UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

BLUEBIRD BIO, INC.
Petitioner

v.

SLOAN KETTERING INSTITUTE FOR CANCER RESEARCH,
Patent Owner

Patent No. 7,541,179

DECLARATION OF INGRID HSIEH-YEE, PH.D.

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I. Introduction

I, Ingrid Hsieh-Yee, Ph.D., do hereby declare as follows:

1. I have been retained as an independent expert witness on behalf of bluebird bio, Inc. ("BLUEBIRD") for proceedings before the United States Patent and Trademark Office ("PTO") regarding the authenticity and public availability of certain prior art references in relation to US Patent No. 7,541,179.

2. I am being compensated for my work in this matter at my customary hourly rate. I am also being reimbursed for any reasonable expenses associated with my work and testimony in this investigation. My compensation is not contingent on the results of my study, the substance of my opinions, or the outcome of this matter.

A. Qualifications and Professional Experience

3. My complete qualifications and professional experience are described in my academic curriculum vitae (**Appendix A**). The following is a brief summary of my relevant qualifications and professional experience.

4. I was a Professor in the Department of Library and Information Science at the Catholic University of America for 32 years and retired at the end of August 2022. I have experience working in an academic library, a medical library, and a legislative library. I hold a Ph.D. in Library and Information Studies from the University of Wisconsin-Madison and a Masters in Library and Information Studies from the University of Wisconsin-Madison.

5. I am an expert on library cataloging and classification and have published two editions on this subject, *Organizing Audiovisual and Electronic Resources for Access*: *A Cataloging Guide* (2000 and 2006). I taught a variety of courses, including Cataloging and Classification, Advanced Cataloging and Classification, Organization of Internet Resources, Organization of Information, Digital Content Creation and Management, Internet Searches and Web Design, Information Literacy Instruction, Advanced Information Retrieval and Analysis Strategies, and The Information Professions in Society. I am familiar with metadata schema design and implementation. In my teaching, I covered the design and implementation of metadata in databases, search engines, digital repositories, digital libraries, and digital archives. I also covered how information organization affects the discovery and access to digital resources on the Internet. My research interests cover cataloging and classification, information organization, metadata, information retrieval, information architecture, digital collections, scholarly communication, user interaction with information systems, and others.

6. I am very familiar with a library cataloging encoding standard known as the "Machine-Readable Cataloging" standard, also known as "MARC," which became the national standard for sharing bibliographic data in the United States by 1971 and the international standard by 1973. MARC is the primary communications

protocol for the transfer and storage of bibliographic metadata in libraries. Experts in my field reasonably rely upon MARC records when forming their opinions.

7. A MARC record consists of several fields, each of which contains specific data about the work. Each field is identified by a standardized, unique, three-digit code corresponding to the type of data that follows. **Appendix B** is a true and correct copy of Parts VII to X of "Understanding MARC Bibliographic: Machine-Readable Cataloging" (http://www.loc.gov/marc/umb/um07to10.html) from the Library of Congress that explains commonly used MARC fields. For example, the personal author of the work is recorded in Field 100, the title is recorded in Field 245, publisher information is recorded in Field 260, the physical volume and characteristics of a publication are recorded in Field 300, and topical subjects are recorded in the 650 fields.

8. The Online Computer Library Center (OCLC) is the largest bibliographic network of the world, with more than 534 million records and thousands of member institutions (many of which are libraries of some type) in more than 100 countries. OCLC was founded in 1967 to promote and support library cooperation. According to the "Third Article, Amended Articles of Incorporation of OCLC Online Computer Library Center, Inc.," OCLC was created "to establish, maintain and operate a computerized library network and to promote the evolution of library use, of libraries themselves, and of librarianship, and to provide processes

and products for the benefit of library users and libraries, including such objectives as increasing availability of library resources to individual library patrons and reducing the rate of rise of library per-unit costs, all for the fundamental public purpose of furthering ease of access to and use of the ever-expanding body of worldwide scientific, literary and educational knowledge and information" (source: https://www.oclc.org/content/dam/oclc/membership/articles-of-incorporation.pdf).

9. OCLC members can contribute original cataloging records in MARC to the system or derive cataloging records from existing records, an activity referred to as "copy cataloging." When an OCLC participating institution acquires a work, it can create an original MARC record for the work in OCLC's Connexion system (a system for catalogers to create and share MARC records), and the system will automatically generate a code for the date of record creation in the yymmdd format, and the creating library's OCLC symbol is recorded in subfield "a" of the 040 field. Once the MARC record is in Connexion, it becomes available to other OCLC members for adoption to their local online catalogs (*i.e.*, copy cataloging).

10. After a MARC record is created in Connexion, it also becomes searchable and viewable on WorldCat, which is a free web portal for users to explore more than 10,000 libraries worldwide. The record in WorldCat, however, is not presented in MARC fields. Instead, the data elements are labeled to help users

interpret the record. Thus, the information stored in MARC records in Connexion is available to the interested public through the user-friendly WorldCat web portal.

11. Library online catalogs gained acceptance in the early 1980s and many libraries migrated their systems to the World Wide Web in the mid-1990s. Library online catalogs are based on MARC records that represent their collections in order to help the public understand what materials are publicly accessible in those libraries. Most libraries with online catalogs have made their catalogs freely available on the Web. These online catalogs offer user-friendly search interfaces. Strong user interest in keyword searches and the popularity of Google have led to the "googlization" of library search systems. As a result, many library catalogs now provide a single search box for users to conduct keyword searches, with additional support for searches by author, title, subject terms, and other data elements such as ISBN (International Standard Book Number). Library catalogs these days also offer features for users to narrow their search results by language, year, format, and other elements. Many libraries display MARC records on their online catalogs with labels for the data elements to help the public interpret MARC records. Many libraries also offer the option to display MARC records in MARC fields.

12. Libraries create MARC records for works they acquire, including books, serials, motion pictures, and publications in other formats. Monograph cataloging is fairly common in libraries, and most libraries make a newly cataloged

monograph available to the public soon after the cataloging work is completed, usually within a week. Libraries can create original cataloging records or use an existing record in OCLC to create a copy cataloging record. As soon as the cataloging record is completed, it is added to the library's online catalog for users. If the record is an original record, it is also entered into OCLC WorldCat. If it is a copy cataloging record, the library's holding symbol is attached to the existing original record in OCLC WorldCat to facilitate searching and interlibrary loan.

13. The cataloging of serials and the serial check-in process are discussed here to show how libraries usually provide access to newly received serial issues. According to the glossary of the *RDA: Resource Description and Access* cataloging standard, a serial is "a mode of issuance of a manifestation issued in successive parts, usually bearing numbering, that has no predetermined conclusion. A serial includes a periodical, monographic series, newspaper, etc." Because the publisher of a serial makes new issues of the serial available successively, a customary cataloging practice is to create one bibliographic record for the serial, and the serial record (encoded in MARC) typically provides information on the beginning date and frequency of the serial, not the dates of individual issues. In other words, libraries typically do not create MARC records for individual issues of a serial. Instead, they rely on a serial check-in system to track the receipt of new issues. A common check-in practice is to date stamp a new issue when it arrives. This practice has

become automated since the late 1990s, and libraries now vary in how they share the receipt date of a new serial issue with the public. Some libraries use a date stamp, some affix a label to indicate the receipt date, some pencil in the receipt date, and some do not provide the information to the public.

14. The serial check-in process usually takes less than an hour, and one of the steps involves placing a date stamp on the new issue to document the date the issue is checked in. After that, the holdings information of the serial is updated in the library's catalog so that users know which issues are available for request or access. After serial check-in is completed, the new issue is placed on the shelf with the previous issues of the serial. Libraries with a public periodical room typically place new issues in the periodical room for easy user access. Because information presented in serials often reflects latest discovery, a general practice of libraries is to make new issues of serials available for user access soon after they are checked in, usually within a week.

15. I am personally familiar with many online catalogs, databases, and search engines. In preparing for this declaration, I used the following authoritative information systems to search for records:

- Google Scholar (https://scholar.google.com)
- Online catalog of the University of Wisconsin-Madison General Library System (https://search.library.wisc.edu/search/system)

- ProQuest Dissertations & Theses Global (https://about.proquest.com/en/dissertations/)
- PubMed (https://pubmed.ncbi.nlm.nih.gov/)
- WorldCat (https://www.worldcat.org)

B. Scope of This Declaration

16. I have been asked to offer my expert opinion on the authenticity and public availability date of the following documents:

> May, C. M. (2001). *Therapeutic hemoglobin synthesis in beta-thalassemic mice expressing lentivirus-encoded human beta-globin* (Order No. 3020182), available from ProQuest Dissertations & Theses Global (304774780), obtained from ProQuest on September 21, 2022, **Ex 1004** ("the *May Thesis*");
>
> May, C., Rivella, S., Callegari, J., Heller, G., Gaensler, K. M. L., Luzzatto, L., & Sadelain, M. (2000). Therapeutic haemoglobin synthesis in β-thalassaemic mice expressing lentivirus-encoded human β-globin, *Nature*, vol. 406, no. 6791, pp. 82-86, obtained by counsel from the University of Wisconsin-Madison Memorial Library on September 28, 2022, **Ex 1005** ("the *May Article*");

May, C., Rivella, S., Callegari, J., Gaensler, K., & Sadelain, M., (May 1, 2000). 683. Lentiviral-mediated Transfer of the Human β-Globin Gene and Large Locus Control Region Elements Permit Sustained Production of Therapeutic Levels of β-Globin in Long-term Bone Marrow Chimeras, in "Stem Cell and Blood—Preclinical and Clinical Applications," *Molecular Therapy*, vol. 1, Issue 5, P. S248-249, obtained by counsel, **Ex 1006** ("the *May Abstract*").

C. Evidence Considered in Forming My Opinions

17. In the preparation of this declaration, I have reviewed the documents referenced below and any other documents I reference herein, and each of these is a type of material that experts in my field would reasonably rely upon when forming their opinions:

(1) The document referenced above in Section I.B;

(2) ProQuest affidavit on the public availability of the *May Thesis*, obtained from ProQuest on September 21, 2022, **Appendix 1004A**;

(3) ProQuest database record for the *May Thesis*, available from the ProQuest Dissertations & Theses Global database (PQDT) at https://www.proquest.com/docview/304774780?pq-origsite=gscholar&fromopenview=true, accessed and obtained on September 19, 2022, **Appendix 1004B;**

(4) Publisher copy of the *May Article*, obtained from counsel on October 6, 2022, **Appendix 1005A**;

(5) Bibliographic and MARC records for *Nature* that contains the *May Article*, available at https://search.library.wisc.edu/serial/999481760902121 from the online catalog of the University of Wisconsin-Madison Library System, accessed and obtained on October 6, 2022, **Appendix 1005B**;

(6) Pubmed metadata record for the *May Article*, available at https://pubmed.ncbi.nlm.nih.gov/10894546/, accessed and obtained on October 3, 2022, **Appendix 1005C**;

(7) Citations to the *May Article*, obtained from Google Scholar, **Appendix 1005D**;

(8) Publisher's copy of "Stem Cell and Blood—Preclinical and Clinical Applications" that contains the *May Abstract*, available at https://www.cell.com/action/showPdf?pii=S1525-0016%2800%2990176-X, accessed and obtained on October 3, **Appendix 1006A**;

(9) Publisher webpage for "Stem Cell and Blood—Preclinical and Clinical Applications" that contains the *May Abstract*, available at https://www.cell.com/molecular-therapy-family/molecular-therapy/fulltext/S1525-0016(00)90176-X?_returnURL=https%3A%2F%2Flinkinghub.elsevier.com%2Fretrieve%2Fpii%2FS1525001600090176X%3Fshowall%3Dtrue, accessed and obtained on October 3, 2022, **Appendix 1006B.**

These records are identified and discussed in this declaration. Experts in the field would reasonably rely on the data described herein to form their opinions.

II. Authenticity and Public Availability of the *May Thesis* (Ex 1004)

A. Authentication

18. **Ex 1004** is a true and correct copy of "Therapeutic hemoglobin synthesis in beta-thalassemic mice expressing lentivirus-encoded human beta-globin," ("the *May Thesis*"), a dissertation completed by Chad M. May in 2001 at the Weill Medical College of Cornell University, that I obtained from ProQuest, the publisher of the ProQuest Dissertations & Theses (PQDT) database, which is the most comprehensive collection of dissertations and theses in the world. When I began preparing this declaration I searched Google Scholar for records for the *May Thesis*, and retrieved a record that led me to a ProQuest database that contains the *May Thesis*. I then requested an affidavit from ProQuest on the date when the *May*

Thesis was added to PQDT and became publicly accessible. The affidavit I received from ProQuest includes a copy of the *May Thesis*, which is presented as **Ex 1004** in this declaration.

