CHAPTER 17

Inherited Defects and Gene Therapy

SUMMARY

In multicellular animals such as humans, the only way a genetic defect can be inherited is if the mutation occurs in the germline cells that give rise to sperm or egg. Also, higher animals are diploid, which means they have two copies of each type of gene. Heterozygotes have only one defective copy and may not necessarily exhibit a genetic defect. However, having two copies of the defect makes an individual homozygous, which usually confers a genetic defect onto that individual, particularly for recessive alleles. Examples of homozygous, recessive genetic diseases include cystic fibrosis, Duchenne's muscular dystrophy, hemophilia, sickle cell anemia, and many others.

Other genetic diseases depend on defects in multiple genes. These include cleft palate, spina bifida, some cancers, and diabetes. Down syndrome is also a defect that relies on multiple genes because this disease is caused by an extra chromosome 21, which increases the number of all the genes on that chromosome.

Haploinsufficiency is also responsible for some genetic defects. Usually, having one functional copy of a gene is sufficient. In some cases, though, two functional copies are necessary. For example, a defect in one gene for elastin causes a defect in the structure of the aorta, which leads to narrowing of the aorta. Either a genetically engineered transcriptional activator or the use of RNA interference is a potential therapy.

Some mutations result in a gain of function for the organism. These mutations are often dominant and might also be negative or positive. Dominant negative mutations cause a mutant protein to lose function but also interfere with the function of other proteins. Positive mutations case a gain in function for the protein of interest, such as a mutation rendering a receptor unable to "turn off," even though the signaling molecule is not present.

Many neurodegenerative diseases with late onset are caused by deleterious tandem repeats, which insert CAG codons within the coding region of a gene. The CAG codon codes for glutamine. Consequently, the protein product will have several glutamine repeats called polyglutamine tracts. These tracts can often propagate into multiple repeats that are unstable and interfere with protein activity, thus causing disease. The tandem repeats are added to an autosomal gene, and the effects of numerous polyglutamine tracts are dominant. One example of a disease caused by tandem repeats is Huntington's disease. Fragile X syndrome causes mental retardation and is a second example for deleterious repeats. The difference in fragile X syndrome is that the repeats are CGG that are inserted within the 5′ UTR of the FMR1 gene, which regulates the synthesis of proteins at nerve junctions. Even though they do not code for protein, they do become methylated since they resemble CG islands. Methylation blocks transcription of the FMR1 gene.

RNA gain of function is an unusual situation in which an altered RNA molecule carrying a triplet repeat sequence is responsible for disease symptoms. These repeat sequences may hinder mRNA processing in eukaryotes and transport into the cytoplasm. Hairpin structures prevent the transport of the mRNA into the cytoplasm. Furthermore, the dsRNA from the hairpin structure interacts with and activates the enzymes involved in antiviral defense, which in turn causes protein synthesis to shut down as well as other damaging effects.

DNA is often methylated to regulate gene expression, which is called genetic imprinting. Methylated genes are often silenced, and the second copy of the gene in a diploid organism provides the protein product. Sometimes, methylated DNA can be inherited from the gametes, which alters gene expression in the offspring. Which disorder an individual has may be dictated by which gamete the methylated copy is inherited from, along with a deletion in the second allele for the gene. An example of this epigenetic effect is with a portion of chromosome 15 called 15q11-q13, which is subject to imprinting. If a deletion or mutation occurs in this area in the male gamete that fertilizes an egg containing a methylated copy of the same region, the result is Prader–Willi syndrome. However, if a deletion or mutation of

the same area is within the egg, and the egg is fertilized with a sperm that contains a methylated copy of the same region, the result is Angleman's syndrome. Those regions that are imprinted will not be expressed, which leads to the syndrome. Basically, one copy of the region is methylated, rendering it nonfunctioning. The other copy of the region is either deleted or mutated, or in either case, nonfunctioning. Both syndromes are characterized by mental retardation. However, Prader–Willi syndrome leads to gross obesity, and Angelman's syndrome leads to growth retardation and hyperactivity.

Mitochondria contain their own sets of chromosomes, which are separate from the nuclear chromosomes. Mitochondria, and also the chloroplasts of plants, are inherited through the maternal line. Some genetic defects can be caused by mutations in mtDNA, particularly for processes such as cellular respiration. Despite the relative small size of mtDNA relative to nuclear DNA, a high number of mutations arise from the mitochondria. This might be due to the levels of free radicals that are produced during respiration within the mitochondria. Or perhaps the absence of many sophisticated DNA repair systems is partly to blame. A majority of mitochondrial genetic defects affects respiration, and hence energy production. The effect on tissues is dependent on the tissues' specific energy needs, with the brain and muscles requiring the most cellular energy. Examples of mtDNA-related genetic defects include neuropathy, ataxia, and retinitis pigmentosa (NARP) syndrome. All of these defects have been traced to a specific site within a gene that encodes a portion of the major energy producing enzyme, ATP synthetase. In addition, other mtDNA defects that lower the amount of energy produced by the organelle also exist.

Identifying and locating defects within genes that cause genetic disorders is sometimes difficult. Candidate cloning examines the genetic disease and makes a hypothesis about the specific types of proteins that might cause the disease. The list of candidates is then further examined to identify a defect–disease match. Another method to identify the defects responsible for a genetic disorder is to use a model organism to investigate the effects of knocking out genes on the organism. Mice, which share a significant amount of homology with humans, are often used in this situation. Functional cloning and positional cloning are also used for identification of the defect.

In functional cloning, the amino acid sequence of a suspect protein is sequenced, and it is then used to deduce the DNA coding sequence. Through various cloning and hybridization processes, the gene on the chromosome is then identified. In positional cloning, the gene responsible for a defect is mapped using genetic markers (RFLP, VNTRs, etc.) to within a region. Once a region has been identified as likely associated with a defect, the region is cloned and analyzed for the presence of a functional gene. One of several features may be present if the localized region is transcribed. The presence of an open reading frame suggests an expressed region. Also, the presence of CG islands indicates a role for methylation, which is involved in regulation. Hybridization to DNA sequences of closely related species indicates a coding region, since noncoding regions often contain more mutations, which corresponds with less hybridization. Examination of mRNA extracts from affected tissues should yield a higher concentration of defective mRNA, thus indicating a role for that region in the disorder. Finally, standard sequencing of the region and comparisons of affected and unaffected individuals provide the final proof.

Genetic screening and counseling for high-risk genetic defects are possible for a wide range of genetic diseases. Usually, the screening consists of PCR or hybridization techniques. Potential parents who test positive for recessive, defective genes have a 25% chance of having a child that is homozygous recessive and who will then have the genetic disease. Screening individuals for diseases that appear later in life allows individuals who test positive to modify their diet and/or lifestyle to help prevent or delay the onset of symptoms. Screening techniques, such as amniocentesis or chorionic villi sampling, are used on embryos to determine if a genetic defect is present.

