Vettori microbici ed applicazioni in terapia genica e cellulare

Gene therapy

A therapeutic approach to preventing and/or treating disease by replacing, removing or introducing genes or otherwise manipulating genetic material.

Examples:

- adding a gene to a cell to produce a specific missing protein
- using antisense molecules to prevent viral replication
- altering CD4 cells to make them resistant to HIV infection

Genes may be introduced by direct injection or using a harmless viral vector to deliver genes into cells.

Gene therapy

Dizionario di tecnologia della scienza (academic press)
È l'introduzione di un gene in una cellula allo scopo di: 1) correggere una malattia ereditaria; 2) migliorare il genoma

It is the introduction of a gene into a cell in order to: 1) correct a hereditary disease; 2) improve the genome

Kenneth Culver
È l'inserzione di un gene funzionante in un paziente per: 1) correggere un errore innato nel metabolismo; 2) fornire una nuova funzione ad una cellula

It is the insertion of a functioning gene in a patient to: 1) correct an innate error in the metabolism; 2) to provide a new function to a cell

Gene therapy definition

The **European Medicines Agency** (EMA) defines that a gene therapy medicinal product is a biological medicinal product which fulfils the following two characteristics:

(a) it contains an active substance which contains or consists of a recombinant nucleic acid used in or administered to human beings with a view to regulating, repairing, replacing, adding or deleting a genetic sequence;

(b) its therapeutic, prophylactic or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence. Gene therapy medicinal products shall not include vaccines against infectious diseases.

The US **Food and Drug Administration** (FDA) defines gene therapy as products: "that mediate their effects by transcription and/or translation of transferred genetic material and/or by integrating into the host genome and that are administered as nucleic acids, viruses, or genetically engineered microorganisms. The products may be used to modify cells *in vivo* or transferred to cells *ex vivo* prior to administration to the recipient"

Somatic and Germ line and GT

Gene therapy can be categorized into two categories:
1) somatic gene therapy
2) germ line gene therapy

Somatic gene therapy genetic material is inserted in some target cells, but the change is not passed along to the next generation, whereas in **germ line gene therapy** the therapeutic or modified gene will be passed on to the next generation

Cell therapy

Cell therapy describes the process of introducing new cells into a tissue in order to treat a disease. Cell therapies often focus on the treatment of hereditary disease, with or without the addition of gene therapy.

There are many potential forms of cell therapy:

- Stem cell treatment; autologous (same donor) or allogenic (other donor). Mesenchymal cells are being proposed as agents for cell-based therapies, due to their plasticity, established isolation procedures, and capacity for *ex vivo* expansion
- Transplantation of mature, functional cells.
- The application of modified human cells that are used to produce a needed substance
- The xenotransplantation of non-human cells that are used to produce a needed substance. For example, treating diabetic patients by introducing insulin-producing pig cells directly into their muscle.
- The transplantation of transdifferentiated cells derived from the patient's own differentiated cells. Example: insulin producing beta cells transdifferentiated from isolated hepatocytes as a treatment for diabetes.

Stem cells

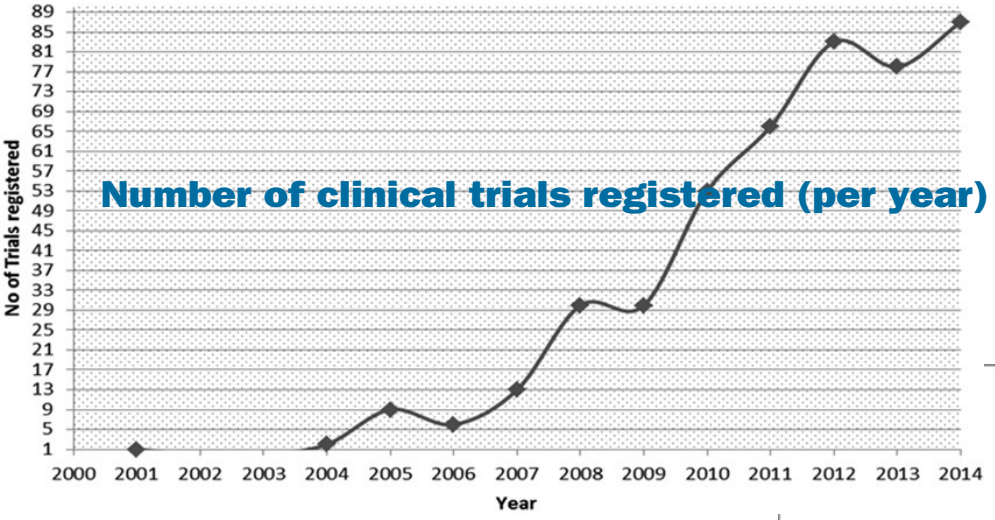
Stem cells are specialized cells, capable of renewing themselves through cell division and can differentiate into multi-lineage cells.
Stem cells include:

- embryonic stem cells (ESCs),
- induced pluripotent stem cells (iPSCs)
- adult stem cells (MSC).
- Mesenchymal stem cells (MSCs) are adult stem cells which can be isolated from human and animal sources.

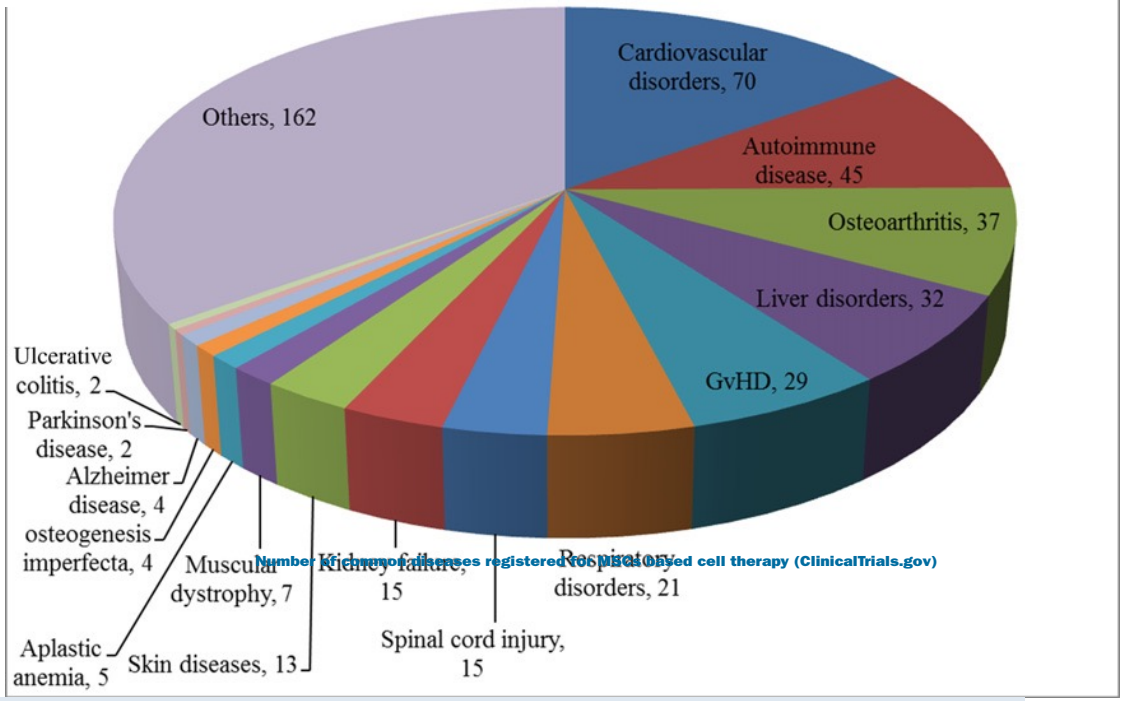
Human MSCs (**hMSCs**) are the non-haematopoietic, multipotent stem cells with the capacity to differentiate into **mesodermal lineage such as osteocytes, adipocytes and chondrocytes** as well **ectodermal (neurocytes)** and **endodermal lineages (hepatocytes)**.

MSCs express cell surface markers like cluster of differentiation (CD)29, CD44, CD73, CD90, CD105 and lack the expression of CD14, CD34, CD45 and HLA (human leucocyte antigen)-DR.

MSC in therapy



Number of clinical trials registered (per year) for MSCs based therapy (ClinicalTrials.gov)