19. Page 1 of **Ex 1004** is the "Information to Users" page that states that "[t]his manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted." This page also shows that UMI of ProQuest Information and Learning of Ann Arbor, MI is the publisher. Page 3 is the title page that shows "Therapeutic hemoglobin synthesis in beta-thalassemic mice expressing lentivirus-encoded human beta-globin" is the title of a doctoral thesis by Chad M. May submitted to "the Faculty of the Graduate School of Cornell University" in "May 2001." Page 4 is the copyright page for the UMI copy of the *May Thesis* that shows the UMI Number for the *May Thesis* is "3020182" and that "UMI Microform 3020182" has a "2001" copyright date, with Bell & Howell Information and Learning Company as the copyright holder. Page 5 shows the original thesis has a "2001" copyright date with the author as the copyright holder. Pages 6 and 7 are the abstract, page 8 (internal page iii) is a "Biographical Sketch" of the author, followed by the dedication page and the acknowledgement page, and pages 11 to 14 (internal pages vi to ix) are the table of contents. **Ex 1004** shows the *May Thesis* has a front matter that ends on internal page xv, the main text has seven

chapters, including an appendix in Chapter 6 and a list of 155 references in Chapter 7, tables and figures, and the last numbered page of the *May Thesis* is page 130.

20. The PQDT copy of the *May Thesis* (**Ex 1004**) is in a condition that creates no suspicion about its authenticity. Specifically, the front matter and the body of the text are not missing any intermediate pages, the text on each page appears to flow seamlessly from one page to the next, and there are no visible alterations to the document. Moreover, **Ex 1004** was provided by ProQuest, the publisher of a database known as the most comprehensive dissertations and theses database, a place where, if authentic, this thesis would likely be found. I therefore see no reason to question the authenticity of this copy of the *May Thesis* (**Ex 1004**).

B. ProQuest Affidavit

21. **Appendix 1004A** is a true and correct copy of a ProQuest affidavit by Ms. LaTonya Morris on the public availability of the *May Thesis*. I personally requested this affidavit from ProQuest and received it on September 21, 2022. This is the type of material experts in my field would reasonably rely upon when forming their opinions.

22. Ms. Morris states in the affidavit that "ProQuest is an information company that collects, organizes publishes and distributes for sale content from a wide range of sources, including dissertations and master's theses." (¶ 2) She also indicates that the sales and publication practices of ProQuest are that "ProQuest

receives dissertations and master's theses from participating degree granting institutions. Shortly after these works are received, ProQuest makes them available for sale to the public … Prior to 2013, a 'Record' of each dissertation and each master's thesis submitted to ProQuest was published in ProQuest's print publication Dissertation Abstracts International or Master's Abstracts International" and such a record typically includes "an abstract, index record, and citation information including title, author name, degree granting institution and degree date." (¶ 3) She further describes how, prior to 1985, ProQuest disseminated records of dissertations and master's theses through print publications, then created the Abstract Database in 1985 to provide access to those records. An important change in 1997 was that "ProQuest made available the contents of the Abstract Databases in its ProQuest Digital Dissertations and Theses database ('PQDT'), which is accessible via the world wide web to authenticated subscribers" (¶ 4) and "PQDT allows for text searching of the Records as well as full text where the full PDF is properly formatted." (¶ 5) She also clarifies that "[o]nce a Record is published on the Abstract Database, the Record is available to the students, faculty, staff and /or patrons of any institutions that subscribe to the Abstract Database." (¶ 6) After reviewing ProQuest's records on the *May Thesis*, which is Dissertation No. 3020182 in PQDT, Ms. Morris states that ProQuest first made the *May Thesis* available for sale in PDF format and placed in PQDT on November 26, 2001 (¶ 8) and that "[a]t

the time of the Dissertation's publication, the Record of the Dissertation was made available directly from ProQuest in electronic format and online via third party vendors, including Dialog, as part of the Abstract Database which was made available via such vendors in the ordinary course of business." (¶ 9) This affidavit confirms my prior knowledge about Dissertation Abstracts International, the Abstracts Database, and the relationship between the Abstracts Database and PQDT. It is therefore my opinion that the *May Thesis* became publicly discoverable and accessible via PQDT on November 26, 2001.

C. ProQuest Database Record for the *May Thesis*

23. The ProQuest Dissertations & Theses Global database ("PQDT") is the most comprehensive collection of dissertations and thesis in the world that is "updated weekly and features 24 indexed and searchable fields, including full text searchability for the entire text of full-text dissertations" (source: https://proquest.libguides.com/pqdt/content). Graduate students typically consult this database to determine if their proposed thesis or dissertation topics have not already been written about; and students, faculty and other researchers search this database for titles related to their research interests. Researchers can access this database online as well as print reference products such as *Dissertation Abstracts International* ("DAI"). This information about PQDT and DAI is supported by a ProQuest webpage "Finding the dissertation or thesis you need" archived by the

Internet Archive on October 21, 2008, and available at https://web.archive.org/web/20081021040200/http://www.umi.com/en-US/products/dissertations/individuals.shtml.

24. **Appendix 1004B** is a database record for the *May Thesis* that I personally located, identified, and obtained from PQDT. This is the type of record experts in my field would reasonably rely upon when forming their opinions.

25. This PQDT database record shows that it represents *Therapeutic hemoglobin synthesis in beta-thalassemic mice expressing lentivirus-encoded human beta-globin* by "May, Chad Michael" of the Weill Medical College of Cornell University, the dissertation was published by ProQuest Dissertations Publishing with a 2001 publication date, and the ProQuest dissertation number for this thesis is "3020182." The database record shows that users can obtain the full text in PDF, preview PDF, and view abstract/details. It also shows an abstract that is identical to the abstract in the *May Thesis*. The record details area shows the subjects of the *May Thesis* are "Molecular biology" and "Immunology" and seven keywords are provided to represent the subjects, including "Health and environmental sciences; Biological sciences; Globin; Hemoglobin; Lentivirus-encoded; Thalassemic; Therapeutic." The record also shows the *May Thesis* has a total of 130 pages, the degree date is "2001," its ISBN is "978-0-493-32698-6," Weill Medical College of Cornell University is the degree granting institution, the

dissertation number is "3020182" and the ProQuest document ID is "304774780." In addition, the database record shows the source of these details is "DAI-B 62/07, Dissertation Abstracts International," meaning the *May Thesis* was indexed in vol. 62, no. 7 of *Dissertation Abstracts International. B, The sciences and engineerin*g. This PQDT record has made the *May Thesis* discoverable by author, title, subjects, keywords, abstracts and full text. After users discover this record, they would have been able to access the *May Thesis* from an institution that subscribes to PQDT or purchase a copy of the dissertation from ProQuest.

D. Summary of My Opinion on the *May Thesis*

26. Based on the ProQuest copy of the *May Thesis* (**Ex 1004**), the ProQuest affidavit (**Appendix 1004A**), and the PQDT database record (**Appendix 1004B**), it is my opinion that the *May Thesis* was published by the Weill Medical College of Cornell University in 2001, and ProQuest made this dissertation discoverable and accessible in PDF no later than November 26, 2001.

III. Authenticity and Public Availability of the *May Article* (Ex 1005)

A. Authentication

27. **Ex 1005** is a true and correct copy of Therapeutic haemoglobin synthesis in β-thalassaemic mice expressing lentivirus-encoded human β-globin, ("*May Article*"), by May et al., *Nature*, vol. 406, no. 6791, pp. 82-86, obtained by counsel from the library system of the University of Wisconsin-Madison. When I

began preparing this declaration I searched WorldCat by the title of the *May Article* for records and retrieved a WorldCat record (https://worldcat.org/en/title/4654005982) that showed the University of Wisconsin-Madison General Library System held the journal containing the *May Article*, so I searched their online catalog for records for this journal, and the search results confirmed the holdings information. I then obtained a copy of the *May Article* from this library system through counsel, who obtained the copy from Wisconsin TechSearch (WTS), a document delivery service based at the University of Wisconsin-Madison. This copy is presented as **Ex 1005** in this declaration.

28. The first page of **Ex 1005** is the cover of *Nature* that shows this journal is an international weekly journal of science that is also available online at www.nature.com, and this print issue has a "6 July 2000" issue date. The cover highlights four articles in this issue, including "β-Thalassaemia: Prospects for gene therapy." It carries an address label of the Memorial Library of the University of Wisconsin-Madison, a date stamp of "Received JUL 18 2000 University Library" and a label stating "Do Not Remove From Current Periodicals Room." Page 2 is the table of content that identifies this issue as "Volume 406, issue no. 6791" and shows an article "β-Thalassaemia: Prospects for gene therapy" that begins on page 82. The bottom of page 2 shows the ISSN of *Nature* is "0028-0836" and the journal "is published weekly on Thursday, except the last week in December," by the Nature

Publishing Group of London. Pages 3 to 7 (internal pages 82 to 86) are the *May Article*, and internal page 82 shows the title, authors, and abstract, and also shows the *May Article* is placed in the "Letters to nature" section of this issue. **Ex 1005** has a total of five pages, including six figures (some color) and 30 references. The publication history is shown on internal page 86 indicating the article was "[r]eceived 10 February; accepted 17 May 2000." A note after the references indicates "[s]upplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*."

29. **Appendix 1005A** is a true and correct copy of the *May Article* obtained by counsel from the publisher. Because the *May Article* is about gene therapy, I searched PubMed to determine if it was indexed. I found a PubMed record (https://pubmed.ncbi.nlm.nih.gov/10894546/) for the *May Article* that showed a full text link to "Nature Publishing Group" that led me to the publisher's webpage for the *May Article* at https://www.nature.com/articles/35017565. It informed users that they could obtain the *May Article* from an institution with a subscription to the journal or purchase the article from the publisher, so I obtained a copy of the *May Article* through counsel. This publisher copy is presented as **Appendix 1005A** in this declaration. I personally located and identified this article, and obtained this copy through counsel. This is the type of material experts in my field would

reasonably rely upon when forming their opinion.

30. **Appendix 1005A** shows the *May Article* has a total of 5 pages, including six figures and 30 references. I have closely compared the University of Wisconsin copy of the *May Article* (**Ex 1005**) with the publisher's electronic copy (**Appendix 1005A**) and found them to have the same content. The only difference is that every page in the publisher's copy shows a "©2000 Macmillan Magazines Ltd" note.

31. The University of Wisconsin copy of the *May Article* (**Ex 1005**) has the same content as the publisher's copy (**Appendix 1005A**). It is in a condition that creates no suspicion about its authenticity. Specifically, this copy is not missing any intermediate pages, the text on each page appears to flow seamlessly from one page to the next, and there are no visible alterations to the document. Moreover, **Ex 1005** was found in the custody of a university library, a place where, if authentic, it would likely be found. Accordingly, I see no reason to question the authenticity of the University of Wisconsin of the *May Article* (**Ex 1005**).

B. Records of the University of Wisconsin-Madison Library System

32. **Appendix 1005B** is a true and correct copy of the bibliographic and MARC records for *Nature* whose vol. 406, no. 6791 contains the *May Article*. I personally located, identified and obtained these records from the online catalog of the University of Wisconsin-Madison General Library System by searching by the

journal title for records. These are the type of records experts in my field would reasonably rely upon when forming their opinions on the public availability of a document.

33. Pages 1 to 6 of **Appendix 1005B** show the bibliographic record and holdings information for *Nature* at the University of Wisconsin-Madison General Library System. Page 1 informs users they can view this journal online, use it in a reading room, or request a copy. Page 2 shows the Memorial Library call number for this journal is "AP N283" and the library keeps "[c]urrent issues in Periodicals Room" and has many older volumes of this journal, including "v.342=no.6245(1989:Nov.2)-v.438=no.7071(2005:Dec.29)" meaning this library holds volume 406, no. 6791 that contains the *May Article*. The "Publication Details" section on page 5 shows this publication's format is "Journals, Magazines, Newspapers," its contributor is "Lockyer, Norman, Sir, 1836-1920, editor" and its ISSNs are "0028-0836, 1476-4787." The notes on page 6 show the journal's cover shows "International weekly journal of science" and it is also "issued online."

34. Pages 6 to 9 are the MARC record for *Nature* that shows the creation date and creator of the record. The first six digits of Field 008 show that this MARC serial record was created on "750901" (*i.e.*, September 1, 1975) and the "c18699999" code following this creation date indicates that the journal is a continuing resource that began publication in 1869, and "9999" indicates that the

journal has no end date of publication. Subfields "a" and "c" of Field 040 show that "MUL" created the original MARC serial record and Field 049 shows "GZMA" used the original MARC record to create their copy cataloging record. According to the *Directory of OCLC Members* (https://www.oclc.org/en/contacts/libraries.html), "MUL" is the OCLC library symbol for MULS, a library network based at the Wilson Library of Minneapolis, Minnesota, and "GZM" is the OCLC library symbol for the University of Wisconsin-Madison General Library system. Because the General Library system did not modify the original MARC record when it created its copy cataloging record, their symbol ("GZM") is not included in subfield "d" of Field 040, and Field 049 shows this MARC record represents a publication in the General Library System. Data from Fields 008, 040, and 049 inform my opinion that MULS created the original serial record for *Nature* on September 1, 1975, and the record was used by the University of Wisconsin-Madison General Library System as the basis for copy cataloging later. As discussed earlier, a common library practice is to create a record for a serial, without creating records for individual issues of the serial. When individual issues are received, they go through the serial check-in process, which usually takes less than an hour. After that, the newly received issues are placed on the shelf or in a periodical reading room for user access, usually on the same date of serial check-in or within a week after serial check-in. I know this is the customary library practice from personal experience as a cataloger,

cataloging instructor and researcher of library catalogs and information systems. The date stamp on vol. 406, no. 6791 of *Nature* that contains the *May Article* (**Ex 1005**) shows the issue was received by the Memorial Library on July 18, 2000. It is therefore my opinion that this issue (and the *May Article* contained therein) would have been publicly accessible as early as July 18, 2000, and no later than July 25, 2000, at the University of Wisconsin-Madison General Library System.