The goal of genetic engineering is to permanently change an organism to contain desirable traits. Gene therapy seeks to cure genetic defects present in specific tissues. The consequences of gene therapy are often only observed in somatic cells of the affected individual and, therefore, not inheritable to the offspring. Germline cells still contain the defect, which could potentially be passed onto offspring.

In replacement gene therapy, a normal copy of a gene is inserted into the cells to overcome the effects of recessive defects. Often, only one good copy is all that is needed for proper function and treatment of the genetic disorder. Replacing a gene in a cell involves properly identifying the gene that corresponds to a particular genetic defect, successfully cloning the gene of interest into a relevant vector, followed by delivering the gene and effectively expressing it into protein.

Adenovirus vectors are useful in delivering therapeutic genes, despite the drawbacks of being short-lived and able to cause serious infections in immunocompromised individuals. The genes can be packaged into the virus, which readily infects animal cells, resulting in delivery of the therapeutic gene directly into the cell. To prevent possible ill effects from an adenovirus in immunocompromised individuals, scientists disarm the virus through genetic engineering by removing the viral gene responsible for replication. The problem is that the therapy is short-lived and a one-shot deal. In subsequent infection, the immune system recognizes the adenovirus and destroys the virus before it can deliver the therapeutic gene.

Cystic fibrosis is a homozygous recessive defect, meaning that two defective copies of genes must be inherited in order for the individual to have the disease. The specific affected gene, called *CFTR*, encodes a chloride ion channel protein used in the transport of these ions across the cell membrane. Even though the defect is within a single gene, the disease affects multiple tissues in the body. The mucus produced in the lungs and digestive tracts is particularly thick because of the absence of chloride ions, which attract water. The thick mucous secretions not only are difficult to eliminate, but also provide a haven for bacteria. Individuals with cystic fibrosis often succumb to respiratory failure because the bacteria destroy the lung tissue, which is then replaced by scar tissue. Several different mutations in the *CFTR* gene can cause cystic fibrosis in homozygous individuals. Heterozygous individuals have decreased chloride ion flow and show a partial phenotype due to the mutation in *CFTR*. These individuals are more resistant to enteric pathogens, particularly those pathogens that cause typhoid and cholera.

Short-term success in treating cystic fibrosis has been accomplished by engineering a normal *CFTR* gene into an adenovirus and then delivering the virus directly to the lungs via an inhaler. The success is short-lived because the host's immune system eliminates the virus. Several drugs built on the molecular biology of CFTR protein have been developed, nut at present, they are either too expensive or still in clinical trials.

Retroviruses infect a wide range of mammalian cells, although they require cells to be actively dividing in order to do so. They also make a good candidate, and have been used successfully, to deliver therapeutic genes into cells. Similar to adenoviruses, retroviruses are attenuated in their ability to cause inflammation and infection. Most of the genes from the retrovirus are removed. So propagation of the virus prior to infection of a mammalian cell is aided by a helper virus, which contains retroviral genes for virus particle development. Therapeutic genes are packaged into the retrovirus, and cells are infected with the virus. As previously discussed for adenovirus, retroviral gene therapies are also integrated permanently into the host cell's genome.

Retrovirus gene therapy has successfully treated children with severe combined immunodeficiency (SCID). SCID is so severe that children are often raised in a sterile bubble environment to prevent infection with even the common cold because they literally have little to no immune system. Most instances of SCID are due to defects in the *Ada* gene, which encodes adenosine deaminase. Without this enzyme, T and B cells are not made. To deliver the gene therapy to the patients, scientists removed bone marrow

and infected the cells with a retrovirus containing the *Ada* gene. The cells were then returned to the body, where they produced healthy immune system cells. However, this production is only short-lived. The use of blood stem cells instead of bone marrow cells has been paramount in overcoming the issue of having to reintroduce engineered bone marrow cells periodically into affected children. Blood stem cells are precursors to many cell types. Using a retrovirus to engineer the *Ada* gene into blood stem cells that already have known deletions in both *Ada* genes has enabled long-term production of white blood cells in these individuals. Since therapy of the SCID defect also involves injections with purified enzymes, it is unclear if the engineered blood stem cells or the purified Ada enzyme is responsible for a majority of the success. Defects in some interleukins also cause SCID. This form has been successfully treated using gene therapy alone, although one patient developed leukemia, perhaps as a result of using a retrovirus in the delivery of the therapeutic gene.

Adeno-associated virus (AAV) requires a helper virus, usually an adenovirus but in some cases a herpesvirus, to infect cells. Mostly harmless, this DNA virus is another candidate for delivering gene therapies. AAV has advantages over adenoviruses. Primarily, AAV does not elicit an immune response. Therefore, it may theoretically be used multiple times without destruction by immune system cells. Nor does it cause inflammation, which reduces symptoms associated with an infection. AAV also has broad host range and tissue tropism, whereas adenovirus is primarily confined to the respiratory system. Finally, AAV is lysogenic, thus integrating its DNA into the host cell's genome permanently. Since the genes are potentially inserted into the host's genome, it is important that the gene is properly regulated by the addition of a regulatory system. Sometimes, these regulatory systems are present on separate vectors that are used simultaneously. Despite the positive features of AAV, there are still some drawbacks. One disadvantage includes the small AAV genome, which allows the virus to carry only short segments of DNA. Additionally, without the helper virus, AAV integrates into the host genome and becomes latent. Clinical trials are already underway for Leber congenital amaurosis (LCA; causes child blindness), hemophilia B, some types of muscular dystrophy, lipoprotein lipase deficiency, Pompe disease, Sanfilippo syndrome, Batten disease, age-related macular degeneration, severe heart failure, Alzheimer's, Canayan disease, and Parkinson's disease.

Nonviral delivery of gene therapies is generally regarded as safer than its viral counterparts and includes the use of naked DNA, DNA/protein hybrids, particle bombardment, and liposomes, as well as many others. Liposomes are small membrane-bound vessels that can be packaged with any number of desirable substances. For gene therapy, liposomes are packaged with the gene of interest and allowed to fuse to cells and release their contents into the cytoplasm, a process called lipofection. The process is nonspecific but provides a promising method for the treatment of cancer by packaging liposomes with either DNA or toxic proteins that target components of cancer cells.