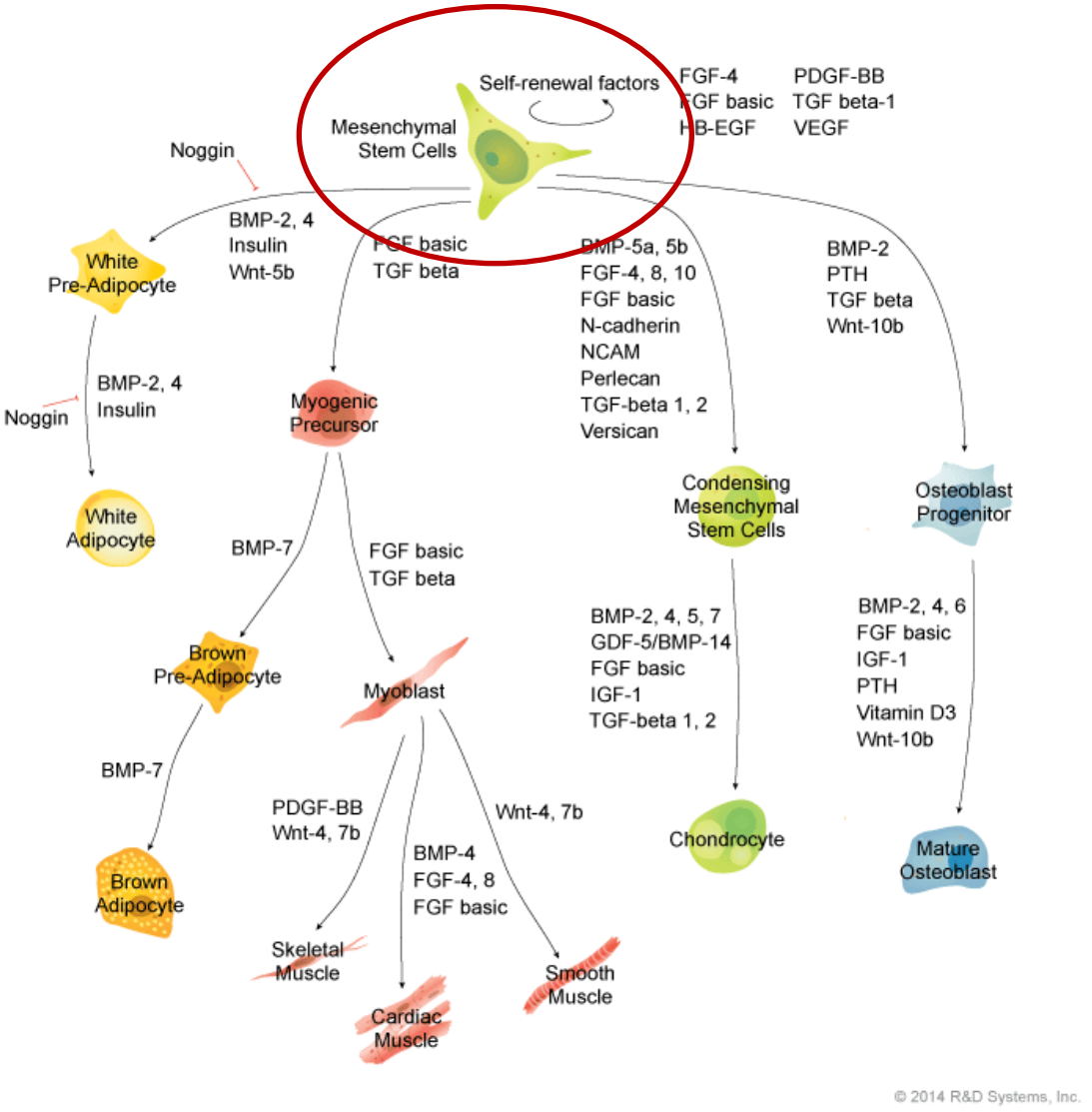
Human mesenchymal stem cells - current trends and future prospective

Biosci. Rep. (2015) / **35** / art:e00191 / doi 10.1042/BSR20150025

Imran Ullah*, Raghavendra Baregundi Subbarao* and Gyu Jin Rho*†[1]

Mesenchymal stem cells

Mesenchymal stem **cells** are multipotent stromal **cells** that can differentiate into a variety of **cell** types, including osteoblasts (bone **cells**), chondrocytes (cartilage **cells**), myocytes (muscle **cells**) and adipocytes (fat **cells** which give rise to marrow adipose tissue).



Enti
Istituto Superiore di Sanità (Per le linee guida vedere: Pubblicazioni → Notiziario)
NIH Office of Biotechnology Activities (OBA)
Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER)
UK Department of Health Gene Therapy Advisory Committee (GTAC)

Database
NCBI - MEDLINE, GenBank, Genomi, ecc

Riviste
Human Gene Therapy
Gene Therapy
Cancer Gene Therapy
Gene Therapy Weekly
Gene Medicine
Molecular Therapy

Siti web, collezioni di link
PhRMA Genomics
Gene Browser
The Virtual Center of Biotechnology for the Americas
DNA vaccine
Biotechnology Resources on the Internet
US Department of Energy genome programs

Società scientifiche
American Society of Gene Tehrapy
European Society of Gene Therapy
Japan Society of Gene Therapy

La terapia genica oggi/the gene therapy today

È un approccio sperimentale più che una pratica terapeutica
Necessita di studi di ricerca di base nei campi della:
It is an experimental approach rather than a therapeutic practice
It requires basic research studies in the fields of

- vettorologia/Vectors development
- trasferimento del DNA/DNA transfer methods
- controllo del sistema immunitario/control of the immune response
- studi di biosafety/biosafety studies

Genetic diseases that can be approached with gene therapy

Disease	**Genetic defect**
hemophilia A	absence of clotting factor VIII
cystic fibrosis	defective chloride channel protein
muscular dystrophy	defective muscle protein (dystrophin)
sickle-cell disease	defective beta globin
hemophilia B	absence of clotting factor IX
severe combined immunodeficiency (SCID)	any one of several genes (ADA) fail to make a protein essential for T and B cell function