35. Field 022 of the MARC record (**Appendix 1005B**) shows "0028-0836" as this journal's ISSN, Field 030 shows "NATUAS" as the journal's CODEN (a six-character alphanumeric unique identifier of a periodical or a non-serial publication), Field 245 shows the journal title, Field 260 shows the publisher information (the square brackets indicate the information is not in the journal and is provided by the cataloger who consulted external sources), Field 362 shows the journal began with volume 1 on November 4, 1869, and Field 310 shows the publication frequency since 1981 has been "[w]eekly, except last week in Dec." Field 510 shows *Nature* is selectively indexed by *Chemical Abstracts*. Field 530 shows this journal is also available online. Field 700 shows the original editor is provided as an additional access point to help users discover this journal.

36. Pages 9 to 11 are the MARC record for the online version of *Nature*. Field 022 shows the ISSN for the online version is "1476-4687," Field 260 shows the publisher is Nature Publishing Group, Field 310 shows the publication frequency

is "Weekly" and Field 710 shows the publisher is included as an additional access point to help users discover this journal.

37. The subject of *Nature* is represented by Library of Congress subject headings in Fields 650. The MARC record for the print version shows "Science" is used as the main heading, followed by a form subheading of "Periodicals" encoded in Subfield "v" to indicate the main topic is treated in a journal. The MARC record for the electronic version shows four subject headings are included in Fields 650 to represent the subjects of *Nature*, including "Science," "Biology," and "Physics" as the main headings, each followed by a form subheading of "Periodicals" encoded in Subfield "v" to indicate the main topics are treated in a journal. In addition, a heading for "Science" is also represented in Field 650. These subject representations enable users to conduct subject searches to locate this journal.

38. This MARC record (**Appendix 1005B**) shows that *Nature* is a long-running journal, and the MARC record has made it searchable in the online catalog of the University of Wisconsin-Madison General Library System by at least July 18, 2000, if not earlier. Users interested in the subject of this journal would have been able to conduct subject searches in the library online catalog by the Library of Congress subject heading in the MARC record to discover this journal. They would also have been able to discover and access *Nature* by the journal title, ISSN, CODEN and Nature Publishing Group.

39. It is therefore my opinion that *Nature* would have been discoverable in the online catalog of the University of Wisconsin-Madison General Library System by at least July 18, 2000, and the physical issue of vol. 406, no. 6791 (and the *May Article* contained therein) would have been publicly accessible no later than July 25, 2000.

C. PubMed Record for the *May Article*

40. Because the topic of the *May Article* is related to life science research I also searched PubMed by the title of the *May Article* to determine if this article was indexed. PubMed is an authoritative system that contains "more than 34 million citations for biomedical literature from MEDLINE, life science journals, and online books" and PubMed records may include links to "full text content from PubMed Central and publisher web sites" (source: https://pubmed.ncbi.nlm.nih.gov). **Appendix 1005C** is a true and correct metadata record for the *May Article* (https://pubmed.ncbi.nlm.nih.gov/10894546/) that I personally located, identified and obtained from PubMed. This is the type of record experts in my field would reasonably rely upon when forming their opinions. I saved the metadata record in PDF and added the MEDLINE format of the record to show the record creation date. These data are presented as **Appendix 1005C** in this declaration.

41. Page 1 of **Appendix 1005C** is the metadata record for the *May Article* that shows a full text link to "nature publishing group" that leads to the publisher's

website for the *May Article* at https://www.nature.com/articles/35017565. The PubMed metadata record shows the title, authors, journal and issue in which the *May Article* appears, and abstract of this article. It also shows the PubMed identifier for this record is "10894546," the DOI of the *May Article* is "10.1038/35017565."

42. Pages 2 to 3 of **Appendix 1005C** are the PubMed record saved in MEDLINE format to show data in specific fields. I use the "MEDLINE/PubMed Data Element (Field) Descriptions" (https://www.nlm.nih.gov/bsd/mms/medlineelements.html) to interpret the record. The "PMID" field shows "10894546" as the PubMed unique identifier for the record for the *May Article*. The "STAT" field shows the status of the record is "MEDLINE" (meaning Medical subject headings were assigned to the article), and the DCOM field shows that the record was completed on "20000731" (*i.e.*, July 31, 2000). The first IS field shows the print journal's ISSN is "0028-0836," Fields VI (volume), IP (issue), DP (date of publication), Ti (title) and PG (pages) show the *May Article* was published in vol. 406, no. 6791 (July 6, 2000) of *Nature*, pp. 82-6. The first FAU (full author) field shows "May, C" as the first author. The JT (journal title) field on page 2 shows the *May Article* appears in *Nature*. The MH (MeSH terms) fields on page 3 show 17 medical subject headings were assigned to indicate the subjects of the *May Article*. The "AID" (article identifier) field shows the doi of the *May Article* is "10.1038/35017565."

43. This MEDLINE record **(Appendix 1005C)** made the *May Article* searchable in PubMed as of July 31, 2000, when the MEDLINE record was completed. Users would also have been able to search for the *May Article* in PubMed by the authors, title, and medical subject headings assigned to this article. Because MEDLINE records are loaded into PubMed daily, it is my opinion that the *May Article* would have become searchable in PubMed no later than August 1, 2000.

D. Usage Record

44. Actual usage of a publication is reflected by the publications that make reference to it. The citation history on Google Scholar shows the *May Article* has been cited 685 times, including ten citations published in 2000. To demonstrate usage, **Appendix 1005D** presents screenshots from Google Scholar to show the initial screen of the 685 citations, the ten citations from 2000, and information on an August 2000 citation by Bodine that I have verified. Page 5 of **Appendix 1005D** shows a screenshot of the publisher's webpage (https://www.cell.com/molecular-therapy-family/molecular-therapy/fulltext/S1525-0016(00)90113-8) for an August 1, 2000 article by D. Bodine, entitled "Globin gene therapy: one (seemingly) small vector change, one giant leap in optimism" in *Molecular Therapy*, vol. 2, no. 2, pp. 101-102, and pages 6 and 7 show the Bodine article itself (https://www.cell.com/action/showPdf?pii=S1525-0016%2800%2990113-8).

Appendix 1005D shows that after the *May Article* was published on July 6, 2000, it

was used by many researchers, and one of the earliest citations appeared online on August 1, 2000.

E. Summary of My Opinion on the *May Article*

45. Taken together, the "JUL 18 2000" date stamp on the University of Wisconsin-Madison copy of the *May Article* (**Ex 1005)**, the bibliographic and MARC records of the General Library System (**Appendix 1005B**), the PubMed record for the *May Article* (**Appendix 1005C**), my understanding of the ordinary and customary cataloging and processing practices of libraries and my knowledge of PubMed inform my opinion that the *May Article* was first published in vol. 406, no. 6791 (July 6, 2000) of *Nature*, the University of Wisconsin-Madison General Library System received their copy of this issue on July 18, 2000, and MEDLINE completed the index record on July 31, 2000. It is therefore my opinion that the *May Article* would have been discoverable by at least July 18, 2000, the physical copy would have become publicly accessible at the University of Wisconsin-Madison General Library System no later than July 25, 2000, and the *May Article* would have been searchable on PubMed no later than August 1, 2000. Citation history of the *May Article* shows one of the earliest citations appeared online on August 1, 2000, further supporting my opinion that the *May Article* would have been publicly accessible by July 18, 2000, and would have been searchable on PubMed no later than August 1, 2000.

IV. Authenticity and Public Availability of the *May Abstract* (Ex 1006)

A. Authentication

46. **Ex 1006** is a true and correct copy of "683. Lentiviral-mediated Transfer of the Human β-Globin Gene and Large Locus Control Region Elements Permit Sustained Production of Therapeutic Levels of β-Globin in Long- term Bone Marrow Chimeras," ("the *May Abstract*"), by May et al., contained in "Stem Cell and Blood—Preclinical and Clinical Applications" that was published in *Molecular Therapy*, Vol. 1, Issue 5. I obtained this copy from counsel. Page 1 shows the title of this document is "Stem Cell and Blood—Preclinical and Clinical Applications" that begins on page S246 and ends on page S250. The bottom of each page shows this work appears in "Molecular Therapy Vol. 1, No. 5, May 2000," which is "Part 2 of 2 Parts" and it is copyrighted with The American Society of Gene Therapy as the copyright holder. Page S248 shows the *May Abstract* is presented as abstract "683" that has "Lentiviral-mediated Transfer of the Human b-Globin Gene and Large Locus Control Region Elements Permit Sustained Production of Therapeutic Levels of B-Globin in Long-term Bone Marrow Chimeras" as the title of a work by Chad May, Stefano Rivella, John Callegari, Karen Gaensler, and Michel Sadelain.

47. To authenticate **Ex 1006,** I searched Google for "Stem Cell and Blood—Preclinical and Clinical Applications" that contains the *May Abstract*, and located the publisher's copy at https://www.cell.com/action/showPdf?pii=S1525-

0016%2800%2990176-X. This copy is presented as **Appendix 1006A** in this declaration. I have closely compared **Ex 1006** with **Appendix 1006A** and concluded that they are the same.

48. The copy of the *May Abstract* (**Ex 1006**) is in a condition that creates no suspicion about its authenticity. Specifically, the body of the text are not missing any intermediate pages, the text on each page appears to flow seamlessly from one page to the next, and there are no visible alterations to the document. Moreover, **Ex 1006** was verified against the publisher's copy and found to be the same as that copy. I therefore see no reason to question the authenticity of this copy of the *May Abstract* (**Ex 1006**).

B. Publisher Webpage

49. **Appendix 1006B** is a true and correct copy of the publisher's webpage for "Stem Cell and Blood—Preclinical and Clinical Applications" that contains the *May Abstract*, available at https://www.cell.com/molecular-therapy-family/molecular-therapy/fulltext/S1525-0016(00)90176-X?_returnURL=https%3A%2F%2Flinkinghub.elsevier.com%2Fretrieve%2Fpii%2FS152500160090176X%3Fshowall%3Dtrue. I personally located, identified and obtained this webpage and saved it as PDF. This is the type of record experts in my field would reasonably rely upon when forming their opinions.

50. Page 1 is a screenshot of the top of the publisher's webpage for "Stem Cell and Blood—Preclinical and Clinical Applications" that presents abstracts in "Volume 1, Issue 5, PS246-S250, May 01, 2000" of *Molecular Therapy*. Page 1 also shows this work is in an "Open Archive" (meaning freely available to the public), its DOI (digital object identifier) is https://doi.org/10.1006/mthe.2000.0176 and users can use the PDF link to obtain a copy of this work. Page 1 shows this work begins with abstract 678. Pages 2 to 19 are the Web version of "Stem Cell and Blood—Preclinical and Clinical Applications" and pages 8 and 9 show the *May Abstract* is presented as abstract 683. I have compared this Web version of the *May Abstract* with the counsel's copy (**Ex 1006**) and the publisher's copy (**Appendix 1006A**) and found them to have the same content.

51. *Molecular Therapy* is "the leading journal for research in the areas of gene transfer, vector development and design, stem cell manipulation" and the journal "is dedicated to promoting the sciences in genetics, medicine, and biotechnology" (Aims and Scope, https://www.sciencedirect.com/journal/molecular-therapy/about/aims-and-scope). This journal is available as a print journal and also as an electronic journal. A common publishing practice of electronic journals is to mark the date of document availability clearly on the document webpage to inform the public of the public availability date of a document. In this case, the webpage shows "Stem Cell and

Blood—Preclinical and Clinical Applications" that contains the *May Abstract* was published on "May 01, 2000." Based on that information, it is my opinion that this work (and the *May Abstract* contained therein) was first publicly available on May 1, 2000. As of that date, interested users would have been able to discover this webpage on the Internet to use the Web version of this work and the *May Abstract*. They would also have been able to follow the PDF link from this webpage to obtain a PDF copy of this work and all the abstracts contained therein, including the *May Abstract*.

C. Summary of My Opinion on the *May Abstract*

52. Based on the publisher's copy (**Appendix 1006A**) and the publisher's webpage (**Appendix 1006B**), it is my opinion that the copy from counsel that contains the *May Abstract* (**Ex 1006**) is authentic and that "Stem Cell and Blood—Preclinical and Clinical Applications" that contains the *May Abstract* was published online on May 1, 2000, in Volume 1, Issue 5 of *Molecular Therapy* and became publicly accessible from that date on.

V. Conclusion

53. I hereby declare that all statements made herein on my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are made under penalty of perjury.

Date: 10-17-2022 Executed: Ingrid Hsieh-Yee

Ingrid Hsieh-Yee, Ph.D.