The approach to treating cancers with gene therapy is multifaceted. In some cases, genes in the cancerous cells that are ultimately responsible for the loss of control over cell division could be replaced with normal genes. One such gene could be p53. Many cancer cells are defective in this gene, which helps control the cell cycle. Another approach is to ramp up the copy number of genes that encode cancer-killing proteins, such as tumor necrosis factor (TNF). Using a drug/gene therapy hybrid system that contains a prodrug, a precursor to a cancer-killing drug, and a gene for an enzyme that converts the drug into a toxic form is also a potential approach in cancer therapy. In this scenario, the gene for prodrug conversion could be administered directly to tumor cells using a gene therapy delivery method described previously. Upon treatment with the prodrug, only the cancer cells would have the enzyme, thus converting the prodrug into toxic form and essentially committing suicide. A final approach aims to increase recognition of the cancer cells by immune system cells through introduction of a gene for cell surface identification tags into the cancer cells. The immune system would then be more efficient at recognizing the cancerous cells.

In the past, the use of RNA in therapy was largely centered around antisense RNA. At present, the main focus with RNA therapy is through RNA interference or microRNA and, to a lesser extent, antisense RNA, artificial aptamers, and ribozymes. Delivery of the RNA is difficult and most likely would involve liposomes or intrinsic generation of therapeutic RNA by transcription. Also, RNA could be stabilized by using modified oligonucleotides.

Antisense RNA binds to target mRNA and decreases translation. Two types of antisense RNA could be used for this application: full-length antisense RNA (anti-gene) to the target mRNA or smaller RNA oligonucleotides. There has been some success using anti-genes in the treatment of a common form of brain cancer, malignant glioma.

It is also possible to block proteins with RNA molecules, such as the prevention of binding by a transcription factor to its target gene. This would be due to the presence of a small oligonucleotide molecule bound to the DNA-binding domain of the protein. Consequently, gene expression is decreased. Aptamers are non-naturally occurring nucleotide sequences that can be used to block the active sites of many proteins, including those that do not normally bind to nucleic acid. Recently, an RNA aptamer has been approved for the treatment of age-related macular degeneration.

Ribozymes are RNA molecules that have catalytic activity. Theoretically, ribozymes could be engineered at the genetic level to recognize specific target mRNAs and cut them into unusable fragments. The ribozyme gene would then get delivered into the target cell, where it would be transcribed into the active form and target specific mRNAs for degradation. Deoxyribozymes could be used in a similar manner to ribozymes, except with the use of DNA nucleotides. One possibility for DNAzymes in the future is to degrade mRNA that codes for proteins involved in antibiotic resistance in bacteria.

RNA interference, or RNAi, is a major focus for RNA therapies and is currently being researched as a therapy for age-related macular degeneration. Just as with other RNA-based therapies, delivery of the RNA to the site is the biggest issue. Liposomes, whole living bacteria, and bacterial vectors are potential delivery methods.

Gene editing involves the repair or patching of defective genes using engineered nucleases targeted to specific locations. The nucleases that so far have been considered for gene editing include zinc-finger nucleases, transcription activator-like effector (TALE) nucleases, and homing endonucleases. The DNA recognition sites must be recognized by the chosen nuclease. Therefore, the nuclease must be engineered to recognize this site. Genome editing involves cutting the dsDNA with the engineered nuclease, followed by insertion of the new DNA sequence and use of the cell's own repair systems to repair the site. Zinc finger nucleases have reached clinical trials, specifically for making human cells resistant to HIV.

Trisomy occurs when three copies of a chromosome are present within the same cell from a diploid organism. Trisomy 21 causes Down syndrome. Genome editing could also be used to correct trisomy. In this case, one of the three copies of chromosome 21 is silenced or eliminated; this is done by either inserting a DNA cassette into one of the copies of chromosome 21 using AAV or through silencing one copy of the chromosome through the action of genetic engineering and Barr body formation.

CRISPR, or clustered regularly interspaced short palindromic repeats, is a bacterial system designed to combat against intruders such as bacteriophage. In essence, this system behaves as a memory or immune type system for the cell against foreign nucleic acids. Short sequences of foreign genetic information are stored within the bacterial cell's own genome and transcribed into guide RNAs that bind to CRISPR-associated nucleases (Cas). When the bacterial cell is exposed to matching sequences, those sequences are destroyed by the Cas nuclease.

The wide variety of gene therapies could be used to alleviate and even cure many inherited defects. Delivery of the systems might include viruses, bacteria, liposomes, and RNA-based technologies.

Case Study Long-Term Stability and Safety of Transgenic Cultured Epidermal Stem Cells in Gene Therapy of Junctional Epidermolysis Bullosa

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Colonies of epidermal stem cells, called holoclones, are required to renew epidermis monthly and repair wounds. Holoclone-forming cells possess long-term regenerative properties and are the only stem cell of human squamous epithelium.

Inherited epidermolysis bullosa (EB) is a genetic disorder that produces fragile epidermis that manifests as blisters and erosions. Patients suffer from low quality of life. Junctional EB (JEB) is blister formation at the lamina lucida due to the absence of alteration of hemidesmosomes. Furthermore, JEB is divided into three categories: Herlitz (JEB-H), non-Herlitz (JEB-nH), and JEB with pyloric atresia (JEB-PA). The three types are differentiated based on mutations present in laminin, collagen, or integrin genes.

In clinical trials, genetically modified holoclones restored normal epidermis to both upper legs of a patient suffering from JEB-nH. In this case study, the authors examine the safety and long-term persistence of the engineered epidermis.

How were the holoclones engineered?

The holoclones were transduced with a murine leukemia virus-based retroviral vector containing a cloned *LAMB3* cDNA. The *LAMB3* cDNA encodes laminin, which helps link integrins to collagen. The epidermal grafts were then transplanted onto the patient's upper thighs.

What observations were made on this patient after a 6.5-year follow-up from the grafting procedure?

Visual inspection of the patient's upper thighs in the follow-up showed normal looking, correctly pigmented skin. No blisters or erosions were observed in the graft region. However, multiple blisters were present in the nongrafted area. Additionally, pain sensitivity was present. When subjected to biopsy, the grafted area healed without producing blisters and erosions.

Upon inspection of the tissue using molecular biology techniques and microscopy, were the cells and tissue normal?

Upon further inspection, only transgenic keratinocytes were found in the regenerated tissue. The stratum corneum was slightly thicker than the control, but the stratification and morphology of the tissue were normal. The density and organization of collagen fibers were normal. There was a reduced number of dermal papillae in the grafted tissue. The basement membrane and hemidesmosomes were consistent with the control sample. Identical amounts of laminin were produced between control and transgenic samples. Keratin and elastin were also produced to normal amounts between samples. There was a difference in organization of elastin fibers, though. In the control sample, elastin fibers showed vertical fine fibers. In the transgenic sample, elastin fibers were diffuse and not well organized. This could be a potential side effect of previous chronic inflammation.

The ΔNp63α transcription factor is highly expressed in holoclones and regulates maintenance of epithelial stem cells. Therefore, this transcription factor is used as a tool for studying long-term persistence of the transgenic graft. Was the transgenic graft persistent?

