor neurons able to establish functional synapses with muscle fibres after transplantation in rats [67,68]. Recently, it was demonstrated how transplanted embryonic stem cell-derived motor neurons can functionally replace *in vivo* those cells destroyed in paralysed adult rats. Transplanted axons reached muscles and formed neuromuscular junctions [69,70].

In conclusion, an effective treatment for SMA will probably depend greatly on the comprehension of the function of the SMN protein in motor neurons and of the pathophysiology of the disease in animal models. Answering these questions it will be useful to better understand this devastating disease and find an adequate treatment.

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WEB ADDRESS

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CFTR GENE TARGETING IN MOUSE EMBRYONIC STEM CELLS MEDIATED BY SMALL FRAGMENT HOMOLOGOUS REPLACEMENT (SFHR)

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1. ABSTRACT

Different gene targeting approaches have been developed to modify endogenous genomic DNA in both human and mouse cells. Simply stated, the process involves the targeting of a specific mutation *in situ* leading to the gene correction and the restoration of a normal gene function.

Most of these protocols with therapeutic potential are oligonucleotide based, and rely on endogenous enzymatic pathways. One gene targeting approach, "Small Fragment Homologous Replacement (SFHR)", has been found to be effective at modifying genomic DNA. This approach uses small DNA fragments (SDF) to target specific genomic loci and induce sequence-specific and subsequent phenotypic alterations.

This study shows that SFHR can stably introduce a 3-bp deletion (deltaF508, the most frequent cystic fibrosis (CF) mutation) into the *Cftr* (CF Transmembrane Conductance Regulator) locus in the mouse embryonic stem (ES) cell genome. After transfection of deltaF508-SDF into murine ES cells, SFHR-mediated modification was evaluated at the molecular levels on DNA and mRNA obtained from transfected ES cells.

The data indicate that the SFHR technique can be used to effectively target and modify genomic sequences in ES cells. Once the SFHR-modified ES cells differentiate into different cell lineages they can be useful for elucidating tissue-specific gene function and for the development of transplantation-based cellular and therapeutic protocols (1,2).

2. INTRODUCTION

Oligonucleotides-mediated gene modification in eukaryotic cells has the potential to correct or introduce specific mutations in the genome while maintaining the integrity of the target gene. These gene targeting strategies will retain the relationship between the protein coding sequences and the gene-specific regulatory elements and make it possible to have a long term, tissue specific, and genetically heritable expression of the modified sequences.

We and others have shown that a gene targeting approach Small Fragment Homologous Replacement (SFHR) efficiently introduces chromosomal gene alterations into mammalian cells either "*in vitro*" and "*in vivo*" (3-10).

SFHR employs small DNA fragments (SDF) that are homologous to the genomic target to catalyze intracellular enzymatic mechanisms that mediate homologous exchange (11-17). SDFs, once introduced into nuclei, facilitate homologous exchange between incoming SDF sequences and endogenous sequences that ultimately result in genotypic and phenotypic changes (18-19). The process can lead to different genomic alterations that include single base substitutions as well as concomitant insertion or deletion of multiple bases. These SFHR-mediated modifications have been observed within the CFTR gene, the human ß-globin gene (Hß-G), the mouse dystrophin (*mdx*) gene, the human SMN (Survival Motor Neuron) gene, and the murine *DNA-PKcs* gene, responsible for SCID disease (7,8,20-23). These findings suggests that SFHR has a broad range of utility both in terms of the target gene and of the cell type.

SFHR gene modification frequency is estimated to be in the range of 1-10% *in vitro* (7) and appears to be influenced by the method with which the DNA is delivered. Recent studies suggest that this efficiency can be significantly increased by nucleofection or by direct nuclear injection of the SDF (10,22,23). However, the enzymatic mechanisms underlying SFHR have yet to be elucidated (24).

This study shows that SFHR is able to stably modify the *CFTR* locus in the genome of mouse embryonic stem (ES) cells and introduce a 3-bp deletion specifically within the mouse equivalent of human exon 10.

SFHR-mediated modification was evaluated at both the DNA and RNA levels.

SFHR application to modify the ES cell genome has important implications for cell and gene therapy in general. ES cells have the ability to differentiate into a variety of tissues that could potentially be used to repair organ damage caused by disease pathology (2,25-27). Furthermore, this novel methodology facilitates the generation of "*in vitro*" modified tissues that can be used as models for genetic diseases and to analyze gene function in specific tissues.