Current gene therapy trials

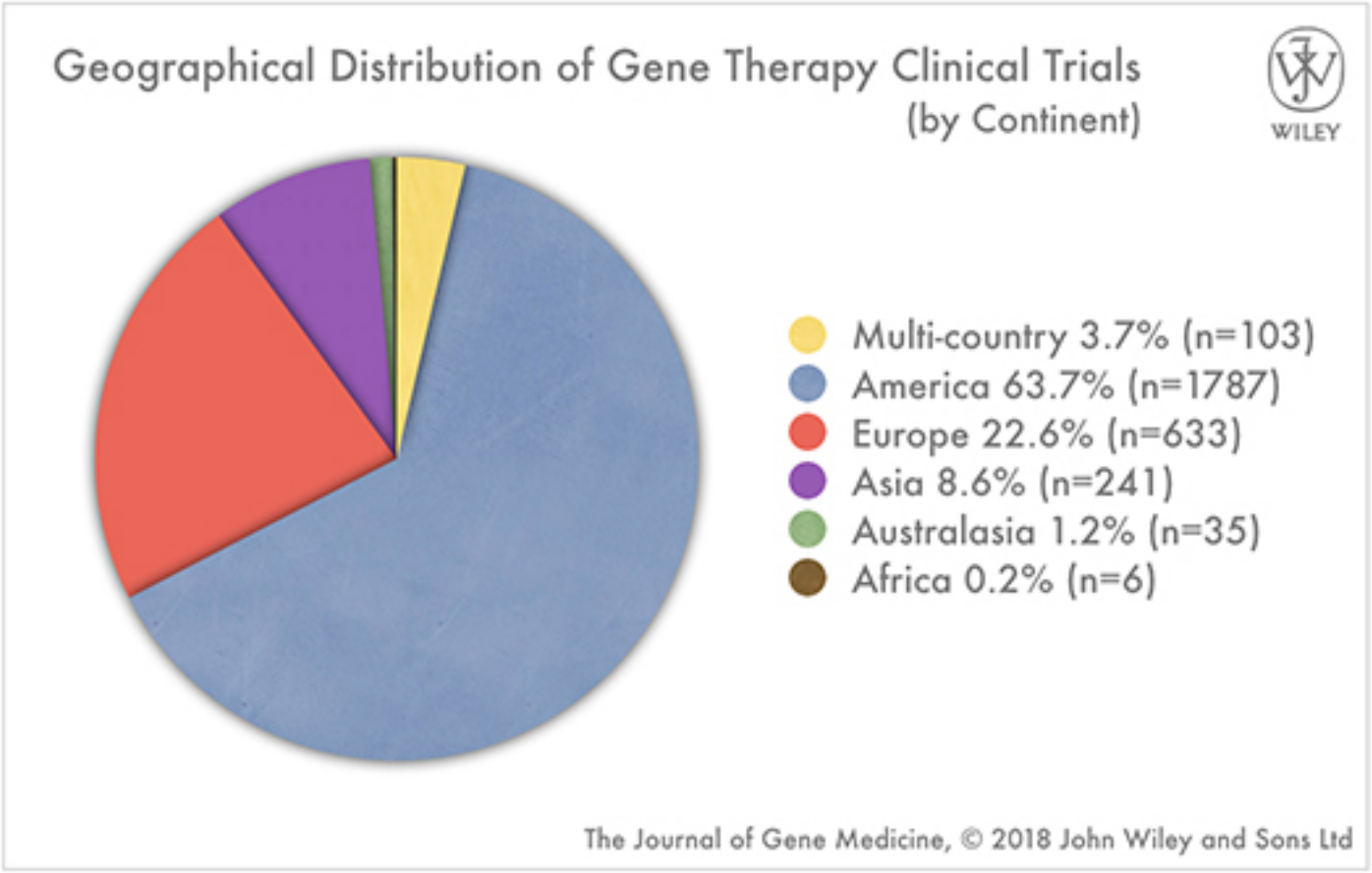
DATI AGGIORNATI AL 2018

Info at

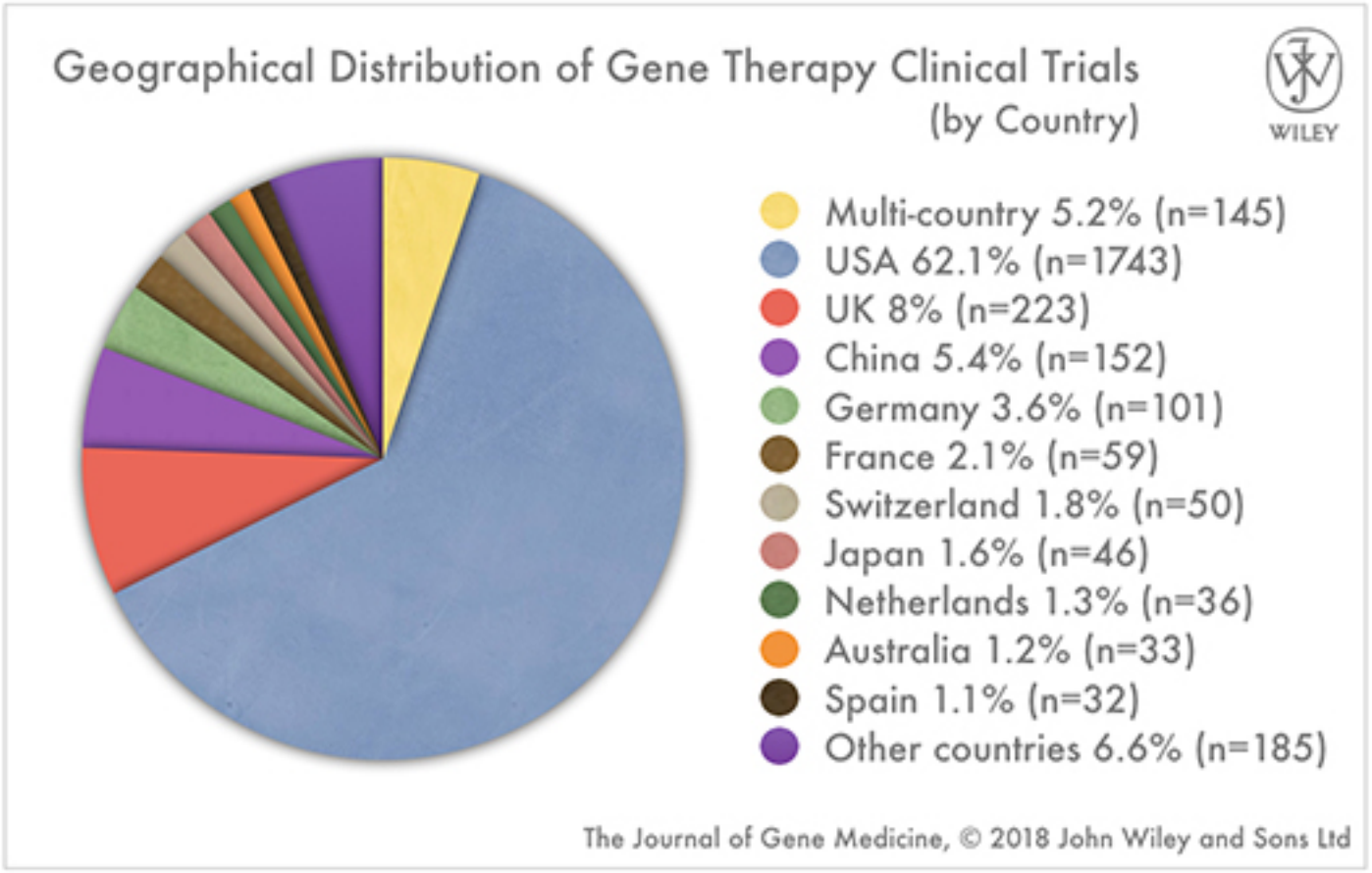
Charts and tables

search

clinical trials in the world



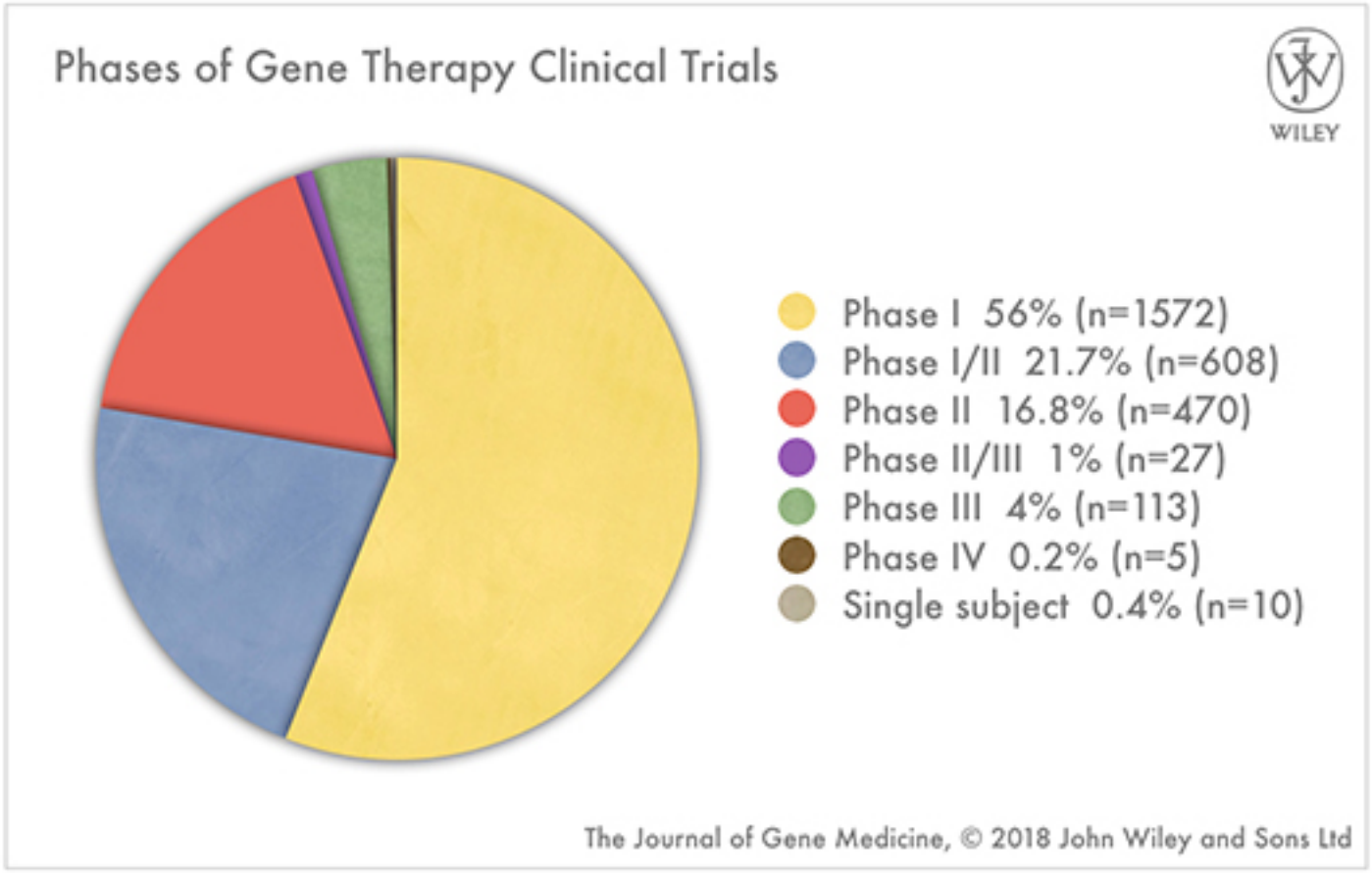
clinical trials by country



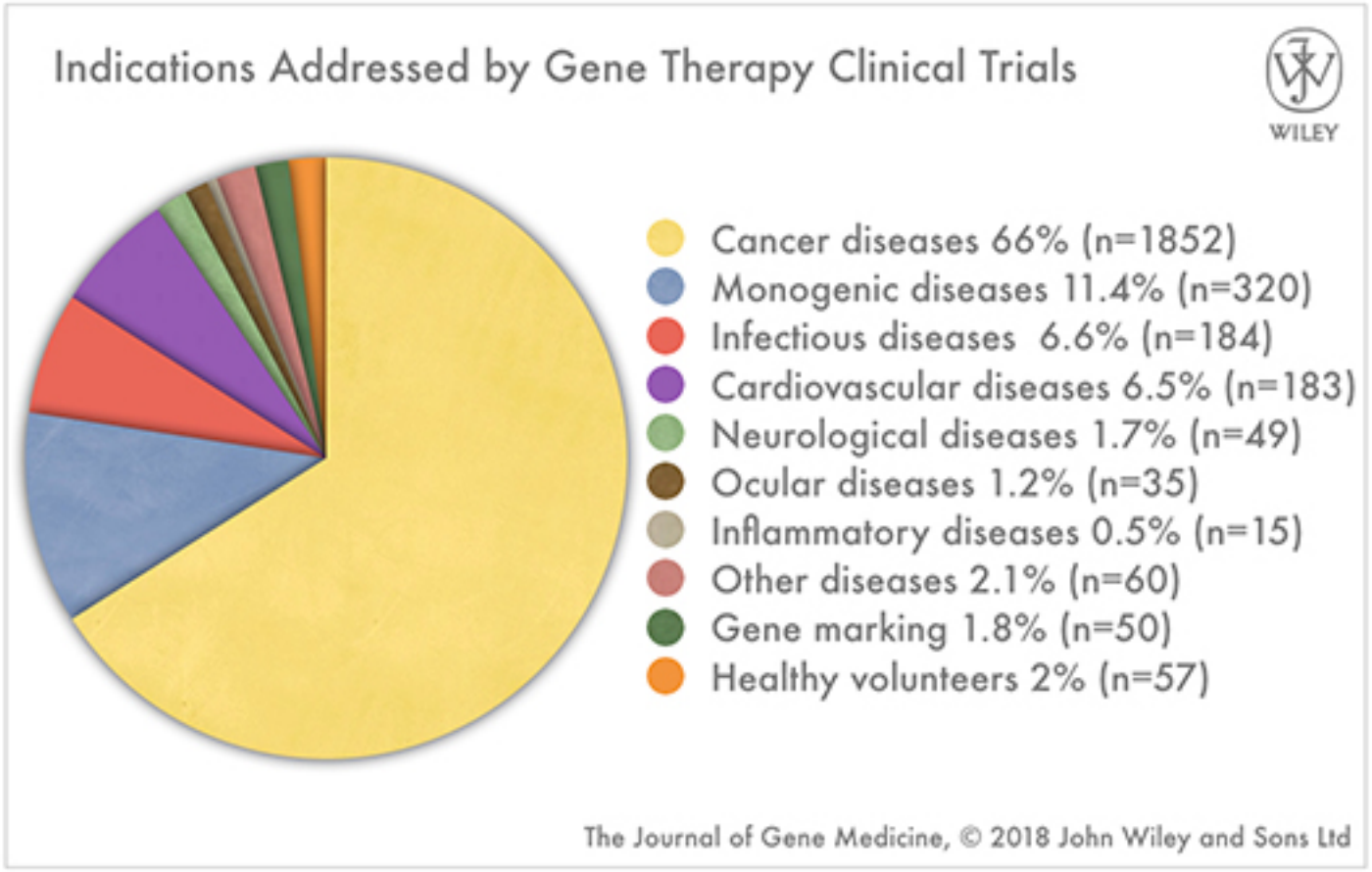
TOTAL NEMBER 2805

Italy 1,2% (n=34)