Appendix A

Ingrid Hsieh-Yee

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Education

Ph.D. 1990. Library and Information Studies, University of Wisconsin-Madison
Minors: Sociology and Psychology

M.A. 1984. Library and Information Studies, University of Wisconsin-Madison.

M.A. 1983. Comparative Literature, University of Wisconsin-Madison.

B.A. 1980. Foreign Languages and Literature, National Taiwan University.

Work Experience

Professor, School/Dept. of Library and Information Science, Catholic University of America, 2004- (Assistant Professor, 1990-1996; Associate Professor, 1997-2004)

Co-Chair, Dept. of Library and Information Science, Catholic University of America, June 2015-August 2016.

Acting Dean, School of Library and Information Science, Catholic University of America, January 2010-June 2012.

Cataloger, Dept. of Legislative Reference Library, Annapolis, Maryland, 1989-1990.

Lecturer, School of Library and Information Studies, University of Wisconsin-Madison, 1988.

Teaching Assistant, School of Library and Information Studies, University of Wisconsin-Madison, 1986-1988.

Cataloger, Health Sciences Library, University of Wisconsin-Madison, 1984-1986.

Areas of Teaching and Research Interests

Information Organization and Access; Metadata; Cataloging & Classification; Information

Architecture; Information Retrieval; Digital Collections; Social Media; Scholarly Communication; Information Behavior; Health Informatics; Human Computer Interaction; Usability Studies

Grants & Honors

Cultural Heritage Information Management Project. IMLS grant. Amount: $498,741. Period: Aug. 2012 to July 2015. Co-PI with Dr. Youngok Choi.

D.C. Health Information Technology (HIT4): Building Capacity & Providing Access in Our Nation's Capital. Dept. of Labor H2B Training Grant. Grant amount: $4,175,500. Grant period: Nov. 2011 to Dec. 2015. Partner with the Metropolitan School of Professional Studies of the Catholic University of America, Children's National Medical Center, D.C. Department of Employment Services, Holy Cross Hospital, Howard University, Center for Urban Progress, Providence Hospital, and Sibley Memorial Hospital.

Capital Health Careers Project. Department of Labor Healthcare Sector and Other High Growth and Emerging Industries Grant. Grant amount: $4,953,999. Grant period: March 2010 – February 2013. Awarded to a group of healthcare organizations and educational institutions in Washington, D.C. Providence Health Foundation of Providence Hospital (Lead institution). Part of the grant supported the development of a Master's degree program in Information Technology with a concentration in Health Information Technology offered by the School of Library and Information Science.

The Washington D.C. School Librarians Project. IMLS grant. Grant amount: $412,660. Grant period: Aug. 2007 – June 2011. The School partnered with the District of Columbia Public Schools (DCPS) and the District of Columbia Library Association to educate and mentor school media specialists for the DCPS system. PI, Jan. 2010 to June 2011.

SIG Member of the Year, American Society for Information Science and Technology (2009).

Most Outstanding Paper of *OCLC Systems & Services* (2001).

ALISE Research Grant (2001).

Most Outstanding Paper of *OCLC Systems & Services* (2000).

Research Grant from ERIC (1999-2000).

Best Research Paper Award; Association for Library and Information Science Education (1998).

Research Grants, Catholic University of America. 1991, 1992, 1993, 1996, 1998, 1999, 2004, 2005, 2006, 2007, 2013-14.

Cooperative Faculty Research Grant, Consortium of Universities in the Washington

Metropolitan Area (1993-1994).

Cooperative Research Grant, Council on Library Resources (1993-1994).

Journal of the American Society for Information Science Best Paper Award (1993).

ASIS/ISI Information Science Doctoral Dissertation Scholarship (1989).

HEA Title IIB Fellowship (Dept. of Education) (1989)

Chinese-American Librarians Association Scholarship (1987).

Beta Phi Mu (1985).

Vilas Fellowship, University of Wisconsin-Madison. 1984

Publications

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Hsieh-Yee, I. (2006). *Organizing Audiovisual and Electronic Resources for Access: A Cataloging Guide*. 2nd ed. Westport, Conn.: Libraries Unlimited.

NISO A Framework of Guidance for Building Good Digital Collections. 2nd ed. Bethesda, MD: National Information Standards Organization, 2004. Framework Advisory Group: Grace Agnew, Liz Bishoff, Priscilla Caplan (Chair), Rebecca Gunther and Ingrid Hsieh-Yee.

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Works in Progress

Hsieh-Yee, I. Actionable lessons for debunking false information: A comparative analysis of literacy lessons in communication and library and information science

Hsieh-Yee, I. Representing diverse voices and communities in cataloging and indexing: The evolution of controlled vocabularies

Hsieh-Yee, I. Information Organization and Retrieval in Practice (a monograph scheduled for completion and publication in 2023)

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Bailey, T. & Hsieh-Yee, I. (February 2020). The Phenomena of Sharing Misinformation and the Need for Information Literacy. Presented at the 2020 Bridging the Spectrum Symposium, Washington, D.C., February 14, 2020.

Hsieh-Yee, I. and Fragan-Fly, J. (May 2018) Trends, Design & Strategies for Digital Scholarship Services. Presented at the 2018 Maryland/Delaware Library Association Conference, Cambridge, MD.

Hsieh-Yee, I. (February, 2018) Research Data Management: What It Takes to Succeed. Presented at the 10th Bridging the Spectrum Symposium, Washington, D.C.

Hsieh-Yee, I. (February, 2017) *Research Data Management: New Competencies and Opportunities for Information Professionals*. Presented at the 9th Bridging the Spectrum Symposium, Washington, D.C.

Hsieh-Yee, I. and Lawton, P. (February, 2017) *Enhancing Catholic Portal Searches with User Terms and LCSH*. Presented at the 9th Bridging the Spectrum Symposium, Washington, D.C.

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Fagan-Fry, J. and Hsieh-Yee, I. (2016, February) *Approaches to Digital Scholarship at Top Universities around the World: Scholarly Publishing in the Digital Age.* Presented at the 8th Bridging the Spectrum Symposium, Washington, D.C.

Hsieh-Yee and Fagan-Fry, J. (2016, January) *Innovative Services for Digital Scholarship at Top 100 Research Libraries of the World*. Poster presented at the 2016 Annual Conference of the Association for Library and Information Science Education, Boston, Mass.

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Hsieh-Yee, I., with the CUA Scholarly Communications Project Team. (2009, January). *Digital Scholarship@CUA: Developing an Institutional Repository for CUA*. Poster presented at 1st Annual Bridging the Spectrum Symposium, Catholic University of America, Washington, D.C.

Wise, M., Cylke, K., and Hsieh-Yee, I. **(**2009, January). *Digital Talking Books: Meeting the Needs of the Blind and the Handicapped*. Paper presented at the Bridging the Spectrum Symposium, Catholic University of America, Washington, D.C.

Hsieh-Yee, I. (2009, January). *User Expectations of MERIC*. Presented at the Information Organization Competencies for the 21st Century Discussion Session of the 2009 Conference of the Association for Library and Information Science Education, Denver, Colorado.

Choi, Y., and Hsieh-Yee, I. (2008,November). *Subject Access for Images in an OPAC.* Annual Meeting of the American Society for Information Science and Technology, Columbus, Ohio. (Also co-organized a panel on Retrieving and Using Visual Resources: Challenges and Opportunities for Research and Education.)

Hsieh-Yee, I. (2008, June). *Educating Cataloging Professionals in a Changing Information Environment*. National Taiwan University, Taipei, Taiwan.

Vellucci, S. L., Moen, W.E., Hsieh-Yee, I., Marson, B., and Wisser, K. (2008, January) *Building a Metadata Education and Research Community through MERIC (Metadata Education and Research Information Commons): Demo and Stakeholder Input*. A panel presented at the 2008 Conference of the Association for Library and Information Science Education, Philadelphia, Pennsylvania.

Hsieh-Yee, I., Choi,Y. and Kules, B. (2007, October). *Searching for Books and Images in OPAC: Effects of LCSH, TOC and Subject Domains.* A poster presented at the American Society for Information Science and Technology Annual Meeting, Milwaukee, Wisconsin.

Hsieh-Yee, I. and Coogan, J. (2007, August) *Google Scholar vs. Academic Search Premier: A Comparative Analysis.* Presented to the Faculty and Staff of the University of the District

of Columbia.

Hsieh-Yee, I. and Coogan, J. (2007, June). *Google Scholar vs. Academic Search Premier: A Comparative Analysis*. Presented to the Washington Research Library Consortium Community, Catholic University of America, Washington, D.C.

Hsieh-Yee, I., Choi, Y., and Kules, B.. (2007, June). *What Users Need for Subject Access: Table of Contents or Subject Headings?* A poster presented at the 2007 American Library Association Annual Conference, Washington, D.C., June 2007.

Choi, Y., Hsieh-Yee, I., and Kules, B. (2007, June). *Retrieval Effectiveness of TOC and LCSH.* A paper presented at the Joint Conference on Digital Libraries 2007, Vancouver, Canada.

Vellucci, S. L., Hsieh-Yee, I., and Moen, W.E. (2007, May). *If We Build It, Will They Come? Building a Community of Practice for Metadata Stakeholders.* A poster presented at the Rutgers University Research Day, Bridgeton, New Jersey.

Hsieh-Yee, I. (2007, May). *Federated Searching: User Experience & Perceptions.* International Conference on Information Organization & Retrieval, National Taiwan University, Taipei, Taiwan.

Hsieh-Yee, I. (2007, May). *Search Performance of Google Scholar and Academic Search Premier.* International Conference on Information Organization & Retrieval, National Taiwan University, Taipei, Taiwan.

Hsieh-Yee, I. (2007, May) *MERIC: Building a Digital Commons for Metadata Education & Research.* International Conference on Information Organization & Retrieval, National Taiwan University, Taipei, Taiwan.

Hsieh-Yee, I., and Coogan, J. (2007, March/April). *A Comparative Analysis of Google Scholar and Academic Search Premier.* Poster presented at the Association of College & Research Libraries 13th National Conference, Baltimore, Maryland.

Vellucci, S. L. and Hsieh-Yee, I. (2007, March/April) *They Didn't Teach Me That in Library School! Building a Digital Teaching Commons to Enhance Metadata Teaching, Learning and Research*. On-site presentation and Webcast by Elluminate. A contributed paper presented at the Association of College & Research Libraries 13th National Conference, Baltimore, Maryland. The acceptance rate for contributed paper was 20%. This paper was one of 10 conference papers chosen for live webcast during the conference.

Moen, W., Hsieh-Yee, I. and Vellucci, S.L. (2007, January) *A DSpace Foundation for a Teaching & Research Commons: The Metadata Education and Research Information Commons.* A poster session presented at the Open Repositories Conference 2007, San Antonio, Texas.

Tang, R., Hsieh-Yee, I., and Zhang, S. (2006, November) *User Perception of MetaLib Combined Search*. Paper presented at the Annual Meeting of the American Society for Information Science and Technology, Austin, Texas, Nov. 2006.

Hsieh-Yee, I. (2006, November). *Federated Searching: User Perceptions, System Design, and Library Instructions.* Paper presented at the Annual Meeting of the American Society for Information Science and Technology, Austin, Texas. (Panel organizer, moderator, presenter).

Hsieh-Yee, I. (2006, November). *Building a Digital Teaching Commons to Enhance Teaching and Learning: The MERIC Experience and Challenges.* Paper presented at the Annual Meeting of the American Society for Information Science and Technology, Austin, Texas. (Panel organizer, moderator, presenter)

Hsieh-Yee, I. (2006, September). *Search Performance of Google Scholar and Academic Search Premier.* Paper presented at the ERIC Publishers Meeting, Washington, D.C.

Hsieh-Yee, I., Zhang, S., and Rong Tang, R. (2006, June). *User Perceptions of a Federated Search System*. Poster presented at Joint Conference on Digital Libraries, Chapel Hill, North Carolina.

Hsieh-Yee, I. and Zhang, S. (2006, June). *Preparing Users for Federated Search: Implications of a MetaLib User Perceptions Study.* Paper presented at the 2006 Ex Libris User Groups of North America Conference, Knoxville, Tennessee.

Hsieh-Yee, I. (2006, January). *MERIC Organizations and Navigation*. Paper presented at the 2006 ALISE Annual Conference, San Antonio, Texas.

Hsieh-Yee, I. (2006, January). *Metadata and Cataloging Education: Recommended Competencies.* Paper presented at the 2006 ALISE Annual Conference, San Antonio, Texas.

Hsieh-Yee, I. (2005, November). *Digital Library Evaluation: Progress & Next Steps.* Presentation at the Annual Meeting of the American Society for Information Science & Technology, Charlotte, North Carolina.

Hsieh-Yee, I. (2005, August). *Providing Access to Digital Content: Issues for DL Managers.* Presentation at MDK12 Digital Library Steering Committee Meeting, Columbia, Maryland.

Hsieh-Yee, I. (2005, April). *Enhancing Teaching and Learning: The Role of School Library Media Specialists.* Presentation at Meeting of the Baltimore County Public School System School Media Specialists, Baltimore, Maryland.