3. MATERIAL AND METHODS

3.1 SDF Preparation

SDF (783-bp) containing the ΔF508 mutation and a unique KpnI restriction site was synthesized by PCR amplification using primers mCF1 and mCF15, (Figure1A) as described previously (4). The KpnI site described for this locus is absent within murine genomic DNA and can be used as a secondary marker to assess SFHR-mediated modification. The single base modification was introduced into the ΔF508-SDF by a modified megaprimer protocol (28). The resultant SDF cloned in a plasmid, was used for large-scale SDF production. Before transfection the SDF was used, always gel and ethanol purified (DNA gel extraction kit; Millipore, Bedford, MA). Briefly, preparative amounts of ΔF508-SDF were generated in a total volume of 50 µl, containing 1X PCR buffer, 1.5 U of *Pfu* DNA polymerase, 20 pmol of each primer, 2 ng of plasmide (ΔF508-SDF) genomic DNA with an initial denaturation at 94°C for 3 min, followed by 30 cycles of: denaturation (94°C for 30 sec), annealing (61°C for 30 sec), extension (72°C for 1 min) and a final extension for 8 min at 72°C.

3.2 Cell Culture

ES-D3 cells were obtained from the ATCC and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% FCS and 1000 U/ml LIF (ESGRO, Chemicon Inc., CA, USA; http:// www.esgro-lif.com) at 37°C under 5% CO_2. The ES cells were adapted to grow off feeders onto gelatin-coated tissue culture dishes, to avoid obscuring the interpretation of the results. The differentiated state of ES cells was routinely

monitored by assaying for the presence of alkaline phosphatase. Under these growth conditions the ES-D3 cells form colonies of 23-25 cells within four days of seeding on glass coverslips.

3.3 ES Nucleofection

Transfection of the D3-ES cells was achieved by electroporation (nucleofection) with the AMAXA Nucleofection System according to the mouse ES cell protocol developed by the manufacturer (AMAXA Biosystems, Köln, Germany). Approximately, $1.5x10^6$ cells were trypsinized, washed in Phosphate Buffer Saline (PBS, Cambrex, NJ, USA) and resuspended in 100 µl of Mouse ES Cell Nucleofector solution (AMAXA Biosystems). ΔF508-SDF (800, 1600, and 2400 µg was equivalent to ~ $6.4 x10^5$, $1.2 x10^6$, $1.9 x10^6$ SDF molecules per cell, respectively) was used to transfect ES cells. SDF concentration was determined spectrophotometrically (ND-1000, Nanodrop Spectrophotometer, Wilmington, Delaware USA). Program A30 was used in conjunction with the Mouse ES Cell Nucleofector solution to transfect the ΔF508-SDF into D3 cells. After electroporation, cells were plated immediately, expanded, and then harvested for analysis. As a control, theD3-ES cells were also transfected with 10^7 SDF per cell of a 498 bp SDF homologous to *Smn* gene (10).

3.4 DNA and RNA Isolation

DNA was isolated using phenol-chloroform.
RNA was extracted with Trizol (Gibco BRL, Gaithersburg, USA), DNAse treated, and then resuspended in DEPC water. All nucleic acids were quantified spectrophotometrically (ND-1000 Nanodrop Spectrophotometer). The mRNA was reverse-transcribed into cDNA according to the manufacturer's instructions (High-Capacity cDNA Archive Kit Applied Biosystems, Foster City, CA USA; http://www.appliedbiosytems.com). Briefly, a 50 µl aliquot of 2X RT Master Mix (2X RT buffer, 2X dNTP mixture, 2X random primers, 5U of MultiScribe RT) was added to tubes containing 50 µl of RNA (500ng-1500ng) and then incubated for 10min at 25°C and 2 hours at 37°C.