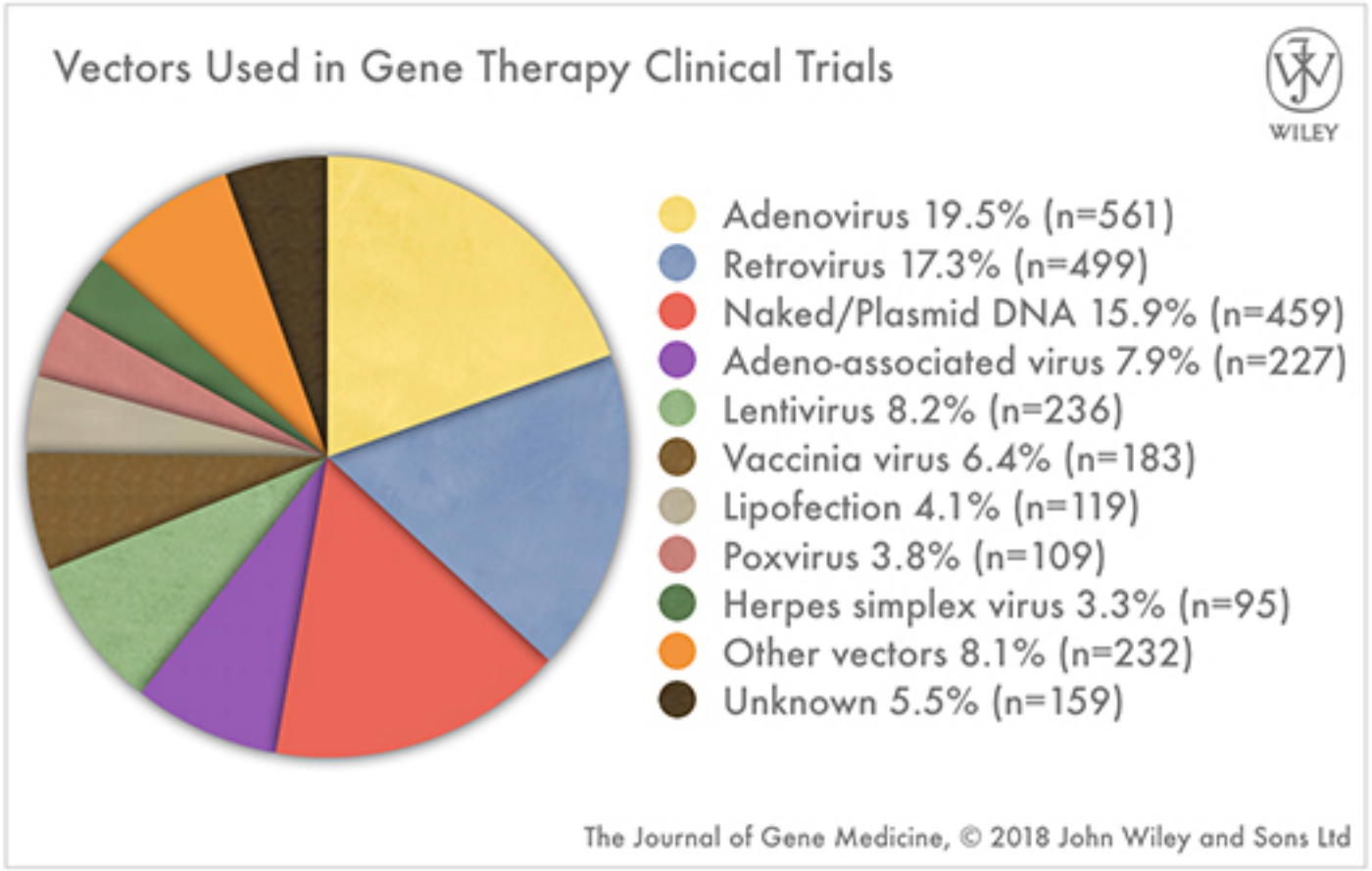
clinical trials in different phases



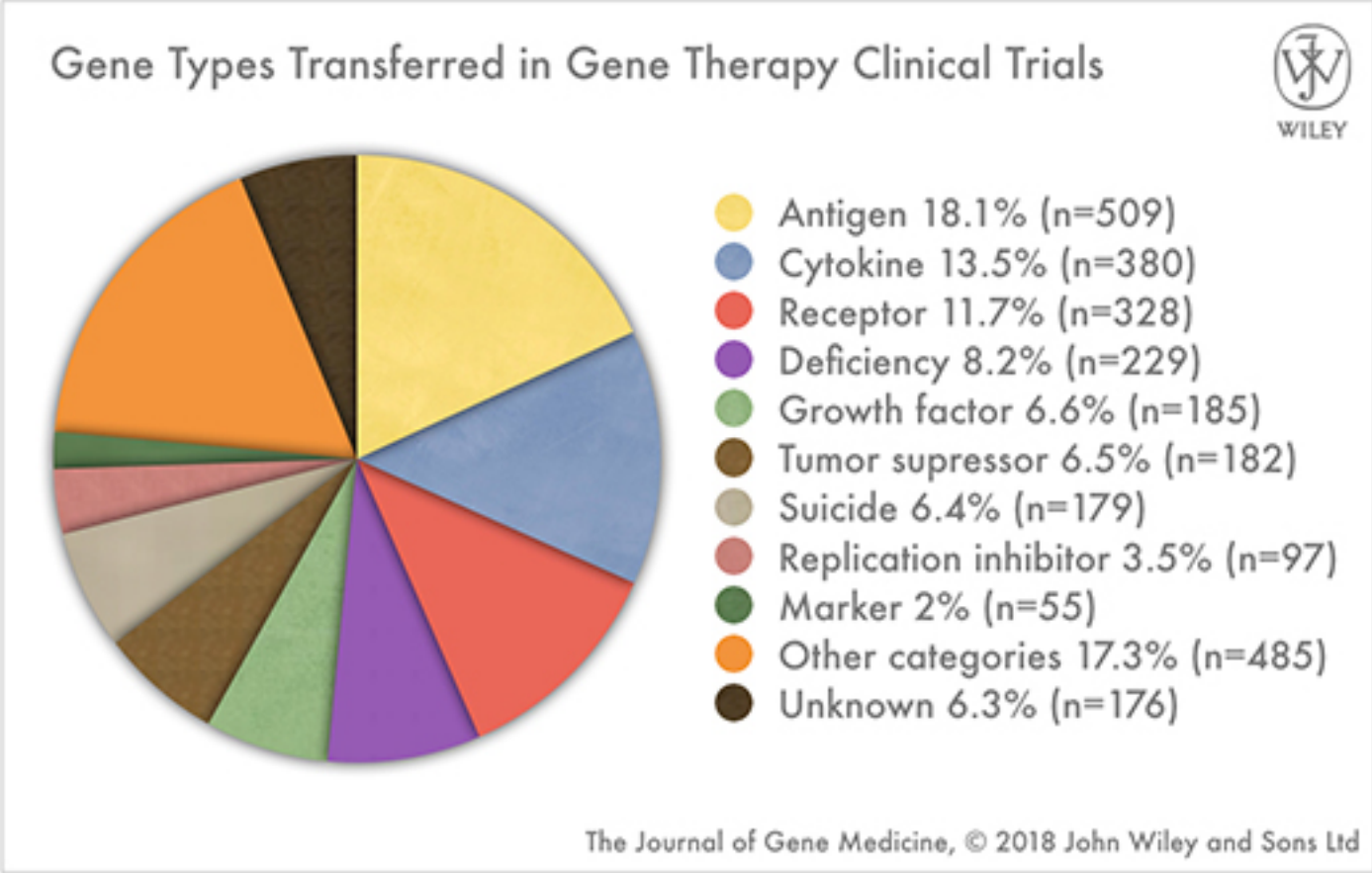
clinical trials by disease



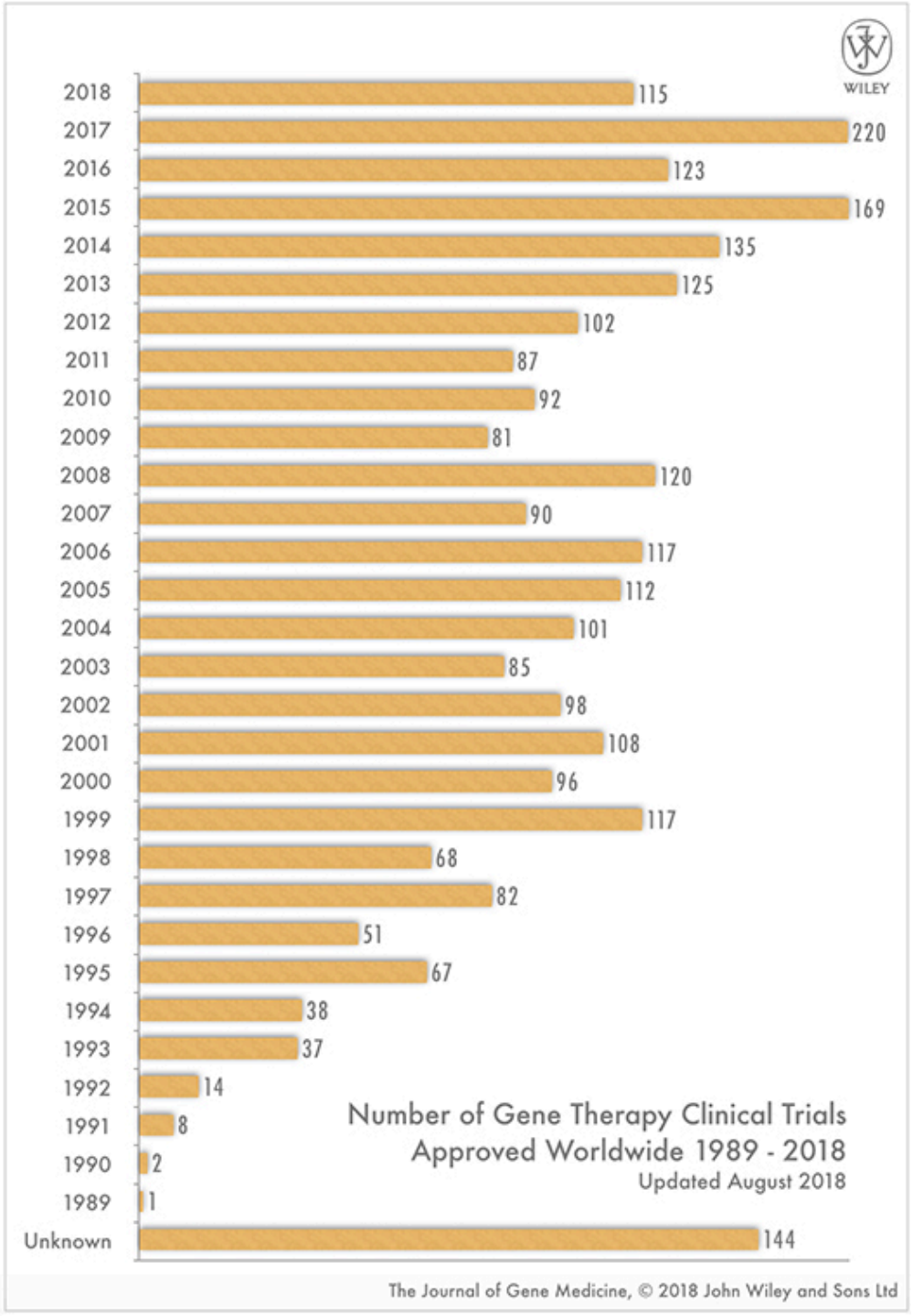
clinical trials by vectors



gene types


Gene Types Transferred in Gene Therapy Clinical Trials
WILEY
Antigen 18.1% (n=509)
Cytokine 13.5% (n=380)
Receptor 11.7% (n=328)
Deficiency 8.2% (n=229)
Growth factor 6.6% (n=185)
Tumor supressor 6.5% (n=182)
Suicide 6.4% (n=179)
Replication inhibitor 3.5% (n=97)
Marker 2% (n=55)
Other categories 17.3% (n=485)
Unknown 6.3% (n=176)
The Journal of Gene Medicine, © 2018 John Wiley and Sons Ltd

years 1989-2018
(updated august)



Review

Human gene therapy: A patent analysis

Wuyuan Zhou [a], Xiang Wang [b,*]

[a] *Zhejiang Academy of Science and Technology Information, Hangzhou 310006, China*
[b] *Key Laboratory for Translational Medicine, First People's Hospital Affiliated, Huzhou University, Huzhou 313000, China*

Gene 803 (2021) 145889

Although seen as a revolution in modern science, gene therapy has been plagued by failed clinical trials and controversial ethics in the last thirty years. Moreover, there is no comprehensive, in-depth, high-quality analysis of global gene therapy patents. This paper proposes a method to correctly retrieve patents to address the issue and use it for the patent landscape. The results show the global patent landscape of gene therapy, with the United States dominating the field, while China has emerged as a leader in recent years. For various reasons, the EU, Korea, and Japan lag in the development of patented technologies. China has edged closer to the US in both live and indefinite patents, with the Chinese Academy of Military Medical Sciences and the Chinese Academy of Sciences leading the way, surpassing primary applicants such as the US