Hsieh-Yee, I. (2005, January). *Subject Access and Users: Insights & Inspirations from Marcia J. Bates.* Paper presented at the Historical Perspectives SIG, 2005 Conference of the Association for Library and Information Science Education, Boston, Massachusetts.

Hsieh-Yee, I. (2005, January). *Electronic Resource Management: Practice, Employer Expectations, & CE Interests.* Paper presented at Technical Services Education SIG, 2005 Conference of the Association for Library and Information Science Education, Boston, Massachusetts.

Hsieh-Yee, I. (2004, October). *Library Professionals for the Digital Age: Competencies & Preparation.* Paper presented at Bibliographic Access Management Team meeting, Library of Congress, Washington, D.C.

Hsieh-Yee, I. (2004, January). *Cataloging and metadata expertise for the digital era.* Presented at Preparing 21st Century Cataloging and Metadata Professionals: A Workshop for Educators and Trainers, San Diego and sponsored by ALCTS, ALISE, LC, and OCLC.

Hsieh-Yee, I. (2004, January). *Educating catalogers for the digital era.* Paper presented at the Technical Services SIG, 2004 Conference of the Association for Library and Information Science Education, San Diego.

Hsieh-Yee, I. (2003, July). *Cataloging Education for the 21st Century.* A presentation at the Library of Congress, Washington, D.C.

Hsieh-Yee, I. (2002, January) *Metadata Education and Research Priorities: A Delphi Study of Metadata Experts.* Presentation at the 2002 Conference of the Association for Library and Information Science Education, New Orleans.

Hsieh-Yee, I. (2001, November). *A Delphi Study of Metadata: Preliminary Findings.* Poster session at the 2001 Annual Meeting of the American Society for Information Science & Technology, Washington, D.C.

Hsieh-Yee, I. (2001, June). *Resources on Asian American Children: Analysis of Retrieval by Search Engines and WorldCat.* Presentation at the National Conference on Asian Pacific American Librarians, San Francisco.

Hsieh-Yee, I. (2001, January). *Delphi Study on Metadata: Project Design.* Presentation at Research Awards Session, Association for Library & Information Science Education, Washington, D.C.

Hsieh-Yee, I. (2000, May). *Web Search Behavior Research: Progress and Implications.* Presentation at the Symposium on Evaluating Library and Information Science Research, University of Wisconsin-Madison, Madison, Wisconsin.

Hsieh-Yee, I. (2000, March). *ERIC User Services: Evaluation in a Decentralized Environment.* Presentation at the National ERIC Joint Directors/Technical Meeting, Arlington, Virginia.

Hsieh-Yee, I. (2000, January). *Enhancing Learning with Web Technology.* Presentation at Faculty Conversations, Catholic University of America, Washington, D.C.

Hsieh-Yee, I. (2000, January). *From Surrogates to Objects: CUA's Approaches to Organizing Electronic Resources.* Paper presentation at the Annual Conference of the Association for Library and Information Science Education, San Antonio, Texas.

Yee, P., and Hsieh-Yee, I. (1997, November). *Individual Differences in Search Behavior on the WWW.* A poster session presented at the 38th Annual Meeting of the Psychonomic Society, Philadelphia, Pennsylvania.

Hsieh-Yee, I. (1997, April). *Research + Marketing + Preparation = Job!* Presented at the "Workshop on Resume and Interview Techniques," Special Libraries Association, Student Chapter, Catholic University of America, Washington, D.C.

Hsieh-Yee, I. (1997, February). *Creating CyberCatalogers: Education and Training.* Presentation at ALA's Midwinter Meeting, Washington, D.C.

Hsieh-Yee, I. (1997, February). *Search Tactics of Web Users in Searching for Texts, Graphics, Known Items and Subjects: A Search Simulation Study.* Presented at the Conference of the Association for Library and Information Science Education, Washington, D.C.

Hsieh-Yee, Ingrid. "Beginning Your Special Library/Information Center Career." Presented at SLA's "Career Day," Jan. 11, 1997, Catholic University of America.

Hsieh-Yee, I. (1996, September). *The Roles of Library and Information Scientists in Managing Electronic Information.* Presentation at Hamilton College, Clinton, New York.

Hsieh-Yee, I. (1996, May). *The Future of Cataloging as a Profession.* Presented at "The Cataloging Forum, Library of Congress, Washington, D.C.

Hsieh-Yee, I. (1994, October). *The Impact of the Internet on OPACs.* Presented at the Third Workshop on User Interfaces for OPACs, Library of Congress, Washington, D.C.

Reports

Hsieh-Yee, I., with Knowledge Management Competencies and Performance Action Group of the Federal Knowledge Management Initiative. "From Knowledge Management Competencies to Improved Organizational Performance." April 9, 2009.

Hsieh-Yee, I., with Knowledge Practices Action Group of the Federal Knowledge Management Initiative. "KM Practice in Government Agencies: Findings and Recommendations." April 9, 2009.

Hsieh-Yee, I. "Delphi Study on Metadata." 2001. Three quarterly reports submitted to the Association for Library and Information Science Education.

Hsieh-Yee, I. "College Students' Information Channels: Patterns of Use and Possible Factors in Channel Selection." 1995. Submitted to the Catholic University of America.

Hsieh-Yee, I. "The Information-Seeking Patterns of Scholars and Their Use of an Online Information System." 1994. Submitted to the Council on Library Resources.

Book Reviews

Review of *The Measurement and Evaluation of Library Services*, by Sharon L. Baker and F. Wilfrid Lancaster. *Information Processing and Management* 30 (1994): 450-52.

Review of *Subject Access to Films and Videos, by Sheila S. Intner and William E. Studwell; and Cataloging Unpublished Nonprint Materials*, by Verna Urbanski with Bao Chu Chang and Bernard L. Karon. *Information Processing and Management* 30 (1994): 449-50.

Review of *Automated Information Retrieval in Libraries: A Management Handbook*, by Vicki Anders. *Journal of Library and Information Science* 19 (1993): 98-100.

Review of *Full Text Databases*, by Carol Tenopir and Jung Soon Ro. *Information Processing and Management* 28 (1992): 667-68.

Review of *Descriptive Cataloging for the AACR2R And USMARC: A How-to-Do It Workbook*, by Larry Millsap and Terry Ellen Ferl. *Information Processing and Management* 28 (1992): 809-11.

Review of *MARC Manual: Understanding and Using MARC Records*, by Deborah J. Byrne. *Information Processing and Management* 28 (1992): 537-38.

Service

Professional Associations and Societies

- Library of Congress. RDA Training Program for the Profession. Co-authored with Tim Carlton. 2013-2014.
- 2014 Digital Preservation Outreach & Education Survey. Contributed to the design of the survey, 2014.
- National Digital Stewardship Alliance. Outreach Committee. 2011-2014.
- National Digital Stewardship Residency Program. Advisory Group, 2012-2013.
- FEDLINK Health Information Technology Advisory Council, 2011-2015.

- 2012 Joint Conference on Digital Libraries. Program Planning Committee, Pre-Conference Proposals Review Committee, 2012
- Catholic Research Resources Alliance. Five-Year Strategic Plan Task Force, 2011-2012
- Institute of Museum and Library Services. Grant reviewer. 2004, 2005, 2010.

- Association for Library and Information Science Education.
  * ALISE Bodan Wynar Research Paper Award Committee, 2015, 2016, 2017
  * ALISE Eugene Garfield Dissertation Award Competition, Jury, 2013, 2014
  * ALISE Research Grant Competition Committee. Chair, 2012
  * Pratt-Severn Faculty Innovation Award. Chair, 2009, 2010
  * ALISE Doctoral Poster Jury, 2012
  * "Information Organization Competencies for the 21st Century" Discussion session leader. 2009 Conference of the Association for Library and Information Science Education.
  * Assisted Technical Services SIG Convener in organizing a program, ""Building a Metadata Education and Research Community through MERIC (Metadata Education and Research Information Commons): Demo and Stakeholder Input" for the 2008 ALISE conference.
  * Association for Library Collections and Technical Services/Association for Library and Information Science Education (ALCTS/ALISE) Metadata Education and Research Information Center (MERIC) Advisory Board, Co-Chair (with Sherry Vellucci), 2005-2007. Chair, 2008-2009 (leading the effort to build MERIC, a repository and collaborative space for metadata educators, practitioners, and researchers)
  * Technical Services SIG, Convener, 2004-2005. Organized a program on "Electronic Resources Management: Current Practices, Employer Expectations, and Teaching Strategies" for the 2005 conference in Boston, Massachusetts.
  * Technical Services SIG, Convener, 2003-2004. Organized a program on "Organizing Information with Metadata: Desired Competencies and Teaching Innovations" for the 2004 conference.
  * Technical Services SIG, Convener, 1999-2000. Organized a program on "Teaching the Organization of Electronic Resources" for the 2000 conference.
  * Curriculum SIG, Co-convener (with Sibyl Moses), 1996-97. Organized a program on "Government Information Policy" for the 1997 conference.

- American Society for Information Science & Technology.
  * Reviewer, Conference program panel submissions and poster submissions, 2005, 2006, 2007, 2009, 2011, 2012, 2013, 2014, 2015, 2016, 2017
  * Nomination Committee, 2009-2011
  * Information Science Education Special Interest Group. American Society for Information Science and Technology. Chair-Elect, 2007-2008. Chair 2008-2009.
  * Committee on Information Science Education. 1999-2006.
  * Committee on Information Science Education. Organizing Committee for an orientation program for students at ASIS annual meetings, 1999-2001
  * Committee on Information Science Education. Sub-committee on Student Welfare (focusing on issues related to master's education), 1998-2001

* SIG ED. Organizing Committee for the "Seminar on Research and Career Development" for junior researchers. 1995-96 (chair), 1997-2001
* ISI Doctoral Dissertation Proposal Scholarship Jury, 1997; 2001, 2002
* Pratt-Severn Best Student Research Paper Award Jury. Chair. 1997
* 1998 Midyear Meeting (referee of contributed papers), 1997
* Organizer and moderator of the ASIS Doctoral Forum and the Doctoral Research Seminar 1994-1995
* SIG Human Computer Interaction. Chair-Elect, Chair, 1993-1995
* Doctoral Forum Award Jury, 1995
* Best Student Paper Award Jury, 1995

- American Library Association.
  * Committee on Accreditation, External Review Panelist, 2009- (site visiting team 2013-2014; site visiting team 2016-2017)
  * Association for Library Collections and Technical Services Task Force on Competencies and Education for a Career in Cataloging, member, 2008-2009
  * Facilitator for "What They Don't Teach in Library School: Competencies, Education and Employer Expectations for a Career in Cataloging," an Association for Library Collections and Technical Services Preconference, June 22, 2007 in Washington, D.C. Also a local liaison for bringing this program to the Catholic University of America.
  * Facilitator for a discussion on "Effect of Electronic Resources on Technical Services" at ALA's Midwinter Meeting held in Feb. 1997 in Washington, D.C.
  * International Relations Committee, Subcommittee Task Force for IFLA and China, 1994-1997

- Virginia Association of School Librarians. Scholarships and Awards Committee. 2010-2012

- Federal Knowledge Management Initiative, Knowledge Management Practices Action Group. Member. 2009 (leading the effort to build a knowledge management repository)
- Federal Knowledge Management Initiative, Knowledge Management Competencies & Learning Action Group. Member. 2009 (developing an action plan for helping government knowledge workers and government agencies to develop knowledge management competencies)
- National Center for Education Statistics. Technical Review Panel. 2008.
- External evaluator for a case of promotion to full professorship. University of Tennessee. 2008.
- National Information Standards Organization (NISO). Advisory Board, Revision of "IMLS Framework of Guidance for Building Good Digital Collections," 2004, 2007.
- Library of Congress, Bibliographic Control of Web Resources: A Library of Congress Action Plan. Principal Investigator of Action Item 5.1, focusing on cataloging and metadata education for students and new librarians, 2002-2003. (worked with the Association for Library Collections and Technical Services, Education Task Force)

- Chinese American Librarians Association
  * Chinese American Librarians Association Outstanding Library Leadership Award in Memory of Dr. Margaret Chang Fung, Award Committee, 2016-2017
  * Achievement Award Jury, 2000-2001
  * CALA Goal 2000 Task Force, 1997
  * Scholarship Committee, 1995, 1996-1997 (chair)
  * Board of Directors, 1994-1997
  * Publication Committee, 1993-1995
  * International Relations Committee, 1993-1996

- Sailor[SM] Assessment Advisory Group (An impact study of Sailor, Maryland's Public Information Network), 1995

- Editorial boards
  Journal of Library & Information Science. Editorial Board, 2012-
  Chinese American Librarians Association, *Occasional Papers Series*. Editorial Board, 2009-2016.
  Library Quarterly. Editorial Board, 2003-2008
  Bulletin of the Medical Library Association, 1994-97
  Newsletter editor for the Chinese American Librarians Association, 1989-92

- Referee for the following journals
  Information Processing and Management
  Journal of Digital Information
  Journal of Education for Library and Information Science
  Journal of Information Science
  Journal of Library & Information Science
  Journal of Library Metadata
  Journal of the American Society for Information Science & Technology
  Library and Information Science Research
  Library Quarterly

- Expert reviewer, "Digital Library" course, Evaluation module, University of North Carolina, Chapel Hill, 2007-2008.
- Expert reviewer, "Information Organization" course, University of Michigan, Ann Arbor. 2007.