3.5 PCR Amplification of DNA and mRNA

Allele-specific PCR (AS-PCR) protocols were used for both DNA and mRNA analysis of the transfected ES cells. The ΔF508 allele was detected by a two-step PCR amplification performed on genomic DNA from transfected and untransfected cells. The first step used primers that were located outside the region of homology defined by the ΔF508-SDF (mCFf: 5'-TTAAAGATGAAAGCAAATTTTCATA-3' and mCFr: 5′-ATTCACTGACCCACCCACTC-3′ and produced a 1080/1077 bp amplicon for both wild type and deleted sequences, respectively (Figure 1B). PCR was performed in a total volume of 50 μL containing 200 mM each of four dNTPs, 2.5 mM $MgCl_2$, 0.25 U Taq polymerase, 20 pmol of each primer, and 150 ng of genomic DNA. The initial denaturation 95°C for 5 min was followed by 35 cycles of denaturation: 94°C for 1 min, annealing: 58°C for 1 min, and extension: 72°C for 1 min, with a final extension step of 72°C for 7 min. Amplicons were gel-purified by spin columns (Millipore, MA, USA, http://www.millipore.com) and used as the template for a second round of amplification . The second step involved a nested PCR amplification in which the primer mCF4: 5'-CACACTCATGTAGTTAGAGCATAGG-3' was located outside of SDF paired with the allele-specific primers mCF3N: 5'-ATCATAGGAAACACCAAA-3' or mCF3ΔF: 5'-ATCATAGGAAACACCGAT -3' (wild type or mutant , respectively) (Figure 1B). PCR was carried out in a total volume of 30 μL of reaction solution described above, using 15 pmol of each primer. Amplification was for 35 cycles as follows; denaturation: 94°C for 30 sec, annealing: 59°C for 30 sec, and extension: 72°C for 30 sec with a final extension cycle at 72°C for 7 min.

Digestion of the 478-bp analytical PCR fragment with KpnI produces two restriction fragments (442-bp and 36-bp) for the ΔF508-specific amplification, while there will be no digestion of the wtCFTR-specific 481-bp . The KpnI restriction site is used as a secondary marker of an SDF-induced homologous exchange. After KpnI digestion, the sample was banded on a 6% polyacrylamide gel.

For analysis of CFTR mRNA, one primer was in exon 9 (mCF11) and was paired with either mCF3N or mCF3ΔF (wild-type and deltaF508, respectively) localized within exon 10(figure 1C). The 234-bp ΔF508-specific amplicon yields a 198 and a36 bp fragment following KpnI digestion if the SDF-derived sequences have been appropriately introduced into the genomic DNA and correctly transcribed into mRNA (4).

3.6 Cloning of PCR amplicons

AS-PCR products from mRNA-derived cDNA were cloned into the pCR 2.1 of the TA cloning system following manufacturer's instructions (InVitrogen, Carlsbad, CA, USA). Each bacterial clone was grown in LB (100 µg/ml ampicillin) at 37°C. The cellular pellet was lysed by heating at 94°C and then directly amplified in 50 µL total volume of 200 mM each of four dNTPs, 2.5 mM $MgCl_2$, 0.25 U Taq polymerase, 20 pmol of each primer, DMSO 1,8 µL with primers M13 forward (5'-GTAAAACGACGGCCAGT-3') and M13 reverse (5'-CAGGAAACAGCTATGAC-3') primers using the following amplification conditions: 35 cycles of; denaturation: 94°C for 30 sec, annealing: 55°C for 30 sec, and extension: 72°C for 30 sec with a 7 min extension on the last cycle. PCR amplicons were then digested with KpnI and run on a 2.5% agarose gel.

Each clone was also sequenced to verify the presence of the deletion and the KpnI restriction site.

3.7 Real-time PCR Analysis of Gene Expression

Real time RT-PCR was performed using a TaqMAN ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA USA) using the following conditions: a 2 min incubation at 50°C (for optimal AmpErase UNG activity) followed by a 10 min incubation at 95°C (for deactivation of AmpErase UNG activity and activation of AmpliTaq Gold). Samples were then amplified for 40 cycles of; denaturation: 15 sec at 95°C and annealing/extension: 1 min at 60°C . Primers were designed using the Primer Express 2.0 software (Applied Biosystems, Foster City, CA). Wild type and mutant alleles were differentiated using MGB probes. CFTR forward primer: 5'-TTTCTTGGATTATGCCGGGTACT-3'; CFTR reverse primer: 5'-GCAAGCTTTGACAACACTCTTATATCTG-3'; CFTR wt–specific MGB probe: 5'-FAM-TATCATCTTTGGTGTTTCC-3'; CFTR ΔF508–specific MGB probe: 5'-VIC-ATATCATCGGTGTTTTCCTAT-3'. A commercially available endogenous gene, phosphoglycerate kinase 1 gene (Pgk1: Mm 00435617_m1) was used as a reference for

the TaqMan assay. This reference gene is assumed to be constant in both transfected and untransfected samples and was used to normalize the amount of cDNA added per sample. A comparative C_T method was used to quantify relative gene expression. All PCR reactions were performed in triplicate. Results are expressed as relative levels of the ΔF508 allele mRNA compared to wtCFTR expression (represented as a 1X expression of the CFTR gene). The samples were calibrated against a sample of untransfected cells that was analyzed on every assay plate with the transfected cells.