Department of Health and Human Services, the University of California, and the University of Pennsylvania. The study also reveals four broad categories of technologies that have been extensively studied in gene therapy: basic biology of the gene and diseases, diseases being treated, gene delivery methods, and potential adverse events. What is more, Adeno- Associated Virus, Retrovirus, and Lentivirus are the most prevalent gene therapy delivery vectors after 2014. The industrial development trend revealed in this paper can provide an evidence-based basis for scientific research management and decision-making.

nascita della terapia genica
birth of gene therapy

da dove nasce l'idea che un frammento di DNA/RNA possa essere inserito in un organismo a fini terapeutici?

where does the idea come from that a DNA / RNA fragment can be inserted into an organism for therapeutic purposes?

Il trasferimento genico orizzontale/Horizontal Gene Transfer

- È un sistema diffuso nei procarioti per incrementare la variabilità genica

- È utilizzato in laboratorio per creare nuove combinazioni geniche che forniscono nuovi ed interessanti caratteri genetici

- È applicabile ad organismi pluricellulari complessi come l'uomo?

Negli anni 70 grazie allo sviluppo delle tecniche di ingegneria genetica e di virologia molecolare furono sviluppati i primi vettori in grado di trasdurre cellule eucariotiche

Le basi molecolari di molte patologie sono ormai note, anche se l'intero pannello di geni responsabili di una patologia (geni modificatori) non è del tutto noto

Si inizia quindi a pensare che sia possibile applicare i protocolli di trasferimento per ripristinare la funzione mancante o alterato (curare il gene malato)

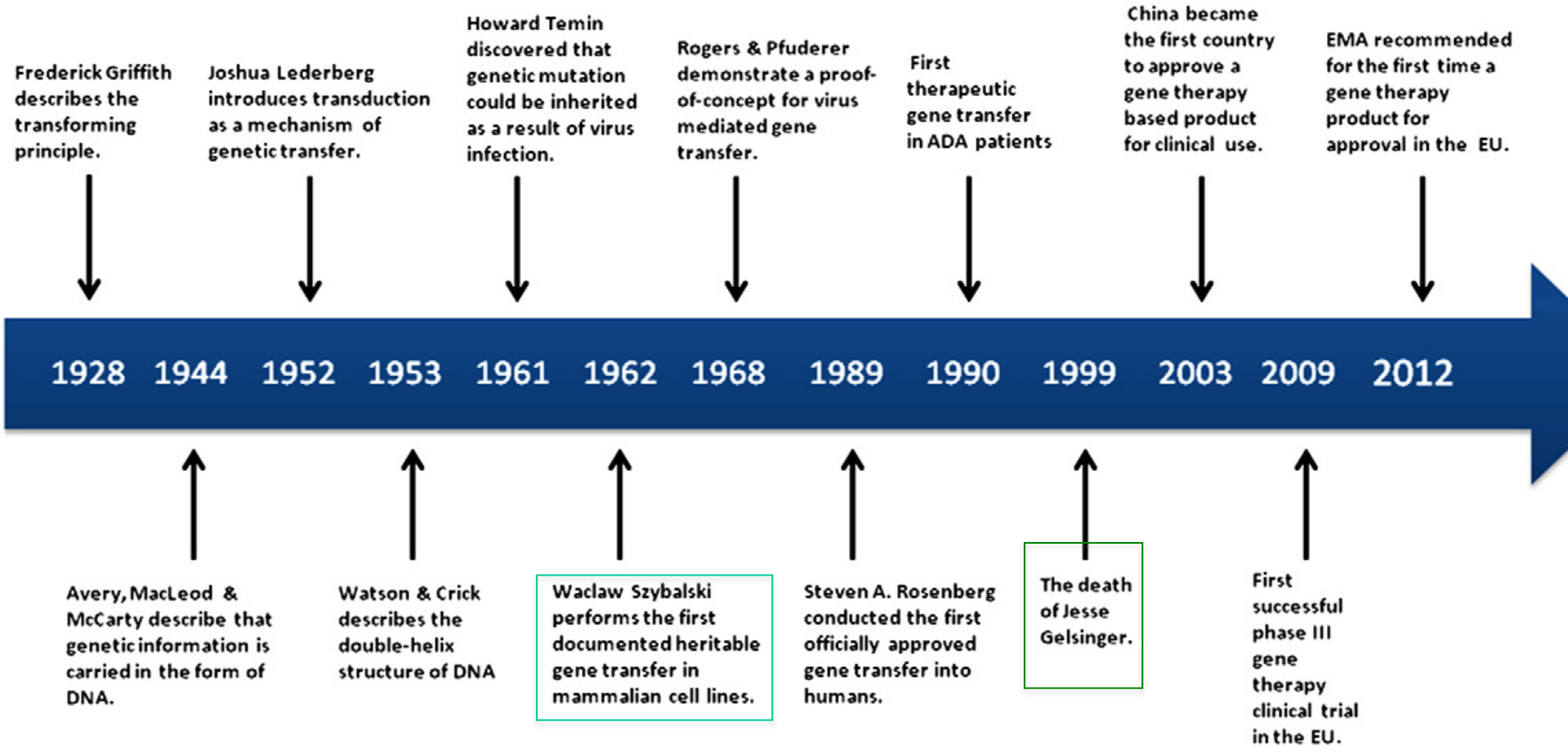


Fig. 1. Timeline highlighting some important milestones of gene therapy.