Yes. The levels of ΔNp63α transcription factor were similar between the control and transgenic samples. This indicates long-term persistence of the transgenic tissue.

Why was the stratum corneum thicker in the transgenic sample than in the control sample?

The epidermal graft was generated using the patient's own palm keratinocytes. Keratin-9 is expressed in palmar keratinocytes but not in other body epidermal cells. The researchers determined that the patient's transgenic graft expressed keratin-9 to similar levels as the control sample from palm epidermis, indicating memory maintenance of their origin, even after approximately 80 renewing cycles *in vivo*. The excess keratin leads to thicker stratum corneum, which was observed in the transgenic graft tissue.

What are the risk factors associated with using a murine leukemia virus-based retroviral vector for any gene therapy?

The authors identified potential risk factors associated with the MLV-RV system. Previous studies have indicated concern for the indiscriminate insertion within the host cell's genome. Insertion could be within proto-oncogenes, which occurred when using the same vector system in gene therapy trials of X-linked severe combined immunodeficiency (X-SCID) and Wiscott–Aldrich syndrome and then caused lymphoproliferative disorders. However, the MLV-RV system was used to transduce hematopoietic stem cells to treat adenosine deaminase SCID. No tumor development was observed in this case study.

The goal of this study was to investigate the safety and long-term persistence of transgenic epidermis in the treatment of JEB-nH. The tissue was produced from the patient's own palm keratinocytes that were transduced with an MLV-based retroviral vector expressing a laminin gene. Through their experimentation in this case study, the authors determined that the transgenic graft is fully functional and behaves as normal epidermis. They also found that although many of the transduced keratinocytes were lost in the grafting process, a few stem cells persisted and were responsible for regenerating the epidermal tissue. This study provides some insight into the use of gene therapy techniques to treat genetic skin disorders.

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Long-Term Stability and Safety of Transgenic Cultured Epidermal Stem Cells in Gene Therapy of Junctional Epidermolysis Bullosa

Laura De Rosa,[1,5] Sonia Carulli,[1,5] Fabienne Cocchiarella,[1] Daniela Quaglino,[2] Elena Enzo,[1] Eleonora Franchini,[1] Alberto Giannetti,[3] Giorgio De Santis,[4] Alessandra Recchia,[1] Graziella Pellegrini,[1] and Michele De Luca[1,*]

[1]Center for Regenerative Medicine "Stefano Ferrari," Department of Life Sciences, University of Modena and Reggio Emilia, 41125 Modena, Italy
[2]Department of Life Sciences, University of Modena and Reggio Emilia, 41125 Modena, Italy
[3]Emeritus of Dermatology, University of Modena and Reggio Emilia, 41125 Modena, Italy
[4]Department of Medical and Surgical Sciences, University of Modena and Reggio Emilia, 41125 Modena, Italy
[5]These authors contributed equally to this work
*Correspondence: michele.deluca@unimore.it
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SUMMARY

We report a long-term follow-up (6.5 years) of a phase I/II clinical trial envisaging the use of autologous genetically modified cultured epidermal stem cells for gene therapy of junctional epidermolysis bullosa, a devastating genetic skin disease. The critical goals of the trial were to evaluate the safety and long-term persistence of genetically modified epidermis. A normal epidermal-dermal junction was restored and the regenerated transgenic epidermis was found to be fully functional and virtually indistinguishable from a normal control. The epidermis was sustained by a discrete number of long-lasting, self-renewing transgenic epidermal stem cells that maintained the memory of the donor site, whereas the vast majority of transduced transit-amplifying progenitors were lost within the first few months after grafting. These data pave the way for the safe use of epidermal stem cells in combined cell and gene therapy for genetic skin diseases.

INTRODUCTION

The human epidermis is renewed monthly, and daily occurring wounds need timely repair. The processes involved in this regeneration and repair rely on epidermal stem cells, which generate colonies known as holoclones (Barrandon and Green, 1987; Pellegrini et al., 1999a; Rochat et al., 1994). Holoclones produce meroclones and paraclones, which have properties expected of transit-amplifying progenitors (Barrandon and Green, 1987; Pellegrini et al., 1999a). The holoclone-forming cell is the only clonal type that possesses long-term regenerative potential, and is the stem cell of all human squamous epithelia (De Luca et al., 2006). Autologous keratinocyte cultures containing holoclones can permanently restore massive epithelial defects such as skin and ocular burns (Gallico et al., 1984; Pellegrini et al., 1997, 1999b, 2013; Rama et al., 2010; Ronfard et al., 2000).

Inherited epidermolysis bullosa (EB) is a family of rare genetic disorders characterized by structural and mechanical fragility of the integuments, leading to recurrent skin and mucosal blistering and erosions that severely impair the quality of life of EB patients (Fine et al., 2008). Junctional EB (JEB) is marked by blister formation at the level of the lamina lucida of the basement membrane and absence (or severe alteration) of hemidesmosomes. JEB has been divided into three categories: Herlitz (JEB-H), non-Herlitz (JEB-nH), and JEB with pyloric atresia (JEB-PA). JEB-H is an early lethal form and is usually due to deleterious mutations in *LAMA3*, *LAMB3*, or *LAMC2* genes causing a total absence of laminin 332 (previously known as laminin 5), a heterotrimeric protein that consists of α3, β3, and γ2 chains, and links α6β4 integrins to collagen VII dermal fibrils. Mutations of the same genes cause JEB-nH, which is characterized by reduced expression of laminin 332. JEB-nH can also arise from mutations in *COL17A1*, the gene encoding collagen XVII, whereas JEB-PA is due to mutations in genes encoding the α6β4 integrin (Fine et al., 2008). There is no cure for EB; treatments are palliative and focused on relieving the devastating clinical manifestations (Carulli et al., 2013).