4. RESULTS

4.1 SDF Nucleofection

A SDF (783-bp) was synthesized as previously described (4,28) introducing the 3-bp deletion (ΔF508) and a silent mutation, that gives rise to a unique KpnI restriction enzyme cleavage site (Figure 1A). The ΔF508SDFs were delivered into cultured ES cells (D3), using the AMAXA Nucleofection System. Preliminary experiments performed in D3 cells with green fluorescent protein plasmid (pEGFP) showed that the optimal electroporation program (A-30), gave a transfection efficiency of ~ 45-50% and survival of ~90% (data not shown).

4.2 DNA Analysis

At 5 days after transfection, cells were harvested to assess whether SDF sequences were correctly incorporated into the genomic DNA. At this time point ~5-6 $x10^6$ cells were present, equivalent to ~8-10-fold population doublings. Given the original SDF dose there should now be a maximum of 3,200 SDF molecules per cell assuming that none have been degraded. Given this SDF dosage the potential of generating a PCR artifact is unlikely (29). No PCR artifact was detectable if a quantity of $\leq 10^4$ (or $\leq 10^6$ depending on the primer) free SDF/cell is mixed within cells and the genomic DNA isolate is amplified.

Transfected cells were analyzed by AS-PCR, and subsequent KpnI restriction enzyme digestion of the PCR amplification products (Figure 1B). SFHR-mediated, site-specific deletion (ΔF508) was detected. KpnI digestion of the PCR amplicons from DNA of electroporated cells was also observed with the different doses of SDF, thus demonstrating SFHR-mediated site-specific modification (Figure 2). Specifically DNA sample amplified with the wild type and ΔF508-specific primers and then digested by KpnI. Only the mutant allele was digested, indicating that SFHR-mediated modification had occurred. To further substantiate the specificity of the molecular analysis, two other control analyses were carried out. First, different amounts of ΔF508-SDF (from 10^6 to 10^{-1} molecules per cell) were mixed with mouse genomic DNA of untransfected cells. Moreover mouse ES-D3 cells were transfected with SDF homologous to *Smn* gene and then extracted and

analyzed (Figure 2). Both samples were used as templates to assay for any potential PCR-mediated artifacts that might arise from the amplification of the SDF. No anomalous PCR amplification products were observed (data not shown) .

4.3 Analysis of mRNA

To evaluate if SFHR-modified DNA was properly expressed, mRNA from transfected cells was converted to cDNA after DNAse treatment, and then amplified by allele specific-PCR (Figure 1C). Two amplicons, 237-bp and 234-bp, were generated for the wild type and ΔF508 CFTR alleles, respectively (Figure 3). These PCR products were subsequently cloned and sequenced to confirm the presence of both modifications (the deletion and the restriction site) within the SDF (TA Cloning, Invitrogen, San Diego, CA; http://www.invitrogen.com). Sequence analysis showed the presence of the expected ΔF508 mutation together with the KpnI restriction site, as indicated by arrows (Figure 3). Both variations were absent in wild-type allele. At the same time, clones were screened for the presence of the KpnI restriction site by PCR amplification and enzymatic digestion.
The ΔF508 transcript was quantified by TaqMan-based real-time quantitative RT-PCR, using the ABI PRISM 7700 Sequence Detection System (Applera; http://www.applera.com). Two oligonucleotide probes homologous to wild type and ΔF508 alleles were designed. Control mRNA was isolated from ΔF508 homozygote, heterozygote, and *CFTR* knockout mouse cells and included in the analysis (data not shown). Real-time PCR analysis of each sample was performed in triplicate and the individual experiments were repeated at least three times. The "recombinant allele", i.e., the ΔF508, was detected at about 12%the frequency of the endogenous wild-type allele (Figure 4). These results indicate that the SDF-modified allele was transcribed and expressed in D3 cells. Cells transfected with *Smn*-SDF and untransfected ones were negative for the expression of the ΔF508 allele.