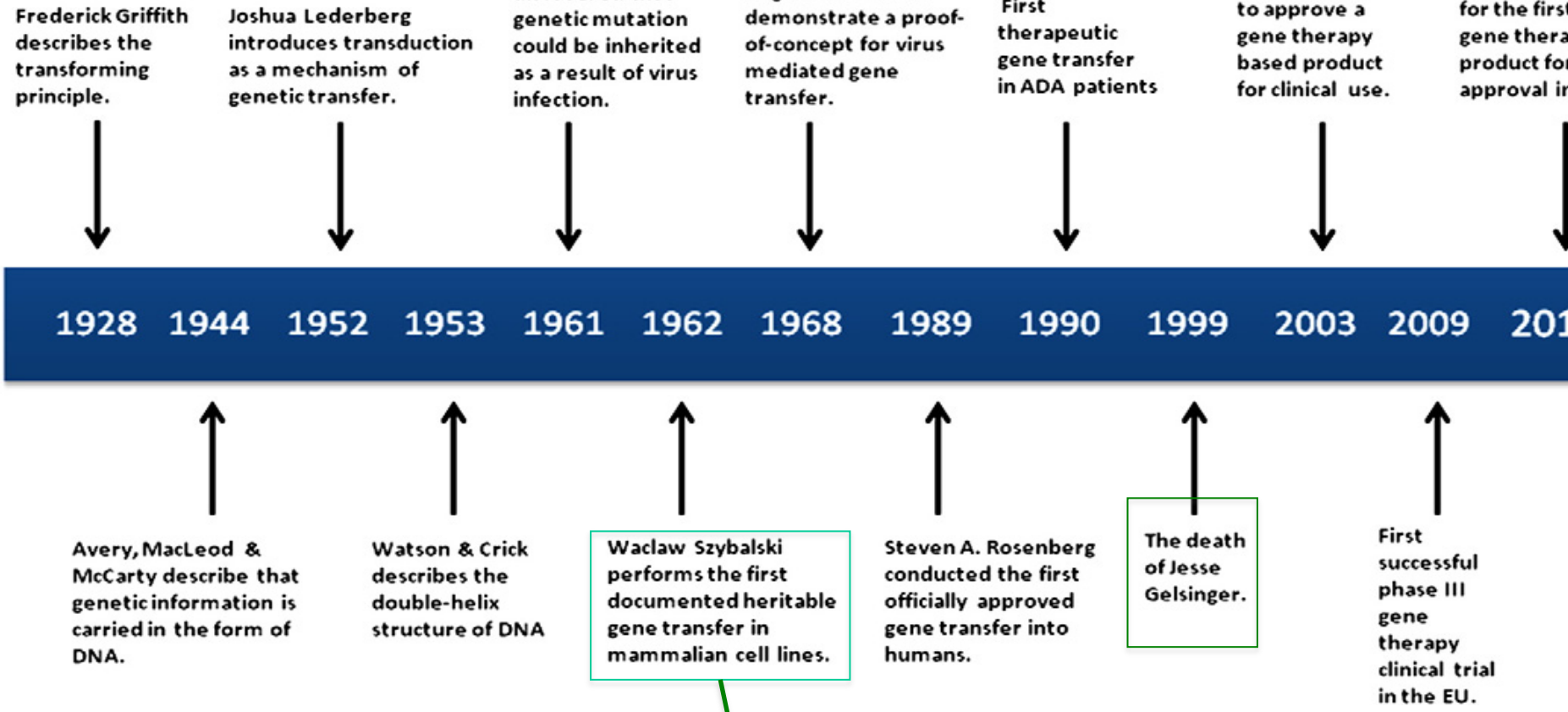


Fig. 1. Timeline highlighting some important milestones of gene therapy.

Szybalski knew that cells are able to take up foreign DNA. However, no one had been successful in demonstrating heritable transformation of a biochemical trait, until 1962, when Szybalski published his study "DNA-mediated heritable transformation of a biochemical trait" (Szybalska and Szybalski, 1962) Szybalski demonstrated that a genetic defect could be rescued by transfering functional DNA from another (foreign) source. Moreover, he demonstrated that the rescued gene could be inherited, as the daughter cells bore the same phenotype, as the transformed parent cells. The results of his study became the first documented evidence of heritable gene transfer in mammalian cells.

PURINE BIOSYNTHESIS

- dihydrofolate reductase (**DHFR**) is required for the de novo synthesis of purine; DHFR is inhibited by **Aminopterin**
- An alternate salvage pathway utilizes the hypoxanthine-guanine phosphoribosyl transferase (**HGPRT**), to convert hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate. HGRPT is a transferase that catalyzes the conversion of hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate, which can be used for purine synthesis
- **This pathway is used by the cells to survive in the presence of aminopterin**.
- HGPRT(-) cells cannot synthesis purine, so they cannot survive in the presence of aminopterin.

Szybalski experimental system

Szybalski set up a selection strategy to isolate genetically modified cells based on their phenotype.

- dihydrofolate reductase (DHFR) is required for the *de novo* synthesis of nucleic acids, purine.
- If DHFR is inhibited (aminopterin) cells utilize the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT).
- HGPRT(-) cells **cannot survive in the presence of aminopterin**

Szybalski established cell lines, derived HGPRT(-) cells from the human bone marrow cell line D98S HGRPT(+).
Aminopterin is a compound that inhibits de novo purine synthesis by inhibiting DHFR.

gene transfer in mammalian somatic cells – HAT selection

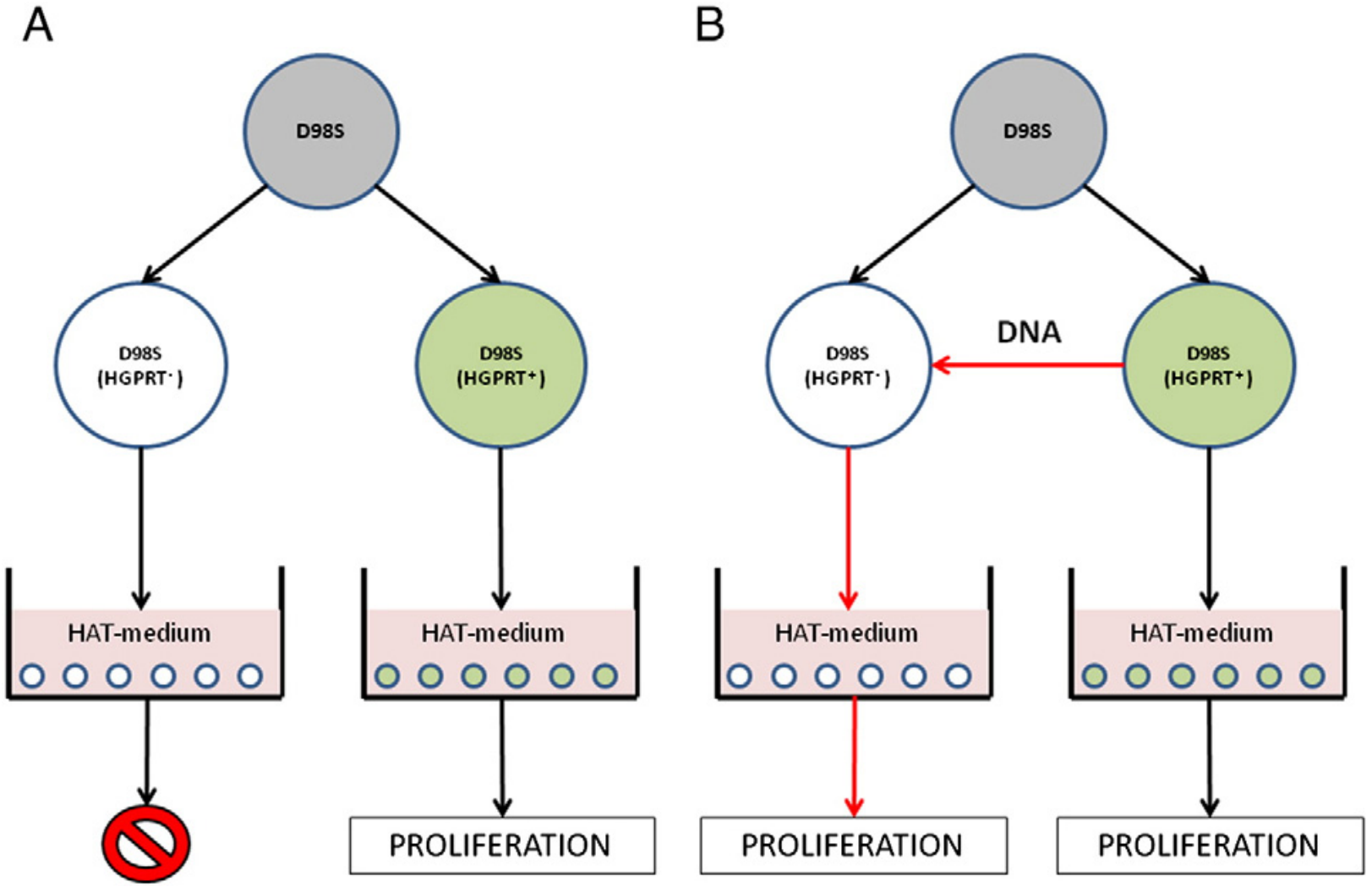


Fig. 2. Principle of the HAT-selection. Dihydrofolate reductase (DHFR) is required for the de novo synthesis of nucleic acids (essential in DNA synthesis during cell proliferation). Aminopterin on the other hand is a compound, present in the HAT-medium that inhibits de novo purine synthesis by inhibiting DHFR. Purines can be provided by the alternate salvage pathway through the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT). However, cells lacking HGPRT activity will die in the presence of aminopterin, as they cannot synthesise DNA (A). HGPRT(−) cells, however, can be rescued by isolating the DNA of HGPRT(+) cells and transferring it to HGPRT(−) cells (B), i.e. the HAT-selection.

GENETICS OF HUMAN CELL LINES, IV. DNA-MEDIATED HERITABLE TRANSFORMATION OF A BIOCHEMICAL TRAIT

BY ELIZABETH HUNTER SZYBALSKA AND WACLAW SZYBALSKI

McARDLE MEMORIAL LABORATORY, UNIVERSITY OF WISCONSIN

Communicated by R. Alexander Brink, October 15, 1962

Szybalski demonstrated that a genetic defect could be rescued by transferring functional DNA from another (foreign) source

First steps of gene therapy: the use of viral vectors

- Howard Temin discovered that in a similar fashion specific genetic mutations could be inherited as a result of virus infection (Temin, 1961). Based on his experimental observations he concluded that chicken cells infected with the Rous sarcoma virus (RSV) stably inherited viral specific gene mutations that contained the information for the generation of RSV progenies.

- In 1966, Edward Tatum published a paper evoking the effectiveness of viruses to be used in somatic-cell genetics and possibly in genetic therapy (Tatum, 1966). Of course, it was also clear that it would be necessary **to strip those viruses from their pathology causing genes** and **replace them with a therapeutic gene or genes.**

- **Rogers and Pfuderer (1968)** demonstrated an initial proof-of-concept of virus mediated gene transfer. In that study, **the tobacco mosaic virus** was used as a vector vehicle to introduce a polyadenylate stretch to the viral RNA.

- Martin Cline: the first to attempt gene therapy using recombinant DNA. Before that, Cline had already succeeded experimentally **in inserting foreign genes (i.e. dihydrofolate reductase and herpes simplex virus thymidine kinase) into mouse bone marrow stem cells (Mercola et al., 1982)**. Furthermore, he was able to demonstrate that **these modified cells were able to partially repopulate the bone marrow of other mice** (Mercola et al., 1982). Cline initiated the study and extracted bone marrow cells from two β-thalassemia patients. One patient was treated in Italy and one in Israel. However, he did so without having received permission to perform those studies

the black side of the moon

In 1999 the worst case scenario for gene therapy became a reality, when 18-year old Jesse Gelsinger took part in a gene therapy clinical trial at the University of Pennsylvania in Philadelphia.
He suffered from a partial deficiency of ornithine transcarbamylase (OTC), a liver enzyme that is required for the removal of excessive nitrogen from amino acids and proteins.
Gelsinger's immune system responded immediately after a very high dose adenovirus administration and he died four days later because of multiorgan failure (Stolberg, 1999).