Catholic University of America

- Academic Senate Committee on Appointments and Promotions, 2005-2008
- Academic Senate Committee on Committees and Rules, 2009-2012, 2019-2022
- Academic Senate representative (for School of Arts & Sciences), 2017-2020
- Academic Senate representative (for School of Library & Information Science) 2003-2012
- Academic Senate Library Committee, Interim Chair (2007), Member, 2008-2012

- Academic Senate Committee on Computing, 1995-2003

- President's Administrative Council, 2010-2012
- Deans' Council, 2010-2012
- Academic Leadership Group, 2010-2012
- Graduate Board, 2010-2012

- School of Arts & Sciences, Committee on Appointments and Promotions, 2015-2019
- School of Arts & Sciences, Academic Council, 2015-2016.
- School of Arts & Sciences, Ordinary Professor Group, 2013-
- Doctoral Dissertation Defense Committee, Chair, Dept. of Psychology, 2016, 2017, 2018, 2019, 2020, 2021, 2022
- Doctoral Dissertation Defense Committee, Chair, Dept. of Education, 2014, 2015, 2017, 2018, 2019
- Doctoral Dissertation Defense Committee, Chair, National Catholic School of Social Services, 2019, 2020, 2021
- Doctoral Dissertation Defense Committee, Chair, School of Nursing, 2006, 2008
- CUA Scholarly Communication Project Team, Member (2007), Chair, 2008-2009
- Dean Search Committee, 1992-1994, 1998-1999, 2002-2003, 2006-2007
- Fulbright Review Panel, 2006
- CUA Service Learning Advisory Board, 2001-2002
- CUA Faculty Conversations on Enhancing Teaching and Learning through Technology, Planning Group, 1999-2001
- CUA Initiative on Technology and Teaching, 1998-2001

Dept. of Library and Information Science

- Admissions Committee, 2007-2009, Chair 2010-2012, Member 2013-2015, Member 2018-2022
- Appointments and Promotions Committee, 1991-2022
- Student advisement, 1990-2022
- 4+1 Bachelor to Master program, Program Advisor, 2014-2022
- LIS Minor, Advisor, 2017-2022
- Curriculum Committee, 2020-21 (Member), 2021-2022 (Chair)
- Scholarship & Awards Committee, 2016-2018, 2020-21
- Comprehensive examination editor, 2020-21
- Symposium, Colloquium, Lecture Series Committee, May 2018-May 2020 (Chair)
- Symposium and Colloquium Committee, fall 2016-May 2018
- Bridging the Spectrum Symposium session moderator, 2018, 2019, 2020, 2021
- Organization of Information Core Course Outcomes Assessment, 2019, 2020
- The Information Professions in Society Core Course Outcomes Assessment, 2019, 2020
- Community Services Librarianship, Course of Study Review and Revision, 2020

- Organization of Information, Course of Study Review and Revision, 2019
- Organization of Information, Competency and Course Offerings Review, 2018-2019
- Accreditation presentation, Chair, June 2015-August 2016
- Interim Co-Chair, June 2015-August 2016.
- Blended/OWL Learning Committee, spring 2016-2018
- Technology Committee, fall 2016-2017
- Comprehensive examination editor, 2016-2017, reader (every year since 1990)
- LIS Advisory Board, 2015-2016 (chair); fall 2016- May 2018 (member)
- Committee on Planning and Assessment, 2015-2016 (chair)
- Senior Faculty Committee, 2014-2016.
- Accreditation Steering Committee, 2014-2016 (Chair, 2015-2016)
- Accreditation Students Standard Committee, co-chair, 2014-2016
- Accreditation Mission, Goals, and Objectives Standard Committee, co-chair, 2014-2016
- Accreditation Curriculum Standard, member 2014-2-16
- Accreditation Administration and Finance Standard, member 2014-2016
- Cultural Heritage Information Management Project (IMLS-funded), Co-PI, 2012-2015
- Cultural Heritage Information Management Forum (scheduled for June 2015), Co-Organizer, 2013-2015
- Health Information Technology Interim Review Committee, 2015 (chair)
- Health Sciences Librarianship Advisory Group, 2015- (chair)
- Comprehensive examination editors, 2013-2014, 2016-2017
- National Digital Stewardship Alliance liaison, 2011-2014
- Advisory Board, Chair 2010-2012
- Academic Honesty Committee, Chair, 2008-2012
- Blended Learning Committee, 2010-2012
- Colloquium Committee, 2010-2012
- Comprehensive Examination Administration, 2010-2012
- Cultural Heritage Information Management Advisory Committee, 2010-2012 (chair), 2013-
- Curriculum Committee, 1991-2003, 2007-2009, Chair 2010-2012, member 2013-
- Curriculum Subcommittee on Comprehensive Examination, Chair 2009-2012
- Health Information Technology Advisory Board, Chair 2010-2012. Member 2013-
- Health Sciences Advisory Committee, 2009, Chair 2010-2012. Member 2013-
- HIT Expert Forum, Chair 2012. Member 2013-2015
- Health Information Technology Student Group Advisor, 2011-2012
- State Council for Higher Education of Virginia, SLIS Representative, 2010-2012
- Symposium Planning Committee, 2010-2012
- Website Management Team, Chair, 2010-2012
- Urban School Librarianship Project (IMLS-Funded), PI, 2007-2011 (chair, 2010-11)
- Failing Grades Committee, 1995-1997 (chair), 2000-2001 (chair), 2004-2005 (chair), 2007 (chair)-2011
- Faculty Search Committee, 1994-1998, 2002-2004, 2006 (chair), Fall 2007-2009, Chair fall 2009-2012

- Recruitment Committee, Chair 2010-2012
- Strategic Planning Committee, Chair 2010-2012
- Technology Committee, 2010-2012
- Accreditation Advisory Committee, 2007-2009
- Accreditation Coordinating Committee, 2007-2009
- Accreditation Steering Committee, 2007-2009
- SLIS Advisory Group, 2007-2009
- Accreditation Curriculum Standard Committee, Co-chair, 2007-2009
- Accreditation Faculty Standard Committee, Co-chair, 2007-2009
- LSC 551 Information Organization Review Team, Co-chair, 2008-2009, 2015-2016.
- Curriculum Subcommittee on Portfolios, 2009
- LSC 555 Information Systems in Libraries and Information Centers Review Team, contributor, 2008-2009
- Redesign of LSC 730 Use and Users of Libraries and Information. 2009-
- Development of a metadata institute that was taught as LSC 715 Organization of Internet Resources in 2008. The institute is being revised and will be offered in 2010 under a new course title.
- Development of lesson plans, assignments, and evaluation rubrics for LSC 606, Cataloging and Classification, for the School's NCATE accreditation. 2008
- Howard and Mathilde Rovelstad Scholarship Committee, Chair, 2004-2007
- Assistant Dean Search Committee, Chair, Fall 2007
- Liaison to the Association for Library Collections and Technical Services to bring its preconference program, Cataloging Education and Employer Expectations, to CUA during the 2007 American Library Association Annual Meeting in Washington, D.C.
- Organizer of the colloquium presentation and reception for Tamar Sadeh of Ex Libris on PRIMO June 2007
- Practicum review and design (work with potential supervisors, such as the American Indian Museum internship description revision) 2006-
- Comprehensive examinations (edits, proctoring, and grading), 1990-
- SLIS Web site redesign: Comments and suggestions. Fall 2007
- Conducted surveys of current students and alumni in preparation for the 2005 re-accreditation, 2004-2005
- Technology Committee, 1992-1999 (chair, 1996-1998), 2002-2003 (member)
- Colloquia Committee 1997-1999, 2002-2003.
- Advisor of the CUA Student Chapter of the American Society for Information Science and Technology, 2002-2003
- Visiting Professor Search Committee, 1999, 2000, 2001
- Leader, Participation in the CORC experiment, 1999-2000
- Advisor of the Special Libraries Association Student Chapter, 1993-1999; the group was recognized for outstanding leadership by SLA in 1999.
- COA planning Committee, Task Force on Electronic Presentation of SLIS Reports (team leader) 1997-1998
- COA Planning Committee, Subcommittee on Technology 1996-1998

- NLM practicum coordinator, 1997-1998
- Computer Literacy Workshops: Assisted with the development and evaluation of the workshops, 1996-1998
- Leader, Participation in the InterCat project, 1995-1997

Appendix B

MARC 21 Reference Materials

Part VII:

A Summary of Commonly Used MARC 21 Fields

This is a summary of the MARC 21 tags used most frequently by libraries in entering their own bibliographic records. For full listings of all MARC 21 tags, indicators, and subfield codes, see *MARC 21 Format for Bibliographic Data*.

In the explanations on these pages:

Tags -- The tags (3-digit numbers) are followed by the names of the fields they represent. In this summary, and in the *MARC 21 Format for Bibliographic Data*, if a tag can appear more than once in one bibliographic record, it is labeled repeatable (R). If it can only be used once, it is labeled non-repeatable (NR). For example, a catalog record can have several subjects, so the tags for subject added entries (6XX) are labeled repeatable (R).

Indicators -- The use of indicators is explained in fields where they are used. Indicators are one-digit numbers. Beginning with the 010 field, in every field -- following the tag -- are two character positions, one for Indicator 1 and one for Indicator 2. The indicators are not actually defined in all fields, however. And it is possible that a 2nd indicator will be used, while the 1st indicator remains undefined (or vice versa). When an indicator is undefined, the character position will be represented by the character # (for blank space).

Subfield codes -- All the data in each field (beginning with the 010 field) is divided into subfields, each of which is preceded by a delimiter-subfield code combination. The most common subfield codes used with each tag are shown. Each subfield code is preceded by the character $, signifying a delimiter. The name of the subfield follows the code.

In general, every field MUST have a subfield 'a' (**$a**). One exception that is often seen is in Field 020 (ISBN), when the ISBN information (subfield **$a**) is unavailable but the price (subfield **$c**) is known. Some subfields are repeatable. In this summary, repeatability is noted for only the more common repeatable subfields.

Examples: Examples follow the explanation for each field. For clarity, one space has

been placed between the tag and the first indicator, one space has been placed between the second indicator and the first delimiter- subfield code, and one space has been inserted between the delimiter-subfield code and the subfield data.

010 Library of Congress Control Number -- (LCCN)
(NR, or Not Repeatable

Indicators undefined.
Subfield used most often:
$a -- Library of Congress control number

```
Example:    010 ##  $a ###86000988#
```

020 International Standard Book Number -- (ISBN)
(R, or Repeatable)

Indicators undefined.
Subfields used most often:
$a -- International Standard Book Number
$c -- Terms of availability (often a price)
$z -- Cancelled/invalid ISBN (R)

```
Example:    020 ##  $a 0877547637
```

040 Cataloging source -- (NR)

Indicators undefined.
Subfields used most often:
$a -- Original cataloging agency
$c -- Transcribing agency
$d -- Modifying agency (R)

```
Example:    040 ## $a DLC
                   $c DLC
                   $d gwhs
```

100 Main entry -- Personal name -- (primary author)
(NR; there can be only one main entry)

Indicator 1: Type of personal name entry element
0 -- Forename
1 -- Surname (this is the most common form)
3 -- Family name
Indicator 2 undefined.
Indicator 2 became obsolete in 1990. Older records may display 0 or 1
Subfields used most often:
$a -- Personal name
$b -- Numeration
$c -- Titles and other words associated with a name (R)
$q -- Fuller form of name
$d -- Dates associated with a name (generally, year of birth)

```
Example:    100 1# $a Gregory, Ruth W.
                   $q (Ruth Wilhelme),
                   $d 1910-
```

130 Main entry -- Uniform title -- (NR)

Indicator 1: Nonfiling characters
0-9 -- Number of nonfiling characters present (for initial articles, including spaces)
Indicator 2 undefined.
Indicator 2 became obsolete in 1990. (See 100 above.)
Subfields used most often:
$a -- Uniform title
$p -- Name of part/section of a work (R)
$l -- Language of a work
$s -- Version
$f -- Date of a work

```
Example:    130 0# $a Bible.
                   $p O.T.
                   $p Psalms.
```

240 Uniform title (NR)

Indicator 1: Uniform title printed or displayed
0 -- Not printed or displayed
1 -- Printed or displayed (most common)
Indicator 2: Nonfiling characters

0-9 -- Number of nonfiling characters present (for initial articles, including spaces)

Subfields used most often:

$a -- Uniform title
$l -- Language of a work
$f -- Date of a work

```
Example:    240 10 $a Ile mystérieuse.
                   $l English.
                   $f 1978
```

245 Title Statement (NR)

Indicator 1: Title added entry

(Should the title be indexed as a title added entry?)

0 -- No title added entry

(indicates a title main entry; i.e. no author is given)

1 -- Title added entry

(the proper indicator when an author given in 1XX; the most common situation)

Indicator 2: Nonfiling characters

0-9 -- Number of nonfiling characters present, including spaces; usually set at zero, except when the title begins with an article; e.g., for *The robe*, the second indicator would be set to 4. The letters *T*, *h*, *e*, and the space following them are then ignored in alphabetizing titles. The record will be automatically filed under "*r*" -- for *Robe*.