5. DISCUSSION

Gene targeting by homologous replacement makes it possible to precisely manipulate genomic DNA and maintains genetic integrity by retaining the relationship between the protein coding sequences and the gene-regulatory elements (7). This aspect of homologous replacement overcomes any potential for inappropriate gene expression either in the amount of protein produced or in the type of cell expressing the gene (30). A recent study suggests that preclinical experimental treatments involving transgenes should include long-term follow-up before they enter clinical trials (31). Authors reports a long latency period before lymphomas develop in mice transplanted with cells that have been transduced with LV-IL2RG. This observation further highlights the need to develop vectors capable of regulated therapeutic gene expression.

Oligonucleotide-mediated modification has been applied by a number of different groups both *in vitro* and *in vivo* to modify both plasmid and genomic DNA targets (32-39). Among the various oligonucleotide-based gene targeting approaches, SFHR has been shown to correct specific mutations at a target locus (7). In a recent study SFHR was shown to restore the *Smn* full length protein in human SMA cells obtained from chorionic villi, demonstrating the feasibility of using this approach to stably correct human fetal cells (10). Another study described genotypic and functional correction of a point mutation in the gene encoding the DNA-dependent protein kinase catalytic subunit (DNA-PKcs)(23). In addition, a number of studies have shown specific modification of the CFTR gene by SFHR (4,6,8,10,19,21,40).

Based on these studies, the potential for of SFHR-mediated modification for “in vivo” or “ex vivo” genetic therapy of monogenic disorders is significant when compared to the cDNA-based "gene complementation" approaches (7,30,41-45).

In fact, targeted gene repair approach has received increasing attention because of the safety and of efficacy issues encountered with more traditional gene therapy strategies, where additional copies of therapeutic genes are delivered to and expressed in transduced cells (gene complementation). The direct conversion of mutant genomic sequences to a wild-type genotype, restoring the normal phenotype, has obvious implications for maintaining genomic integrity and cell-specific expression. By preserving the integrity of the targeted gene, the relationship between the coding sequences and regulatory elements

remains intact, and therefore targeted gene would be expressed at physiological levels in the appropriate cell type. Consequently, cell-specific expression is not altered. Therefore, once adequately developed, gene targeting strategies will result in less random mutagenesis of the genome and lead to fewer mutagenic side effects than do methods that randomly insert genes into the genome (30).

This study showed that it was possible to insert a 3-bp (ΔF508) deletion into the genomic DNA *CFTR* gene of mouse embryonic stem cells by SFHR following electroporation (nucleofection) of a783-bp ΔF508 fragment containing the unique KpnI restriction site. As a result, the SDF-derived ΔF508 mutant mRNA was expressed. Furthermore potential PCR artifacts that could result from the presence of free SDF within the cell (22,29,46,47) was not detected . To minimize the potential for artifact, the PCR primers used were outside the region of homology defined by the SDF. In addition, the SDF copy number at the time of analysis was about 3200 molecules per cell, assuming that there was no degradation or loss of the transfected SDF. This number is less than that required to give rise to any PCR artifacts as already reported in DNA mixing reconstitution analyses (22,29). Moreover, the treatment of the isolated RNA with DNase eliminates any contaminating SDF that might be present in the crude RNA isolate.

In addition to its role as a tool for developing an *in vitro* means for understanding the pathophysiology of monogenic disorders, SFHR can be applied to ES cells for therapeutically correcting genetic mutations and repairing disease dependent tissue damage (7). SFHR has already been used for modifying hematopoietic stem cells (7,22-25) that have been shown to have the capacity to differentiate into human airway epithelial cells (48). Mouse ES cells have also been shown to generate a fully differentiate and functional tracheobronchial airway epithelium (49-51) and could also potentially be applied to repair damaged CF airways.

Moreover, mutating genes in ES cells by homologous recombination has been a powerful research tool for developing animal models of human disease. The approach described here could potentially augment these classical homologous recombination strategies in mice to develop a range of animal models through nuclear transfer (2,7,22,52).

In conclusion, the present study represents the basis for developing innovative cell and gene-based therapeutic strategies for CF or other monogenic disease. While it has not yet been possible to effectively to carry out somatic cell nuclear transfer in human oocytes, the

potential of generating patient derived stem cells with corrected mutant genes could conceivably translate into a significant improvement and possible cures for many inherited diseases.