A phase I/II clinical trial showed that autologous epidermal cultures containing genetically modified holoclones restored a normal epidermis on both upper legs of a patient (Claudio) suffering from a severe form of laminin 332-β3-dependent JEBnH (Mavilio et al., 2006; the phase I/II clinical trial was authorized by the Italian Ministry of Health and approved by the ethics review board of the University of Modena). Epidermal keratinocytes were taken from his palm skin, which, at variance with other affected body sites, contained an appropriate number of holoclones (Mavilio et al., 2006). Cells were transduced ex vivo with a murine leukemia virus (MLV)-based retroviral (RV) vector expressing long terminal repeat (LTR)-driven *LAMB3*

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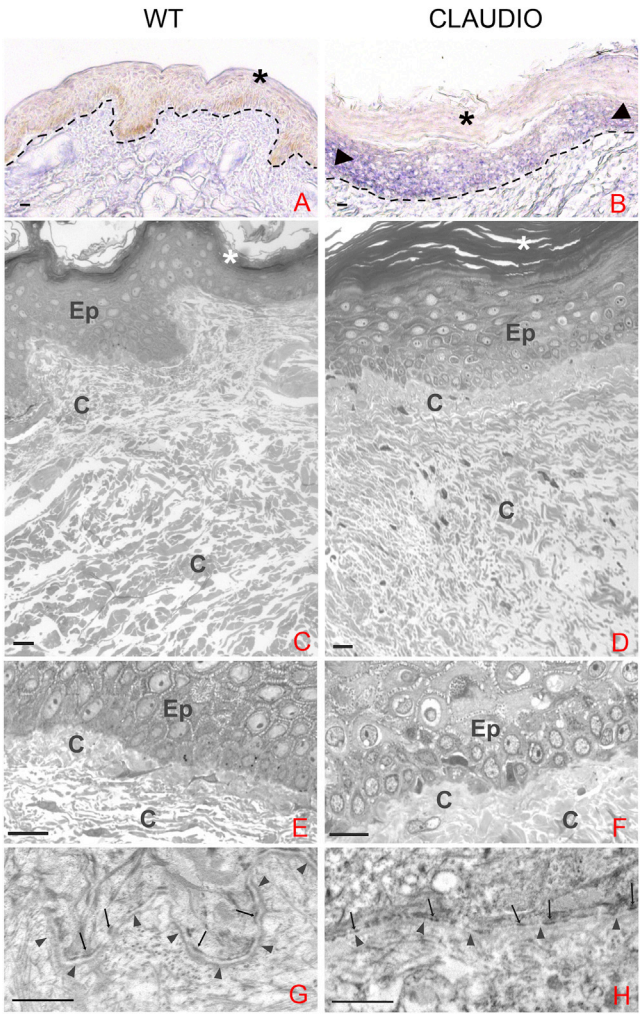

Figure 1. Regeneration of a Functional Transgenic Epidermis

(A and B) In situ hybridization with a vector-specific probe on 20-μm-thick skin sections shows the homogeneous expression of laminin 332-β3 transcripts in all epidermal layers (B, arrowheads). Sections from normal skin were used as a control (A). Dotted lines indicate the basal lamina. Asterisks mark the stratum corneum. Scale bars, 10 μm.

(C–F) Light microscopy of 0.5 μm sections from a skin biopsy of the upper leg of a healthy donor (C and E) and Claudio (D and F) were stained with toluidine blue. In both cases, normal-looking epidermis (Ep) and dermis with well-organized collagen bundles (c) are evident. Asterisks mark the stratum corneum, which is thicker in the regenerated epidermis. Scale bars, 10 μm.

(G and H) Transmission electron microscopy of 70 nm skin sections shows that basement membranes (arrowheads) and hemidesmosomes (arrows) are clearly evident in both control (G) and transgenic (H) skin. Scale bars, 1 μm.

cDNA and used to prepare transgenic epidermal grafts, which were transplanted onto surgically prepared regions of Claudio's upper legs. Synthesis of normal levels of functional laminin 332 was observed together with the development of a firmly adherent epidermis that remained stable for 1-year follow-up in the absence of blisters, infections, inflammation, or immune response (Mavilio et al., 2006).

The critical goals of this trial were to evaluate the safety and long-term persistence of the transgenic epidermis. Assessment of these parameters was crucial for continuing the trial, which has been halted since 2007 to allow our cell-culture facility to conform to the 2007 EU directive 1394 imposing Good Manufacturing Practices for any advanced therapy, and to develop ex vivo gene therapy for other forms of EB. We therefore analyzed the epidermis that regenerated on Claudio's upper legs after a very long (6.5 years) follow-up, during which time the transgenic epidermis underwent a minimum of 80 complete renewing cycles.

RESULTS AND DISCUSSION

Clinical Evaluation of the Transplanted Upper Legs

During the 6.5-year follow-up, the epidermis of both of Claudio's upper legs was normal looking, normally pigmented, and robust, and did not itch or form blisters, either spontaneously or after induced mechanical stress (such as biopsy withdrawal). Tactile and pain sensitivity was present in both legs. In contrast, blisters were consistently observed around the transplanted area. Approximately 3 years after grafting, the patient received a strong contusion on his right upper leg, which would have caused severe blistering in the diseased skin. Blisters did not appear on the bruised area. Three punch biopsies, representative of the whole transplanted area, were taken from Claudio's upper legs and used for further analyses.

Long-Term Restoration of a Normal Functional Epidermis and Dermal-Epidermal Junction

In situ hybridization using vector-specific laminin 332-β3 probes showed that the regenerated epidermis consisted only of transgenic keratinocytes (Figures 1A and 1B, arrowheads). Of note, the stratum corneum of the transgenic epidermis was thicker than that observed in control leg skin (Figures 1A and 1B, asterisks).

As shown in Figures 1C–1F, the morphology and stratification of the transgenic epidermis (Figures 1D and F) were virtually indistinguishable from those of a normal control (Figures 1C and E), with the exception of the stratum corneum, which was thicker in the transgenic epidermis (Figure 1D, asterisks) as compared with a normal upper leg (Figure 1C, asterisks). The density and organization of collagen bundles in the papillary dermis were consistent with restoration of mechanical strength. Although the

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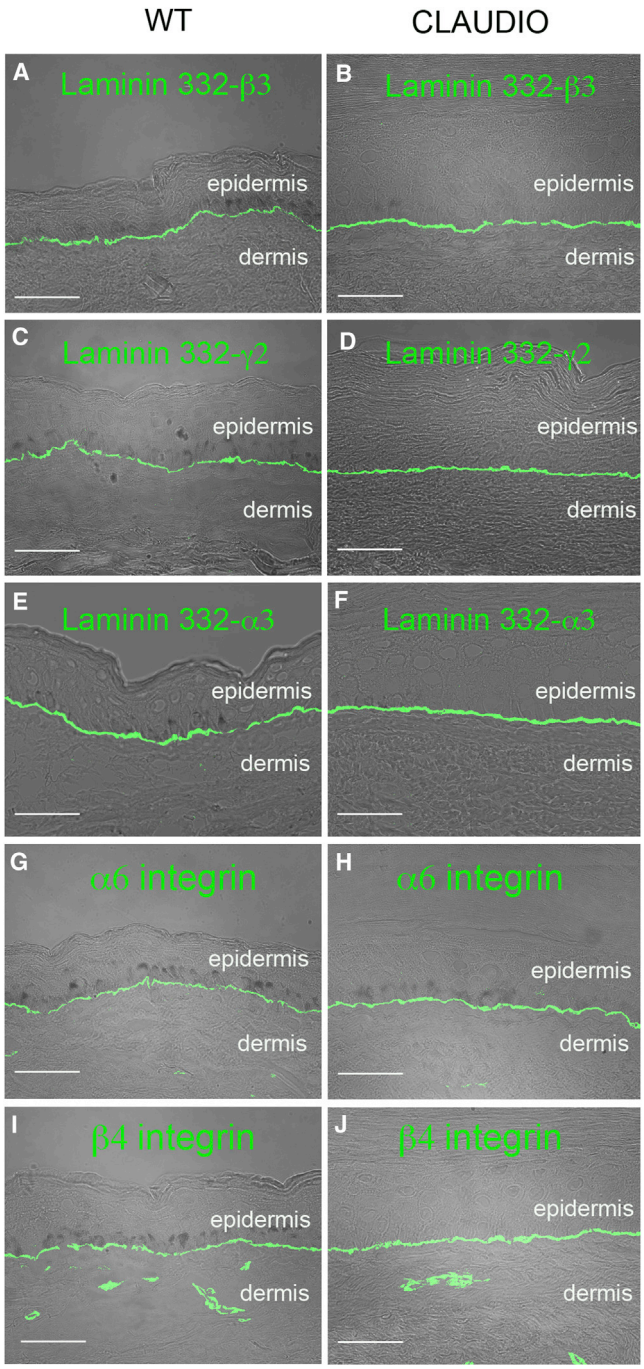