Subfields used most often:

$a -- Title proper
$h -- Medium (often used for non-book media)
$p -- Name of part/section of a work (R)
$b -- Reminder of title (subtitles, etc.)
$c -- Remainder of title page transcription/Statement of responsibility

```
Example:    245 14 $a The DNA story :
                   $b a documentary history of gene
                      cloning /
                   $c James D. Watson, John Tooze.
```

246 Varying form of title (R)

Indicator 1: Note/title added entry controller
1 -- Note, title added entry
3 -- No note, title added entry
Indicator 2: Type of title
-- No information provided
0 -- Portion of title
1 -- Parallel title
4 -- Cover title
8 -- Spine title
Subfield used most often:
$a -- Title proper

```
Example:    246 3# $a Four corners power review
```

250 Edition statement (NR)

Indicators undefined.
Subfield used most often:
$a -- Edition statement

```
Example:    250 ## $a 6th ed.
```

260 Publication, distribution, etc. (Imprint) (R)

Indicator 1: Sequence of publishing statements
-- No information provided
Indicator 2: Undefined
Subfields used most often:
$a -- Place of publication, distribution, etc. (R)
$b -- Name of publisher, distributor, etc. (R)
$c -- Date of publication, distribution, etc. (R)

```
Example:    260 ## $a New York :
                   $b Chelsea House,
                   $c 1986.
```

300 Physical description (R)

Indicators undefined.
Subfields used most often:
$a -- Extent (number of pages) (R)
$b -- Other physical details (usually illustration information)
$c -- Dimensions (cm.) (R)
$e -- Accompanying material (for example, "teacher's guide" or "manual")

```
Example:    300 ## $a 139 p. :
                   $b ill. ;
                   $c 24 cm.
```

440 Series statement / Added entry--Title

This field was made obsolete in 2008 to simplify the series statement. See 490 and 830.

490 Series statement (No added entry is traced from field) (R)

Indicator 1: Specifies whether series is traced (whether an 8XX tag is also present)
0 -- Series not traced
1 -- Series traced (8XX is in record)
Indicator 2 undefined.
Subfield used most often:
$a -- Series statement (R)
$v -- Volume number (R)

```
Example:    490 1# $a Colonial American craftsmen
```

500 General note (R)

Indicators undefined.
Subfield used most often:
$a -- General note (Used when no specialized note field has been defined for the information. Examples: Notes regarding the index; the source of the title; variations in title; descriptions of the nature, form, or scope of the item.)

```
Example:    500 ##  $a Includes index.
```

504 Bibliography, etc. note (R)

Indicators undefined.
Subfield used most often:
$a -- Bibliography, etc. note

```
Example:    504 ## $a Includes bibliographical
                      references.
```

505 Formatted contents note (R)

Indicator 1: Type of contents note
0 -- Complete contents
1 -- Incomplete contents (used with multivolume set when some volumes are not yet published)
2 -- Partial contents
Indicator 2: Level of content designation
-- Basic
Subfield used most often:
$a -- Formatted contents note

```
Example:    505 0# $a Pride and prejudice -- Emma
                      -- Northanger Abbey.
```

520 Summary, etc. note (R)

Indicator 1: Display constant controller
-- Summary
1 -- Review
2 -- Scope and content
3 -- Abstract
Indicator 2 undefined
Subfields used most often
$a -- Summary, abstract, or annotation
$b -- Expansion of summary note

```
Example:    520 ## $a This basic guide to parliamentary
                      procedure tells how to conduct
                      and participate in a meeting
                      properly.
```

600 Subject added entry -- Personal name (R)

Indicator 1: Type of personal name entry element
- 0 -- Forename
- 1 -- Surname (this is the most common form)
- 3 -- Family name

Indicator 2: Subject heading system/thesaurus (identifies the specific list or file which was used)
- 0 -- Library of Congress Subject Headings
- 1 -- LC subject headings for children's literature
- 2 -- Medical Subject Headings
- 3 -- National Agricultural Library subject authority file
- 4 -- Source not specified
- 5 -- Canadian Subject Headings
- 6 -- Répertoire de vedettes-matière
- 7 -- Source specified in subfield $2

(Note regarding Sears subject headings: The MARC 21 format does not provide an assigned indicator for Sears subject headings. Therefore, an indicator of 7 is used, and the MARC defined code "sears" is placed in subfield $2.)

Subfields used most often:
- **$a** -- Personal name (surname and forename)
- **$b** -- Numeration
- **$c** -- Titles and other words associated with a name (R)
- **$q** -- Fuller form of name
- **$d** -- Dates associated with a name (generally, year of birth)
- **$t** -- Title of a work
- **$v** -- Form subdivision (R)
- **$x** -- General subdivision (R)
- **$y** -- Chronological subdivision (R)
- **$z** -- Geographic subdivision (R)
- **$2** -- Source of heading or term (used with 2nd indicator of 7)

```
Example:    600 10 $a Shakespeare, William,
                   $d 1564-1616
                   $x Comedies
                   $x Stage history.
```

```
Example:    600 10 $a Shakespeare, William,
                   $d 1564-1616
                   $x Knowledge
```

```
                        $z Rome
                        $v Congresses.
```

Notice that subfields $v, $x, and $z in the 600 field are repeatable. Subfields $v, $x, $y, and $z do not have to be in alphabetical order. They will be in the order prescribed by the instructions given by the subject heading system.

610 Subject added entry -- Corporate name (R)

Indicator 1: Type of corporate name entry element
- 0 -- Inverted name (not used with AACR2)
- 1 -- Jurisdiction name
- 2 -- Name in direct order

Indicator 2: Subject heading system/thesaurus.
- See indicator 2 under 600

Subfields used most often:
- **$a** -- Corporate name or jurisdiction name as entry element
- **$b** -- Subordinate unit (R)
- **$v** -- Form subdivision (R)
- **$x** -- General subdivision (R)
- **$y** -- Chronological subdivision (R)
- **$z** -- Geographic subdivision (R)
- **$2** -- Source of heading or term (used with 2nd indicator of 7)

```
Example:    610 10 $a United States.
                   $b Army Air Forces
                   $v Biography.
```

650 Subject added entry -- Topical term (Most subject headings fit here.) (R)

Indicator 1: Level of subject
- # -- No information provided

Indicator 2: Subject heading system/thesaurus
(identifies the specific list or file which was used)
- 0 -- Library of Congress Subject Headings
- 1 -- LC subject headings for children's literature
- 2 -- Medical Subject Headings
- 3 -- National Agricultural Library subject authority file
- 4 -- Source not specified
- 5 -- Canadian Subject Headings
- 6 -- Répertoire de vedettes-matière
- 7 -- Source specified in subfield $2

Note regarding Sears subject headings: The MARC 21 format does not provide an assigned indicator for Sears subject headings. Therefore, an indicator of 7 is used, and the MARC defined code "sears" is placed in subfield $2.)

Subfields used most often:
$a -- Topical term
$v -- Form subdivision (R)
$x -- General subdivision (R)
$y -- Chronological subdivision (R)
$z -- Geographic subdivision (R)
$2 -- Source of heading or term used with 2nd indicator of 7)

```
Example:    650 #0 $a Theater
                   $z United States
                   $v Biography
                   $v Dictionaries.
```

Notice that subfields $v, $x, and $z in the 650 field are repeatable. Subfields $v, $x, $y, and $z do not have to be in alphabetical order. They will be in the order prescribed by the instructions given by the subject heading system.

651 Subject added entry -- Geographic name (R)

Indicator 1: undefined.
Indicator 2: Subject heading system/thesaurus.
See indicator 2 under 600
Subfields used most often:
$a -- Geographic name
$v -- Form subdivision (R)
$x -- General subdivision (R)
$y -- Chronological subdivision (R)
$z -- Geographic subdivision (R)
$2 -- Source of heading or term (used with 2nd indicator of 7)

```
Example:    651 #0 $a United States
                   $x History
                   $v Chronology.
```

Notice that subfields $v, $x, and $z in the 651 field are repeatable. Subfields $v, $x, $y, and $z do not have to be in alphabetical order. They will be in the order prescribed by the instructions given by the subject heading system.

700 Added entry -- Personal name (R)

Indicator 1: Type of personal name entry element
0 -- Forename
1 -- Surname (this is the most common form)
3 -- Family name
Indicator 2: Type of added entry
-- No information provided (most common; co-authors, editors, etc.)
2 -- Analytical entry (The values for Indicator 2 changed in 1994 with Format Integration, and older records may display additional values. An analytical entry involves an author/title of an item contained in a work.)
Subfields used most often:
$a -- Personal name
$b -- Numeration
$c -- Titles and other words associated with a name (R)
$q -- Fuller form of name
$d -- Dates associated with a name (generally, year of birth)
$e -- Relator term (such as ill.) (R)
$4 -- Relator code (R)

```
Example:    700 1# $a Baldridge, Letitia.
```

710 Added entry -- Corporate name (R)

Indicator 1: Type of corporate name entry element
0 -- Inverted name (not used with AACR2)
1 -- Jurisdiction name
2 -- Name in direct order
Indicator 2: Type of added entry.
See Indicator 2 under 700
-- No information provided
2 -- Analytical entry
Subfields used most often:
$a -- Corporate name or jurisdiction name as entry element
$b -- Subordinate unit (R)

```
Example:    710 2# $a Sunburst Communications (Firm)
```

740 Added entry -- Uncontrolled related/analytical title (R)

Indicator 1: Nonfiling characters

0-9 -- Number of nonfiling characters present (for initial articles, including spaces)

Indicator 2: Type of added entry. See Indicator 2 under 700

-- No information provided

2 -- Analytical entry

(This field was redefined in 1994 with Format Integration. Prior to 1994, the field was also used for variant titles, such as a different wording on a spine title. In records created since Format Integration, those variant tiles appear in a 246 field.)

Subfield used most often:

$a -- Title

```
Example:    740 02 $a Uncle Vanya.
```

800 Series added entry -- Personal name (R)

Indicator 1: Type of personal name entry element

0 -- Forename

1 -- Surname

3 -- Family name

Indicator 2 undefined.

Subfields used most often:

$a -- Personal name

$b -- Numeration

$c -- Titles and other words associated with a name (R)

$q -- Fuller form of name

$d -- Dates associated with a name (generally, year of birth)

$t -- Title of a work (the series)

$v -- Volume number

```
Example:    800 1# $a Fisher, Leonard Everett.
                   $t Colonial American craftsmen.
```

830 Series added entry -- Uniform title (R)

Indicator 1 undefined.

Indicator 2: Nonfiling characters

0-9 -- Number of nonfiling characters present (for initial articles, including spaces)

Subfield used most often:

$a -- Uniform title

$v -- Volume number

```
    Example:    830 #0 $a Railroads of America (Macmillan)
```

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Part VIII:

A List of Other Fields Often Seen in MARC Records

001 Control number
003 Control number identifier
005 Date and time of latest transaction
006 Fixed-length data elements -- additional material characteristics
007 Physical description fixed field
008 Fixed length data elements (See Part X)
022 International Standard Serial Number (ISSN)
037 Source of acquisition
041 Language code
043 Geographic area code
050 Library of Congress call number
060 National Library of Medicine call number
082 Dewey Decimal classification number (the one recommended by the Library of Congress; locally-assigned call numbers may appear elsewhere)
110 Main entry -- Corporate name (less frequent under AACR2 rules)
256 Computer file characteristics
263 Projected publication date
(indicates a CIP -- Cataloging in Publication -- record)
306 Playing time
508 Creation/production credits note
510 Citation/references note (review sources)
511 Participant or performer note
521 Target audience note (first indicator: 0 = reading grade level, 1 = interest age level, 2 = interest grade level, 3 = special audience characteristics, 4 = motivation interest level)
530 Additional physical form available note
538 System details note
586 Awards note
656 Index term -- Occupation
730 Added entry -- Uniform title
852 Location

856 Electronic location and access
9XX Reserved for local use. (They are used by vendors, systems, or individual libraries to exchange additional data)

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Part IX:

The Leader

There are 24 positions in the Leader, numbered from 00 to 23. For fuller explanation, see the *MARC 21 Format for Bibliographic Data*.