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Abbreviations: CFTR: cystic fibrosis transmembrane conductance regulator, CF: cystic fibrosis, SDF: small DNA fragment, *Smn*: survival motor neuron gene , HR: homologous recombination, SFHR: small fragment homologous replacement, ES cells: embryonic stem cells

Key Words: homologous replacement; real-time PCR; SFHR; embryonic stem cells; CFTR

Running title: Gene modification in embryonic stem cells by SFHR

Legend to figures

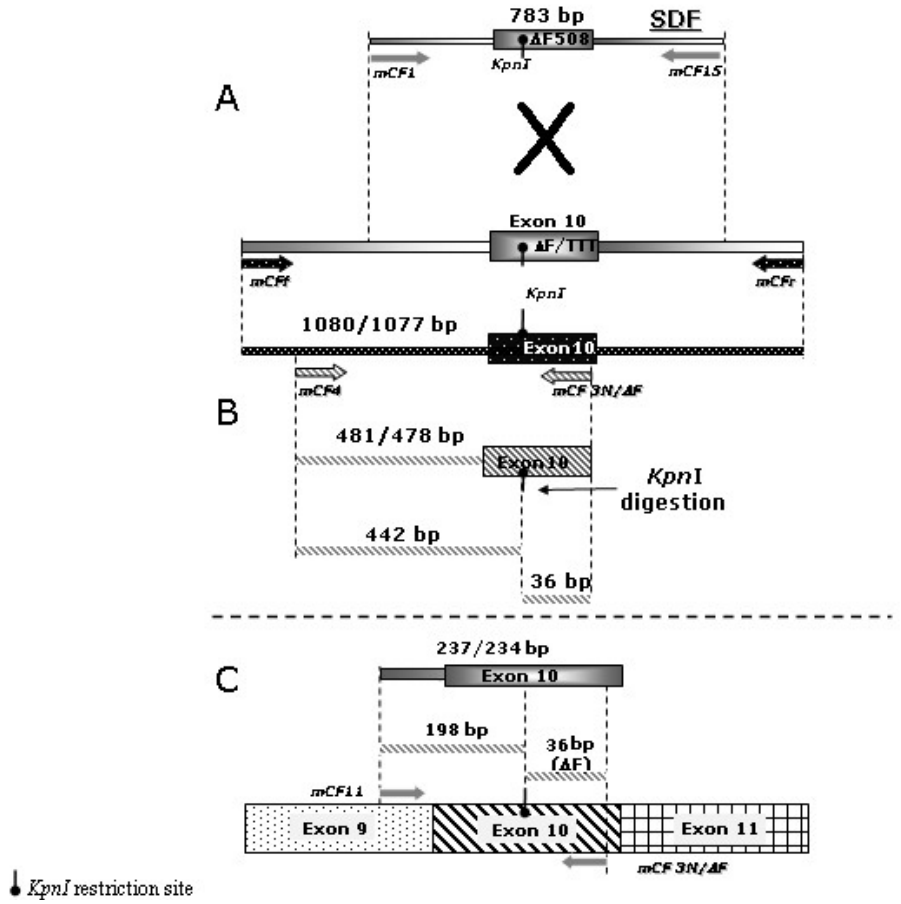

Figure 1. Schematic of small DNA fragment (SDF) generation and PCR analysis of SFHR (A) SDF (783bp) containing the ΔF508 mutation and a KpnI restriction enzyme cleavage site was synthesized using primers mCF1 and mCF15, localized within introns 9 and 10 of *Cftr* gene respectively (2). The unique KpnI site is a secondary marker for assessment of SFHR-mediated modification.

(B) Analysis of genomic DNA using two successive rounds of PCR amplification. The first round of PCR used primers (mCFf/mCFr) were located outside the region of homology defined by the SDF and resulted in a 1080- or 1077-bp amplicon (wild tyoe of ΔF508, respectively). The second round of amplification used the amplicon generated in the first round as a template and allele specific primers (mCF4/mCF3N or mCF4/mCFΔF for wild type and ΔF508 sequence respectively). The 478-bp fragment was then digested with KpnI

to determine whether the 442- and 36-bp restriction fragments, indicating SFHR mediated replacement, were present.

(C) Allele specific RT-PCR for analysis of transfected ES cells using primers located within exon 9 (mCF11) and exon 10 (mCF ΔF or mCF3N, wild type and ΔF508 respectively). The 237- or 234-bp amplicon was then digested with KpnI to assay for restriction fragments of 198-bp and 36-bp indicating expression of SDF-derived sequences.