Figure 2. Expression of LAM332 and α6β4 Integrins

(A–J) IF analysis of laminin 332-β3 (A and B), 332-γ2 (C and D) 332-α3 (E and F), α6 integrin (G and H), and β4 integrin (I and J) in control (WT) and transgenic (Claudio) skin sections. The transgenic epidermis expresses normal amounts of laminin 332 and α6β4 integrins properly located at the epidermal-dermal junction. Scale bars, 40 μm.

transgenic skin had a reduced number of dermal papillae, no blisters, ruptures, and/or detachment of the epidermis from the underlying dermis were ever observed. Transmission electron microscopy (Figures 1G and 1H) showed that the thickness and continuity of the basement membrane (arrowheads) and the number and morphology of hemidesmosomes (arrows) were virtually indistinguishable between control (Figure 1G) and transgenic (Figure 1H) skin, clearly demonstrating that a functional epidermal-dermal junction had been restored.

Laminin 332-β3 was undetectable in the affected skin of Claudio, including the palm keratinocytes that were used to establish cell cultures. A tiny amount of the protein was detected only after immunoprecipitation on cultured cells (Mavilio et al., 2006). In contrast, control and transgenic epidermis expressed virtually identical amounts of laminin 332-β3, which was properly located at the epidermal-dermal junction (Figures 2A and 2B). The absence of laminin 332-β3 is associated with a decrease of α3 and γ2 chains in the protein (and its α6β4 integrin receptor) due to both reduced transcription and increased protein degradation (Matsui et al., 1998; McMillan et al., 1997; Ryan et al., 1999). As shown in Figure 2, the expression of laminin 332-γ2 (Figures 2C and D), 332-α3 (Figures 2E and 2F), α6 integrin (Figures 2G and 2H), and β4 integrin (Figures 2I and 2J) was identical in transgenic (Figures 2B, 2D, 2F, 2H, and 2J) and normal (Figures 2A, 2C, 2E, 2G, and 2I) epidermis.

As shown in Figures 3A–3D, the transgenic epidermis contained normal amounts of keratin 14 (K14, a marker of the epidermal proliferative compartment) and involucrin (INV, a keratinocyte differentiation marker), properly located in basal and suprabasal cells, respectively, suggesting that the balance between proliferation and differentiation had been restored. Elastin (ELN) was equally expressed in normal dermis (Figure 3E) and in the reticular dermis underlying the transgenic epidermis (Figure 3F). However, whereas the control dermis showed peculiar vertical fine fibers (elaunin [arrowheads] and oxytalan [asterisks]; Uitto et al., 2013), Claudio's dermis contained a diffuse, not well organized elastin fiber network. Previous chronic inflammation and/or continuous wound healing may have caused this alteration of the fine morphology of the elastic fibers.

The ΔNp63α transcription factor (Mills et al., 1999; Yang et al., 1998, 1999), which is an essential regulator of epithelial stem cell maintenance (Senoo et al., 2007), is highly expressed in holoclones (Di Iorio et al., 2005; Pellegrini et al., 2001), and is instrumental in the clinical performance and long-term persistence of epithelial cultures (Pellegrini et al., 2013; Rama et al., 2010), was expressed at similar levels and in a comparable number of cells in control (Figure 3G) and transgenic (Figure 3H) epidermis.

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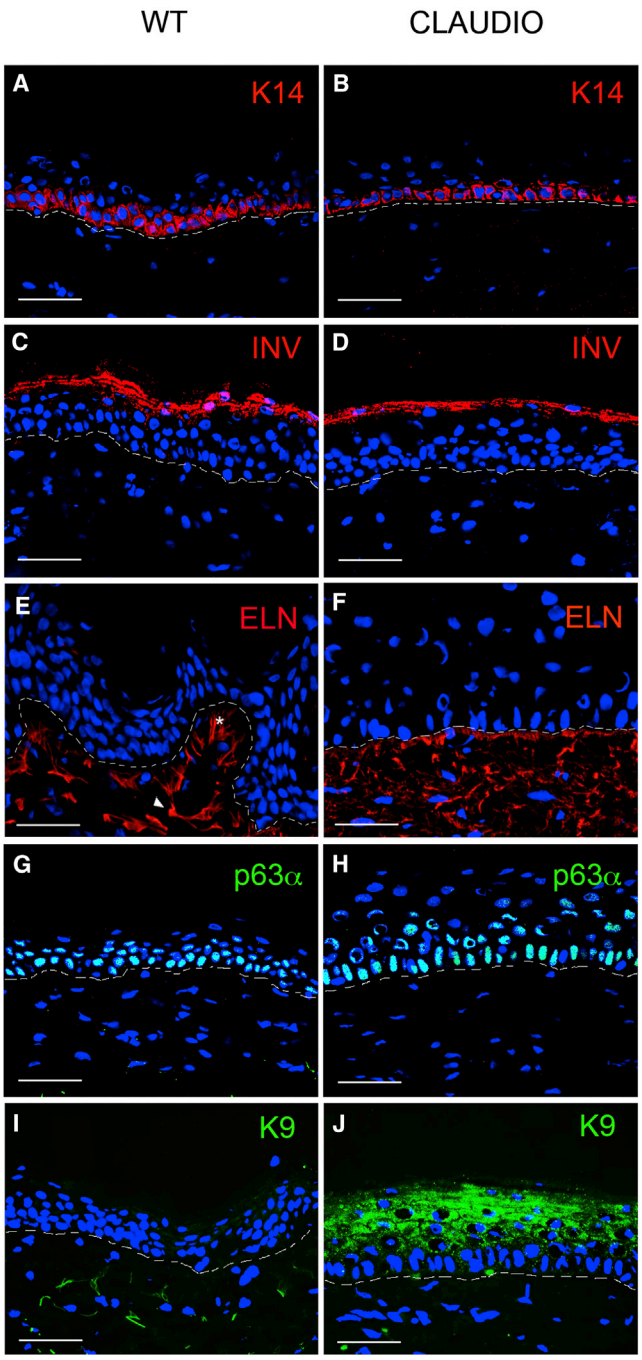

Figure 3. Expression of Epidermal Markers

(A–D) IF analysis of K14 (A and B) and involucrin (C and D) in control (WT) and transgenic (Claudio's) epidermis.