Important!!!

the first patient in whom death could be directly linked to the viral vector used for the treatment.

First pilot experiment in 1989

"The first officially approved clinical protocol to introduce a foreign gene into humans was approved by the Recombinant DNA Advisory Committee (RAC) in December 1988. In that, no actual therapy was proposed, but instead, S.A. Rosenberg aimed at using gene marking techniques to track the movements of tumour-infiltrating (TIL) blood cells in cancer patients (**Rosenberg et al., 1990**)"
Rosenberg extracted the TILs **(tumor infiltrating lynphocytes**) from metastatic melanoma patients and performed retroviral gene transfer to introduce a marker gene (bacterial NeoR gene, which leads to neomycin resistance) to these cells after which he re-administered them back to the patients (Rosenberg, 1992; Rosenberg et al., 1990, 1993). The aim was to clarify, whether there is a clinical correlation between the infiltration of TILs and their effectiveness against tumours.

Based on this initial trial he obtained subsequently permission to treat two patients with advanced melanoma with ex vivo modified TILs expressing tumour necrosis factor (Rosenberg, 1992)

Terapia genica - Terapia cellulare

the team

S. Rosenberg - M Lotze - WF Anderson - RM Blaese

Negli anni 80 due ricercatori WF Anderson e RM Blaese unirono le loro competenze a quelle di due oncologi, S. Rosenberg e M Lotze

Quale modello utilizzare/which model disease?

Basandosi sulle loro competenze

- Sapevano prelevare da pazienti i linfociti T e coltivarli
- Sapevano trasdurli utilizzando un virus come vettore
- avevano isolato il gene responsabile della la malattia

Decisero di

•Trattare una patologia ereditaria del sistema immunitario

•Utilizzare vettori virali per veicolare il DNA terapeutico

il primo protocollo di terapia genica

Michael R. Blaese was the first to conduct a trail using a therapeutic gene (Blaese et al., 1995).
In September 14th 1990 the FDA approved the first time a gene therapy trial with a therapeutic attempt in humans. Two children suffering from adenosine deaminase deficiency (ADA-SCID), a monogenetic disease leading to severe immunodeficiency, were treated with white blood cells taken from the blood of these patients and modified ex vivo to express the normal gene for making adenosine deaminase.

Deficiency of adenosine deaminase (ADA) results in severe combined immunodeficiency disease (SCID). The cause for this is believed to be the accumulation of one of the substrates for ADA, 2'-deoxyadenosine to which T cells are hypersensitive. This disease, treated with exogenous ADA, can be treated successfully with bone marrow transplantation, if a suitable donor is available. Alternatively, the human ADA gene could be introduced into the autologous bone marrow.

ADA-SCID
severe combined Immunodeficiency due to ADA deficiency

•È caratterizzata da una funzionalità anomale dei linfociti T e B con conseguente predisposizione ad infezioni da patogeni opportunisti.
•La malattia è monofattoriale e causata da mutazioni nel gene ADA (deossiadenosima deaminasi)
•Nei pazienti si osserva accumulo di deossiadenosina (il substrato di ADA) che nelle cellule T viene convertito in un composto tossico che disabilita il sistema immunitario

the first gene therapy protocol for the tratment of a genetic disease

Michael R. Blaese was the first to conduct a trail using a therapeutic gene (Blaese et al., 1995).
In September 14th 1990 the FDA approved the first time a gene therapy trial with a therapeutic attempt in humans. Two children suffering from adenosine deaminase deficiency (ADA-SCID), a monogenetic disease leading to severe immunodeficiency, were treated with white blood cells taken from the blood of these patients and modified ex vivo to express the normal gene for making adenosine deaminase.

La cura

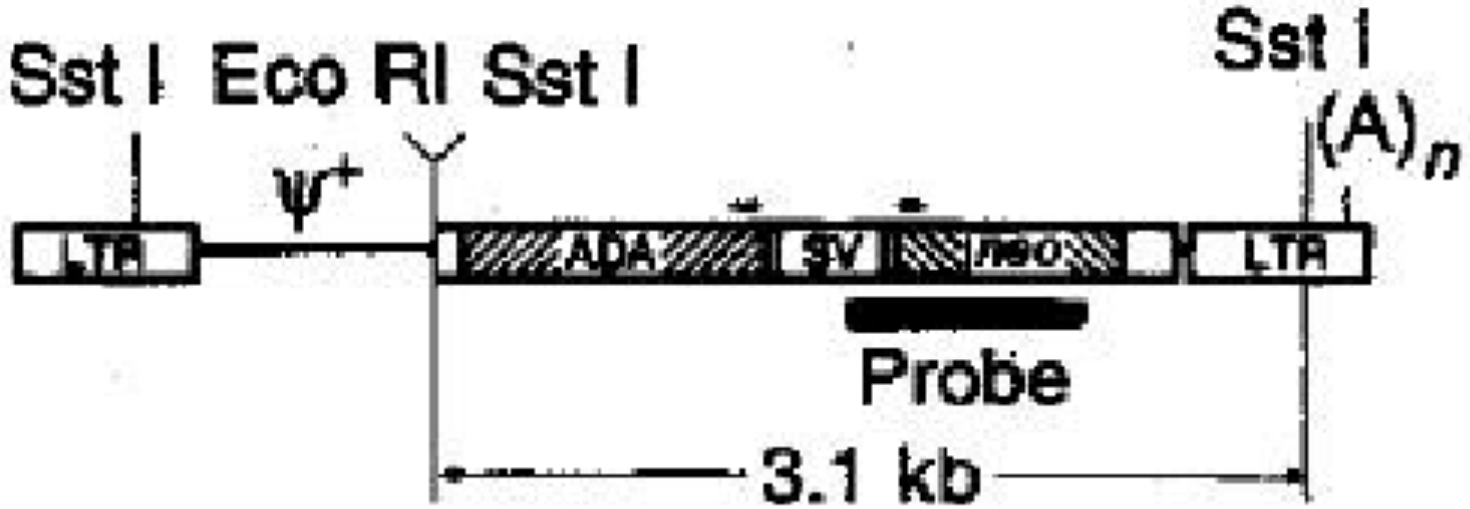
1. Trapianto di midollo allogenico
2. Terapia sostitutiva con PAG-ADA (enzima bovino purificato e coniugato con PEG)

Da esperimenti di trapianto allogenico si osservò che il recupero del difetto era associato alla persistenza dei linfociti T del donatore

Era quindi sufficiente questa popolazione cellulare per ripristinare il difetto

La presenza di una terapia sostitutiva efficace permetteva l'applicazione di un protocollo di Terapia Genica ristretto ad una specifica popolazione cellulare, i linfociti T

il vettore retrovirale contenente il gene ADA

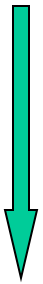


•Il cDNA di ADA (1,5 kb) clonato in un vettore retrovirale
•Linfociti T trasfettati esprimevano valori normali di ADA
•Linfociti T trasfettati in topi, conigli e primati mostravano una sopravvivenza normale
•Se inoculati con linf T ada-, essi mostravano un vantaggio selettivo rispetto ai linfociti non trattati

Nel 1990 si diede inizio ad un trial clinico su due bambini affetti da ADA-SCID in trattamento con PEG-ADA da 9 mesi

I linfociti T venero prelevati, coltivati e trasfettati con un vettore retrovirale-ADA, non venne applicata una selezione

Dopo 9-12 gg vennero reinfusi nei pazienti

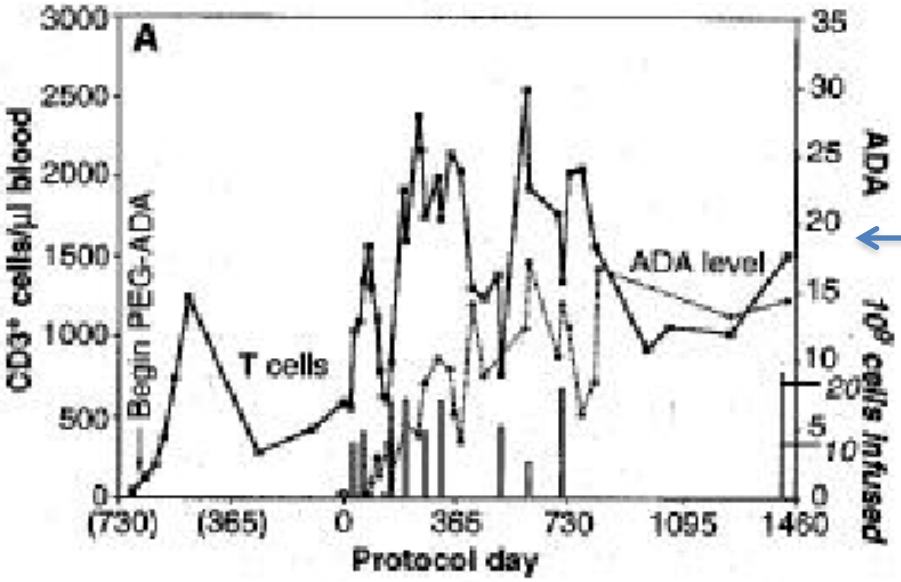


Effetti
- Aumento dei linfociti T nel sangue
- Aumento di attività ADA nei linfociti