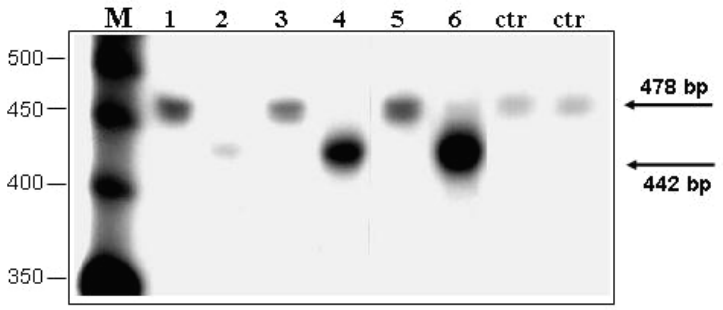

Figure 2. Polyacrylamide gel analysis of allele-specific PCR amplification products generated from the genomic DNA of transfected cells and digested with KpnI. Lane M: 50-bp DNA ladder (Invitrogen, Carlsbad, CA). Lanes 1-6: amplicons derived from ES cells transfected with different quantities of SDF; lane 1 and 2 correspond to cells transfected with 6.4 x 10^5 SDF/cell, lane 3 and 4 with 1.2 x 10^6 SDF/cell, and lane 5 and 6 with 1.9 x 10^6 SDF/cell. Lanes 1, 3 and 5 are amplicons obtained with primers mCF4/mCF3N while lanes 2, 4 and 6 with primers mCF4/mCF3ΔF. *Ctr* sample is derived from ES cells transfected with *Smn*-SDF (8). All samples amplified with primers mCF4/mCF3N and mCF4/mCF3ΔF and digested by KpnI. The 442-bp band is the result of KpnI digestion of SFHR-modified genomic DNA. Arrows indicate the molecular weight of amplicons and of its digestion products.

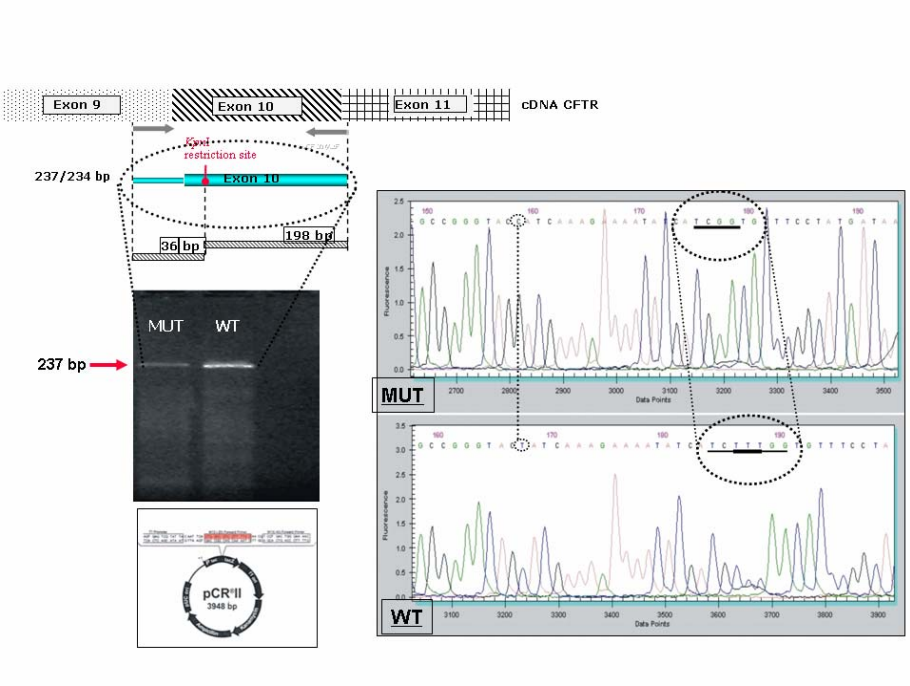

Figure 3. Gel electrophoresis analysis of AS-PCR performed on mRNA-derived cDNA from transfected ES cells. Wild type and ΔF508 amplicons were generated using primers mCF11/mCF3N and mCF11/mCF3ΔF, respectively (see Figure 1 C). These amplicons were cloned into vectors which were then isolated clones and sequenced. Sequencing of mCF11/mCF3ΔF (MUT) exclusively showed the presence of both the ΔF508 allele and the KpnI restriction site (arrows) indicating that the SDF-derived sequences were expressed as CFTR mRNA.