(E and F) IF analysis of elastin fibers. Network fibers (oxytalan [asterisk] and elaunin [arrowhead]) are expressed at comparable levels, but are differently organized in WT skin (E) and Claudio's skin (F).

(G and H) IF analysis shows that the ΔNp63α transcription factor is expressed at similar levels and in a comparable number of cells in control (G) and transgenic (H) epidermis.

(I and J) IF analysis shows that K9 is expressed in the upper layers of the transgenic epidermis (J), but is not detected in normal body epidermis (I).

Scale bars, 40 μm. Dotted lines indicate the basal lamina.

A Defined Number of Transduced Stem Cells Sustain the Regenerated Epidermis

The human epidermis is renewed monthly; hence, Claudio's epidermis underwent ~80 complete renewing cycles in 6.5 years. The long-term maintenance of the regenerated epidermis must be determined by the engraftment of self-renewing transduced epidermal stem cells.

A genome-wide analysis of RV integration sites was performed on DNA extracted from ~10 mm^2 of transgenic epidermis. Libraries of vector-genome junctions, generated by linker-mediated (LM) nested PCR and sequenced to saturation, retrieved six independent integrations unambiguously mapped on the human genome (Table 1). Proviruses were classified as intergenic when they occurred at a distance of >50 kb from any "known gene" (UCSC definition), perigenic when they occurred ≤50 kb upstream or downstream of the transcription start site (TSS) of a known gene, and intragenic when they occurred within the transcribed portion of at least one known gene (Table 1). Three out six integrations were intergenic. One of the three intragenic integrations landed in a gene-dense region, since it was surrounded by five genes in a less than ±40 kb window. None of these integrations belong to a comprehensive compilation of proto-oncogenes and genes associated with common insertion sites (CIS) in mouse tumors (http://microb230.med.upenn.edu/protocols/cancergenes.html). Three of the integrations, whose TSS is more proximal to the MLV integration site, are expressed (according to Affymetrix GeneChip analysis) on keratinocytes cultured under the same conditions used for transduction. The two intragenic integrations landed in the first and second introns of expressed genes, confirming the known integration preferences of γ-RV vectors in human cells (Cattoglio et al., 2010a; Cattoglio et al., 2010b).

Considering an average of two proviral copies per genome and an overall cloning efficiency of ~30%, we estimate the presence of approximately five to ten independently transduced stem cells in 10 mm^2 of epidermis. Since virtually all keratinocytes contain LAM332-β3 transcripts (Figure 1B), it is clear that the entire regenerated epidermis is sustained only by those few engrafted stem cells.

A 10 mm^2 sample of cultured epidermis contains ~15,000 keratinocytes, ~3,000 of which are clonogenic and the vast majority of which (>95%) are transit-amplifying progenitors. Thus, <150 stem cells are usually contained in 10 mm^2 of a cultured graft. Despite years of clinical applications of epidermal cultures, we have no

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Table 1. List of Retroviral Integration Sites in Skin Biopsies 6.5 Years after Grafting

ID #	Chromosome	Position	Target Gene	Gene ID	RefSeq	Location	Distance from TSS (kb)	Orientation	Expression
1sx	17q21.31	42286063	*UBTF*	7343	NM014233.3	intron 17	11.0	rev	intermediate
			ATXN7L3	56970	NM020218.1	upstream	−10.6	rev	intermediate
			TMUB2	79089	NM024107.2	downstream	21.7	for	intermediate
			ASB16-AS1	339201	NR049729.1	upstream	−22.0	rev	absent
			ASB16	92591	NM080863.4	downstream	38.0	for	absent
2sx	7q21.11	80704124				intergenic			
1dx	1p21.1	103642610				intergenic			
2dx	15q22.2	59637265	*MYO1E*	4643	NM004998.3	intron 1	27.8	for	high
3dx	9p22.3	16062571				intergenic			
4dx	10q22.2	75313406	*USP54*	159195	NM152586.3	intron 2	22.0	rev	intermediate

For each integration, columns indicate (from left to right) the identification number and biopsy of origin, chromosomal location, nucleotide position, target gene symbol, target gene identification, RefSeq identifier, position with respect to the hit gene, distance from the TSS, provirus orientation (for, forward; rev, reverse), and expression level in cultured keratinocytes as determined by Affymetrix microarray analysis. Expression values are classified as absent, low, intermediate, or high (Mavilio et al., 2006).

sense of the number of stem cells that can engraft on the wound bed. It is possible that many stem cells are lost during engraftment owing to a hostile in vivo microenvironment. A slightly higher, though comparable, number of stem cells (i.e., 36 and 26 per 10 mm^2) was identified at 1- and 4-month follow-up, respectively (Mavilio et al., 2006). However, the initial genome-wide analysis performed after such a short-term follow-up cannot rule out the presence of residual transit-amplifying cells that are still endowed with a significant proliferative potential and/or stem cells at the end of their natural lifespan. That said, the presence of approximately five to ten stem cells per 10 mm^2 of epidermis is not far from the estimated stem cell content of a normal epidermis (Pellegrini et al., 1999b; Rochat et al., 1994) and is compatible with the presence of an almost normal repertoire of genetically corrected epidermal stem cells in the regenerated skin. The remarkable proliferative and self-renewal potential of epithelial stem cells (Barbaro et al., 2007; Rochat et al., 1994) is likely to sustain the regenerated transgenic epidermis for the lifetime of the patient.