Paziente 1

Infusioni totali 11

CD3-bearing T cells determined by FACS

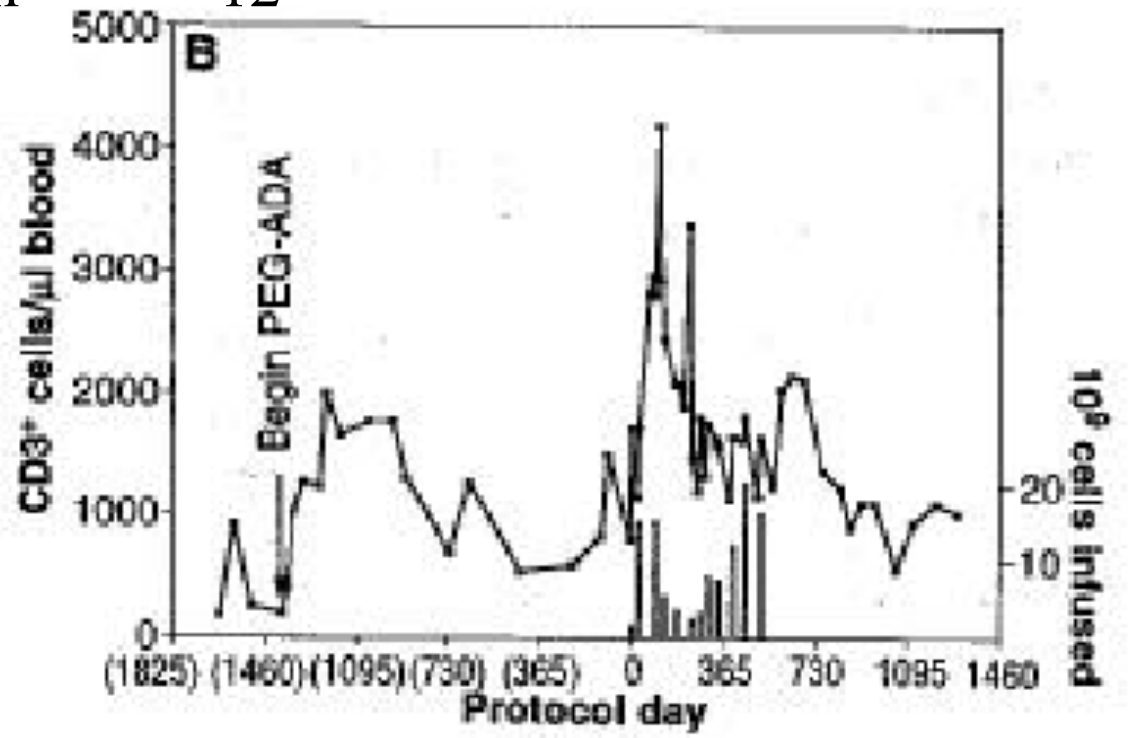


ADA activity, nanomoli di adenosina deaminata/10^8 cellule

Peripheral blood T cell counts since the time the diagnosis of ADA deficiency was made, date of treatment and the total number of cells infused for each patient. ADA level is measured in namomoles of adenosine deaminated per minute per 10^8 cells. ed cellsvertical bars indicate the date of cell infusion and their hight represents the total numbed of non select cells infused at each treatment. The T cell number represent total CD3-bearing T cells determined by FACS. A) Patient 1 began gene therapy on 14 september 1990 (protocol day 0) and received a total of 11 infusion. Ada activity was determined. Values shown are the mean of duplicate sample and represent ADA enzyme activity. B) Patient 2 began gene therapi on 31 January 1991 (protocol day 0) and received a total of 12 infusions.

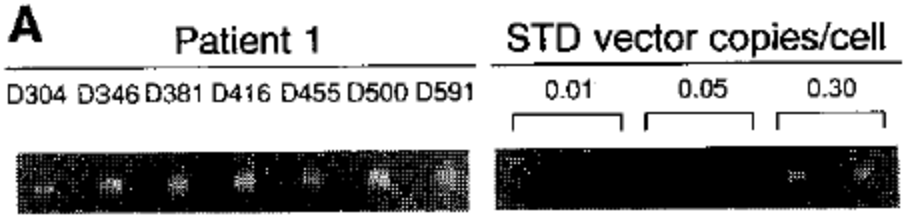
Paziente 2

Infusioni totali 12

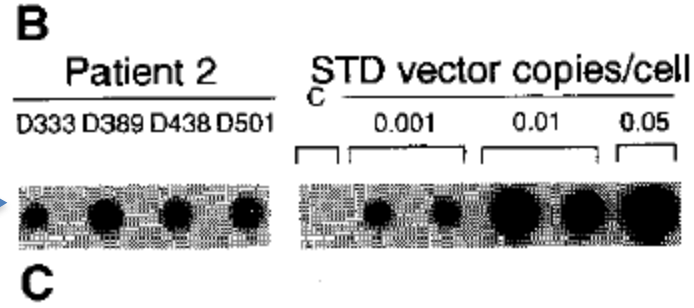


Peripheral blood T cell counts since the time the diagnosis of ADA deficiency was made, date of treatment and the total number of cells infused for each patient. ADA level is measured in namomoles of adenosine deaminated per minute per 10^8 cells. ed cellsvertical bars indicate the date of cell infusion and their hight represents the total numbed of non select cells infused at each treatment. The T cell number represent total CD3-bearing T cells determined by FACS. A) Patient 1 began gene therapy on 14 september 1990 (protocol day 0) and received a total of 11 infusion. Ada activity was determined. Values shown are the mean of duplicate sample and represent ADA enzyme activity. B) Patient 2 began gene therapi on 31 January 1991 (protocol day 0) and received a total of 12 infusions.

Monitoraggio del vettore nel tempo

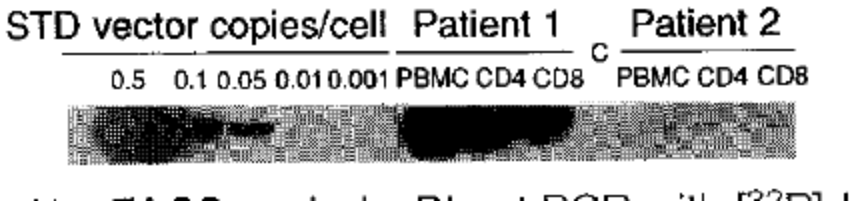


PCR



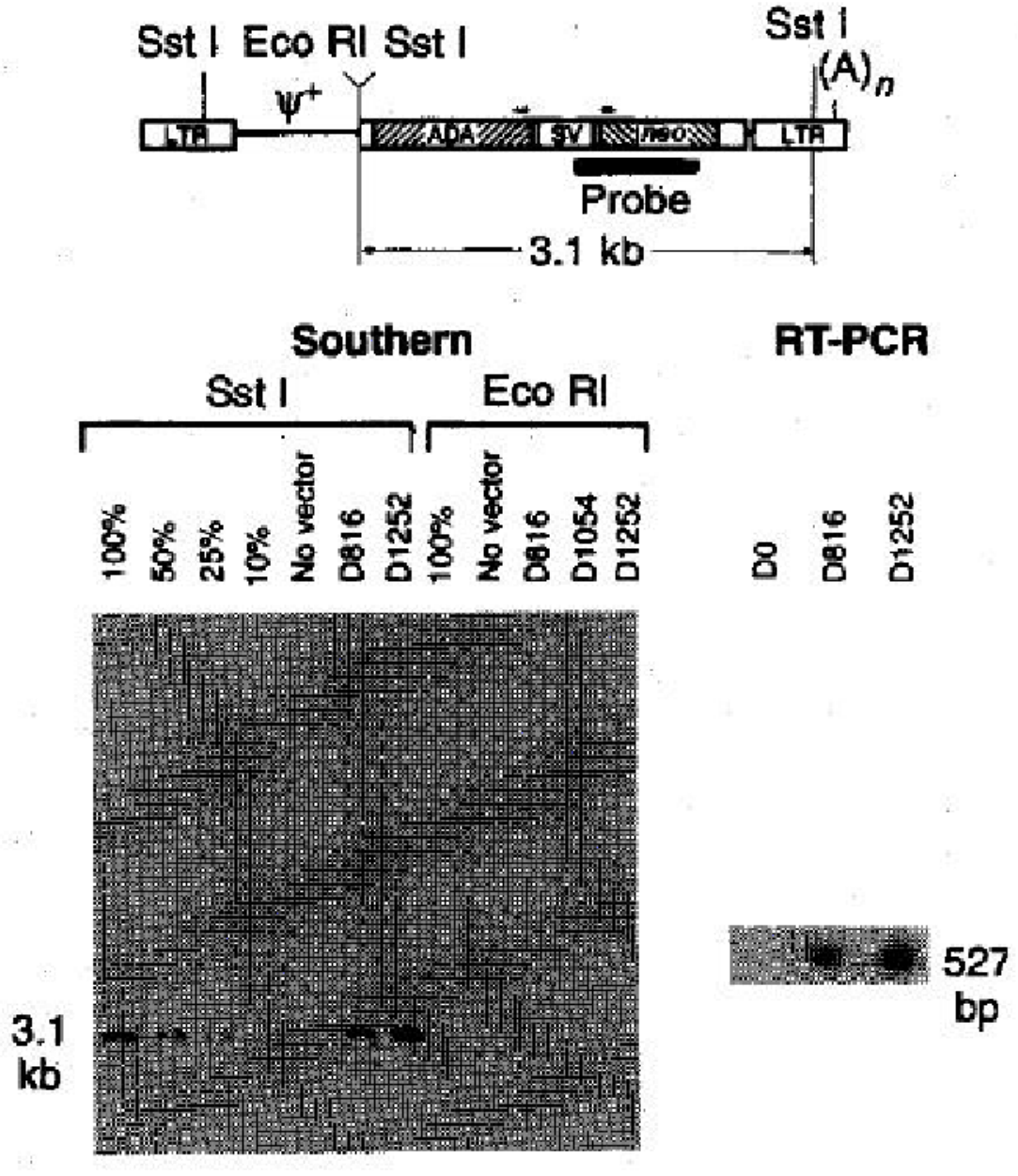
dot blot set up

copy number (dot blot)



Linfociti T purificati (CD4+, CD8+)

PCR, ibridazione dei prodotti con sonda neo



D0 day from GT
D816 day from GT
D1252 day from GT

Da consultare:

Wirth T, Parker N, Ylä-Herttuala S “History of gene therapy” Gene 2013, pp 162-169

Blaese et al. 1995, T Lymphocyte-directed gene therapy for ADA SCID: initial trial results after 4 years.