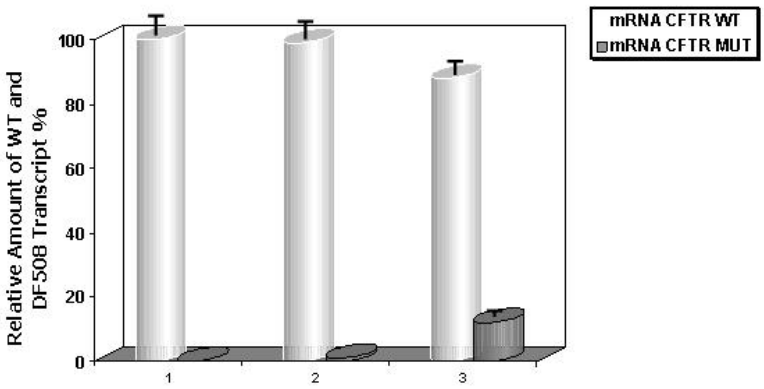

Figure 4. Quantitative PCR analysis of the ΔF508 and wild type *Cftr* transcript in transfected D3 ES cells. Open columns represent the wild type transcript, while shaded columns represent the ΔF508 transcript. Sample 1 corresponds to cells transfected with no SDF, sample 2 to cells transfected with SDF homologous to *Smn* gene, and samples 3 to cells transfected with 1 x 10^6 SDF/cell. The values obtained from treated cells represent the mean of at least three independent experiments that were performed in triplicate. Values were significantly different from those obtained from cells transfected with no SDF (sample1) and from cells transfected with *Smn* SDF (sample 2) . Error bars indicate the SD. A p value of < 0.05 was considered statistically significant.

SFHR GENE TARGETING STRATEGY OPTIMIZATION:

USEFUL TOOLS AND INCREASED EFFICIENCY OF REPAIR

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SFHR (Small Fragment Homologous Replacement) is a gene targeting strategy which uses small DNA fragments (SDF) to induce homologous replacement into the genomic DNA of recipient cells. We and others have previously demonstrated that this targeting approach is capable of inducing chromosomal gene alterations in mammalian cells *in vitro* and *in vivo.* Consequentially, *t*he SFHR technique has been successfully applied to modify genomic loci, as the *Cftr* gene, the *dystrophin* gene, the *DNA-PKcs* gene responsible of the SCID mouse, the hSMN2 gene and the hβ-globin gene.

The lack of a detailed knowledge of the mechanism of action of the SDF at the molecular level sets a limit to a precise reproducibility of this strategy. The efficiency of modification is very variable and depends from different conditions. In fact the transfection protocol, the kind of fragment ,its length and also its concentration (in terms of molecule number transfected) and the chromatin structure, which changes throughout the cell cycle, may be key factors underlying these variations in efficiency. These hypothesis prompted us to systematically investigate SFHR-mediated gene repair (process) at various phases of the cell cycle using different kinds of SDF. To this purpose, we design and constructed an *"assay system"* that can be used to optimize SFHR protocol *in vitro* in order to improve the SFHR efficiency in eukaryotic cells. A mutated copy of EGFP gene were stably integrated within immortalized embryonic fibroblasts (MEF), obtained from Sma mice model ($Smn^{-/-}$; SMN2). Different parameters such as the concentration, the length and the nature of SDF, but most importantly different phases of cell cycle (G0/G1-S-G2/M) were tested to understand which was the exact mechanism underlying the SDF-mediated integration within genomic DNA. The efficiency of modification was quantitatively evaluated by FACS analysis, measuring the percentage of EGFP-positive cells obtained. Transfection protocol was optimized by nucleofection (Nucleofector Amaxa). While in phase G0/G1 (mimosine treated) the efficiency of correction was estimated about 0.01%, this value double in phase S (thymidine treated) (0.02%). The best results was achieved after synchronizing cells in G2 phase (vinblastine treated) and transfecting $7{,}5*10^6$ molecules of SDF/cell. In fact, during this phase the efficiency of correction increased 10 times, up to 0.1%. We also tested the effect of the methylation status of the "corrective" fragment, comparing PCR-amplified SDF versus plasmid-digested one. The latter gave best results in terms of recombination efficiency thanks to its lower methylation grade. After transfecting plasmid-digested SDF in G2 synchronised cells the percentage of fluorescent cells increased 50 times respect to the standard condition. After cell sorting , the stability of the correction after several doublings was also assessed by Southern blot and by microscopy analyses. This study allows us a better comprehension of SDF molecular way of action (G2 phase and homologous recombination) and consequently open up new perspective for considering SFHR as a gene targeting strategy applicable to the *ex vivo* protocols.

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