MLV-RV vectors raised some concerns about the genotoxic risk associated with their uncontrolled insertion into the genome. Insertional activation of a T cell proto-oncogene has been correlated with the occurrence of lymphoproliferative disorders in gene therapy trials of X-linked severe combined immunodeficiency (X-SCID) (Hacein-Bey-Abina et al., 2003, 2008) and Wiscott-Aldrich syndrome (WAS) (Aiuti et al., 2012; Boztug et al., 2010). Such adverse events were not reported when MLV-RV-transduced hematopoietic stem cells were used to treat adenosine deaminase (ADA)-SCID (Aiuti et al., 2009). Thus, specific risk factors may have contributed to the malignant progression observed in X-SCID or WAS. Although MLV-RV integrates preferentially into active regions of the genome (Bushman et al., 2005; Maruggi et al., 2009), insertional mutagenesis might require other oncogenetic factors, which may be related to the cell type, patient's genetic background, disease, and transgene or other mutations, to determine the onset of a tumor (Cavazza et al., 2013; Howe et al., 2008).

We did not observe tumor development or obtain any evidence of clonal expansion in vivo. Although every biopsy has a unique pattern of integration, the notion that the transgenic epidermis is sustained by only a few engrafted stem cells (five to ten per 10 mm^2) indeed minimizes the potential (theoretical) risk of insertional oncogenesis, which has never been reported in human epidermal keratinocytes. Furthermore, transforming human keratinocytes in vitro is quite an awkward task. In evaluating the risk/benefit ratio, one should also consider that severely affected EB patients usually develop aggressive skin cancer as a consequence of the progression of the disease (Fine et al., 2008), and the epidermis can be easily removed if necessary.

Epidermal Stem Cell Plasticity

Epidermal grafts were prepared from palm-derived keratinocytes (Mavilio et al., 2006). Keratin 9 (K9) is expressed in palm and sole keratinocytes, but not in the epidermis covering all other body sites (Langbein et al., 1993). Semiquantitative PCR analysis using K9-specific primers showed that K9 transcripts were equally expressed in control palm keratinocytes and in the transgenic epidermis at 4-month follow-up (not shown). As shown in Figure 3, K9 was still expressed in the upper layers of the transgenic

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epidermis (Figure 3J) after 6.5 years, whereas it was undetectable in normal body skin (Figure 3I). These findings are consistent with the presence of a thick stratum corneum, which is another hallmark of palm and sole epidermis, and demonstrate that epidermal stem cells maintain the memory of their origin even after 80 complete renewing cycles in vivo, even if they have been transplanted onto a virtually undamaged dermis.

This observation is relevant to all somatic human stem cells. It has been suggested that some somatic stem cells might be capable of differentiating across tissue lineage boundaries and hence might represent versatile effectors of therapeutic tissue regeneration. However, studies proposing such "plasticity" remain very controversial, and existing evidence suggests that such transformations are exceedingly rare (if they occur at all) in vivo and can be accounted for by alternative explanations (Bianco et al., 2013). The notion that palm-derived epidermal stem cells do not possess sufficient plasticity to generate a body epidermis makes one reconsider the supposed plasticity of any somatic stem cell. It formally confirms that the in vivo potential of a stem cell is system restricted and cell autonomous, and strengthens the concept that a stem cell's function should be verified by its ability to reconstitute a tissue in vivo (Bianco et al., 2013).

CONCLUSIONS

In summary, these data demonstrate that (1) the regenerated transgenic epidermis is fully functional and virtually indistinguishable from a normal epidermis, (2) the vast majority of transduced keratinocytes are transit-amplifying progenitors that are lost within a few months after grafting, and (3) the regenerated epidermis is sustained by a discrete number of engrafted, long-lasting, self-renewing transgenic stem cells. These data pave the way for the safe use of epidermal stem cells in combined cell and gene therapy for genetic skin diseases.

EXPERIMENTAL PROCEDURES

Light Microscopy, Transmission Electron Microscopy, and Immunofluorescence

Skin biopsies were fixed in 2.5% glutaraldehyde in Tyrode's saline pH 7.2 (24 hr at 4°C), postfixed in 1% osmium tetroxide (Electron Microscopy Sciences) for 2 hr at room temperature, dehydrated in ethanol and propylene oxide, and embedded in Spurr resin (Polysciences) as previously described (Quaglino et al., 1991). Semithin sections were stained with toluidine blue and observed with a Zeiss Axiophot light microscope. Ultrathin sections were collected on copper grids, stained with uranyl acetate and lead citrate, and observed with a Jeol 1200 EXII (Jeol) electron microscope.

For immunofluorescence (IF), skin samples were embedded in optimal cutting temperature compound, frozen, and sectioned. IF was performed on 7 μm skin sections as previously described (Mavilio et al., 2006) using laminin 332-β3 6F12 monoclonal antibody (mAb; Acris Antibodies), 332-γ2 D4B5 mAb (Chemicon), 332-α3 BM165 mAb (a gift from Patricia Rousselle, IBCP), 332-α6 450-30A mAb and 332-β4 450-9D mAb (Thermo Fisher Scientific), rabbit purified anti-p63α immunoglobulin G (IgG; PRIMM) (Di Iorio et al., 2005), K10 and K14 guinea pig antisera (Progen), K9 sc-58743 mAb (Santa Cruz Biotechnology), elastin MAB2503 mAb (Millipore), and human involucrin mAb (Leica Biosystems). Alexa Fluor 488 goat anti-mouse or Alexa Fluor 568 goat-anti rabbit (Life Technologies) conjugated secondary antibodies were used for detection. Cell nuclei were stained with DAPI.

Fluorescent signals were monitored under a Zeiss confocal microscope LSM510meta with a Zeiss EC Plan-Neofluar × 40/1.3 oil immersion objective, and analyses were done with the LSM510 Confocal Analyzer (Zeiss). Elastin staining was monitored using an Axio Imager A1 with a Zeiss EC-Plan Neofluar x40, and analyses were done using Axiovision Rel. 4.8 software.

In Situ Hybridization

The probe sequence was obtained by PCR reaction on Claudio's genomic DNA using 5′-AGTAACGCCATTTTGCAAGG-3′ and 5′-AACAGAAGCGAGAAGCGAAC-3′ primers cloned in pCRII-topoVector (TOPO TA cloning kit; Promega). In situ hybridization was performed as previously described (Brancaccio et al., 2004). Digoxigenin-labeled cRNAs were synthesized using the DIG RNA labeling kit (Roche) according to the manufacturer's instructions. The antisense RNA probe was transcribed with T7 polymerase, and the control sense probe was transcribed with SP6 polymerase.

Analysis of RV Integration Sites

Integration sites were cloned by LM-PCR as previously described (Recchia et al., 2006). Genomic DNA was digested with MseI and PstI, and ligated to an MseI double-strand linker. LM-PCR was performed with nested primers specific for the LTR and the linker. PCR products were shotgun cloned by the TOPO TA cloning kit (Invitrogen/Life Technologies) into libraries of integration junctions, which were sequenced to saturation. Sequences were mapped onto the human genome by the BLAT genome browser (UCSC Human Genome Project Working Draft, Feb 2009, hg19; http://www.genome.ucsc.edu).

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