00-04 Record length (calculated by the computer for each record)
05 Record status
- a = increase in encoding level
- c = corrected or revised
- d = deleted
- n = new
- p = increase in encoding from prepublication (previous CIP)

06 Type of record
- a = language material
- c = printed music
- d = manuscript music
- e = cartographic material
- f = manuscript cartographic material
- g = projected medium
- i = nonmusical sound recording
- j = musical sound recording
- k = 2-dimensional nonprojectable graphic
- m = computer file
- o = kit
- p = mixed materials
- r = 3-dimensional artifact or naturally occurring object
- t = manuscript language material

07 Bibliographic level
- a = monographic component part
- b = serial component part
- c = collection
- d = subunit
- i = integrating resource
- m = monograph/item
- s = serial

08 Type of control
- # = no specified type
- a = archival

09 Character coding scheme
# = MARC-8
a = UCS/Unicode
10 Indicator count (always "2")
11 Subfield code count (always "2")
12-16 Base address of data (calculated by the computer for each record)
17 Encoding level
# = full level
1 = full level, material not examined
2 = less-than-full level, material not examined
3 = abbreviated level
4 = core level
5 = partial (preliminary) level
7 = minimal level
8 = prepublication level (CIP)
u = unknown
z = not applicable
18 Descriptive cataloging form
# = non-ISBD
a = AACR2
i = ISBD
u = unknown
19 Multipart resource record level
# = Not specified or not applicable
a = Set
b = Part with independent title
c = Part with dependent title
20 Length of the length-of-field portion (always "4")
21 Length of the starting-character-position portion (always "5")
22 Length of the implementation-defined portion (always "0")
23 Undefined (always "0")

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Part X:

Field 008 for Books

Field 008 is used for Fixed Length Data Elements ("Fixed Field Codes"). There are 40 character positions in field 008, numbered from 00-39. Undefined positions must contain either a blank (#) or a fill character (|). Positions 00-17 and 35-39 are defined the same way for all media.

The information shown here for positions 18-34 applies only to books. For explanation of all the positions below and for positions 18-34 for other media, see the *MARC 21 Format*

for Bibliographic Data.

Note that field 008 has no indicators or subfield codes.

00-05 Date entered on file (YYMMDD),
where Y=year, M=month, and D=day

06 Type of date/publication status:
- b = no dates given; B.C. date involved
- e = detailed date
- s = single known date/probable date
- m = multiple dates
- r = reprint/reissue date (Date 1) and original date (Date 2)
- n = dates unknown
- q = questionable date
- t = publication date and copyright date
- | = no attempt to code

07-10 Date 1/beginning date of publication

11-14 Date 2/ending date of publication

Date fields contain the year(s) of publication. The type of date(s) in these elements are specified in fixed field element 06: Type of date/publication status. (For further details, see the field 008 description in the *MARC 21 Format for Bibliographic Data*.)

15-17 Place of publication, production, or execution
For example:
- pk# = Pakistan
- cau = California (US)

(For a full list of codes used in these positions, see the *MARC Code List for Countries*.)

18-21 Illustrations (up to 4 codes):
- # = no illustrations
- a = illustrations
- b = maps
- c = portraits
- d = charts
- e = plans
- f = plates
- g = music
- h = facsimiles
- i = coats of arms
- j = genealogical tables
- k = forms
- l = samples
- m = phonodisc, phonowire, etc.
- o = photographs
- p = illuminations
- | = no attempt to code

22 Target audience:
- # = unknown or not specified
- a = preschool
- b = primary
- c = pre-adolescent
- d = adolescent
- e = adult
- f = specialized
- g = general
- j = juvenile
- | = no attempt to code

23 Form of item:
- # = none of the following
- a = microfilm
- b = microfiche
- c = microopaque
- d = large print
- f = braille
- r = regular print reproduction
- s = electronic
- | = no attempt to code

24-27 Nature of contents (up to 4):
- # = no specified nature of contents
- a = abstracts/summaries
- b = bibliographies (is one or contains one)
- c = catalogs
- d = dictionaries
- e = encyclopedias
- f = handbooks
- g = legal articles
- i = indexes
- j = patent document
- k = discographies
- l = legislation
- m = theses
- n = surveys of literature
- o = reviews
- p = programmed texts
- q = filmographies
- r = directories
- s = statistics
- t = technical reports
- u = standards/specifications
- v = legal cases and notes
- w = law reports and digests
- z = treaties
- | = no attempt to code

28 Government publication:
- # = not a government publication
- i = international intergovernmental
- f = federal/national
- a = autonomous or semi-autonomous component
- s = state, provincial, territorial, dependent, etc.
- m = multistate
- c = multilocal
- l = local
- z = other type of government publication
- o = government publication -- level undetermined
- u = unknown if item is government publication
- | = no attempt to code

29 Conference publication:
- 0 = not a conference publication
- 1 = conference publication
- | = no attempt to code

30 Festschrift:
- 0 = not a festschrift
- 1 = festschrift
- | = no attempt to code

31 Index:
- 0 = no index
- 1 = index present
- | = no attempt to code

32 Undefined (since 1990) (Earlier records may contain the values 0 or 1)
- # = Undefined
- | = no attempt to code

33 Literary form:
- 0 = not fiction (not further specified)
- 1 = fiction (not further specified)
- c = comic strips
- d = dramas
- e = essays
- f = novels
- h = humor, satires, etc.
- i = letters
- j = short stories
- m = mixed forms
- p = poetry
- s = speeches
- u = unknown
- | = no attempt to code

34 Biography:
- # = no biographical material
- a = autobiography
- b = individual biography

c = collective biography
d = contains biographical information
| = no attempt to code

35-37 Language:
A three-letter code. For example: eng fre ger spa rus ita

(For a full list of codes used in these positions, see the *MARC Code List for Languages*.)

38 Modified record:
# = not modified
x = missing characters (because of characters unavailable in MARC character set)
s = shortened
d = "dashed-on" information omitted
r = completely romanized/printed cards in script
o = completely romanized/printed cards romanized
| = no attempt to code

39 Cataloging source:
# = national bibliographic agency
c = cooperative cataloging program
d = other sources
u = unknown
| = no attempt to code

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Library of Congress
Library of Congress Help Desk (10/27/2009)

Appendix 1004A

DECLARATION

I, **LaTonya Morris,** declare that the following statements are true and correct to the best of my knowledge, information and belief:

1. I am over 18 years of age and have personal knowledge of the facts set forth in this declaration, based upon practices and training encountered in the course of my work, including review of available business records and documentation maintained by my employer, ProQuest LLC ("ProQuest").

2. I am a **Technical Support Team Leader** employed by ProQuest in Ann Arbor, Michigan. I have worked for ProQuest since **2018** (**3+** years). ProQuest is an information company that collects, organizes publishes and distributes for sale content from a wide range of sources, including dissertations and master's theses. I am familiar with ProQuest's sales and publication practices with respect to the publication of dissertations and master's theses as well as the related Abstract Database (defined in in paragraph 3).

3. ProQuest receives dissertations and master's theses from participating degree granting institutions. Shortly after these works are received, ProQuest makes them available for sale to the public, unless specifically restricted from sales by the dissertation author or the degree granting institution. Prior to 2013, a "Record" of each dissertation and each master's thesis submitted to ProQuest was published in ProQuest's print publication Dissertations Abstracts International or Master's Abstracts International, respectively (the print editions ceased being distributed in print as of volume 73-06 published in December 2012). Beginning in 1985, each Record is also published in the Dissertation Abstracts International Database ("Abstract Database").[1] A "Record" consists of an

[1] The Abstract Database has been maintained exclusively by ProQuest under various corporate names, including Bell & Howell Information and Learning Company, ProQuest Information and Learning Company, UMI Company and University Microfilms, Inc. and has been licensed to subscribers under various product names including "Dissertation Abstracts Online" and "ProQuest® Digital Dissertations." ProQuest LLC (and its predecessor entities) is and at all times has been a U.S. Company based in Ann Arbor, MI.

abstract, index record, and citation information including title, author name, degree granting institution and degree date.

4. Prior to 1985, Records were published only in the print publications Dissertations Abstracts International and Master's Abstracts International. In 1985, the Records in these print publications were combined to create the Abstract Database which was made available online through third party vendors, such as Dialog, Data-Star, and OCLC. In 1987, ProQuest began to offer the Abstract Database on CD ROM. In 1997, ProQuest made available the contents of the Abstract Database in its ProQuest® Digital Dissertations and Theses database ("PQDT"), which is accessible via the world wide web to authenticated subscribers. The Abstract Database allows for text searching of the Records, including the title, author, index terms and the abstract.

5. The **PQDT** database includes the **Abstract Database** and is supplemented by the addition of certain select dissertations and master's theses in PDF format. Whether a dissertation is represented in full PDF format varies based on a number of factors, including the format in which the dissertation or theses was originally submitted for publication (digital, paper or microfiche) or whether the dissertation or theses was part of a retrospective digitization effort. PQDT allows for text searching of the Records as well as full text where the full PDF is properly formatted.

6. At the time of publication of the Dissertation (the full citation is provided in paragraph 9 below), ProQuest published in the **Abstract Database** a Record of each dissertation and each master's thesis that ProQuest offers for sale with new Records added on a **monthly** basis. Once a Record is published on the Abstract Database, the Record is available to the students, faculty, staff and/or patrons of any institutions that subscribe to the Abstract Database. At the time of publication the Abstract Database was available throughout the United States, through third party vendors as well as in CD-ROM format directly from ProQuest.

7. At the time of the Dissertation's publication, the Record and full PDF of the Dissertation was added to PQDT as specified below.

8. I have reviewed ProQuest's records regarding the Dissertation cited as:

Dissertation No.	3020182
Title:	*Therapeutic hemoglobin synthesis in beta-thalassemic mice expressing lentivirus -encoded human beta-globin*
Author:	May, Chad Michael
Degree Date:	2001, PhD
University:	Weill Medical College of Cornell University

Full Text Availability

First Made Available for Sale in PDF format and placed in PQDT	2001-11-26

9. At the time of the Dissertation's publication, the Record of the Dissertation was made available directly from ProQuest in electronic format and online via third party vendors, including Dialog, as part of the Abstract Database which was made available via such vendors in the ordinary course of business.

10. The attached print copy of the Dissertation is a true and complete copy of the Record and the full Dissertation as originally published and kept by ProQuest in the ordinary course of its business.

I make this Declaration on personal knowledge and, if called as a witness at trial, I am competent to testify.

I declare under penalty of perjury that the forgoing is true and correct.

Executed on September 21, 2022

LaTonya Morris

LaTonya Morris
Technical Support Team Leader

rev. 02April2021

Appendix 1004B

Full Text | Dissertation or Thesis

Therapeutic hemoglobin synthesis in beta-thalassemic mice expressing lentivirus -encoded human beta-globin

May, Chad Michael.

Weill Medical College of Cornell University ProQuest Dissertations Publishing, 2001. 3020182.

Abstract

The stable introduction of a functional globin gene in autologous hematopoietic stem cells is a potentially powerful approach to treat β-thalassemia. The challenge facing this approach is to stably express high levels of the human β-globin gene in an erythroid-specific, regulated, and sustained fashion. Low level expression, position effects and transcriptional silencing have hampered until now the effectiveness of viral transduction of the human β-globin gene linked to minimal regulatory sequences. I show here that the use of recombinant lentiviruses enables efficient transfer and faithful integration of the human β-globin gene together with large segments (3.2 kb) of its locus control region (LCR). Studies comparing a vector containing a 3.2 kb LCR, termed TNS9, to one with a minimal 1.0 kb LCR, termed RNS1, demonstrate both a higher mean level of human β-globin expression by TNS9, and a higher fraction of cells expressing human β-globin following vector integration of random sites. In long-term studies in recipient mice engrafted with TNS9-transduced bone marrow cells, production of lentivirus-encoded β-globin is substantially augmented, owing to an increase in both the level of globin expression, as shown by RNA analysis, and the fraction of red cells expressing human β^A, as demonstrated by immunostaining. Murine α_2 : human $\beta^A{}_2$ tetramers account for up to 13% of total hemoglobin in mature red cells in normal long-term bone marrow chimeras. Most importantly, higher levels are obtained in β-thalassemic mice, ranging from 17 to 24% fifteen weeks after transplant, resulting in a substantial increase in hemoglobin concentration and hematocrit levels, with a concomitant reduction in reticulocyte

counts. Red cell morphology (anisocytosis and poikilocytosis) is markedly corrected. Therapeutic benefits are stable up to 40 weeks resulting in reduced extramedullary erythropoiesis in the spleen. My findings demonstrate that high-level, tissue-specific gene expression can be achieved in the progeny of unselected genetically modified stem cells. Therefore, a genetic approach could be successful in treating β-thalassemia. Furthermore, these findings provide a paradigm for stem cell therapy requiring regulated expression of a tissue-specific transgene in the progeny of genetically modified stem cells.
Less

Details

Subject	Molecular biology; Immunology
Classification	0307: Molecular biology 0982: Immunology
Identifier / keyword	Health and environmental sciences; Biological sciences; Globin; Hemoglobin; Lentivirus-encoded; Thalassemic; Therapeutic
Title	**Therapeutic hemoglobin synthesis in beta-thalassemic mice expressing lentivirus-encoded human beta-globin**
Author	May, Chad Michael
Number of pages	130
Degree date	2001
School code	0967
Source	DAI-B 62/07, Dissertation Abstracts International
Place of publication	Ann Arbor
Country of publication	United States
ISBN	978-0-493-32698-6
University/institution	Weill Medical College of Cornell University
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Appendix 1005A

Therapeutic haemoglobin synthesis in β-thalassaemic mice expressing lentivirus-encoded human β-globin

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The stable introduction of a functional β-globin gene in haematopoietic stem cells could be a powerful approach to treat β-thalassaemia[1] and sickle-cell disease[2]. Genetic approaches aiming to increase normal β-globin expression in the progeny of autologous haematopoietic stem cells[3] might circumvent the limitations and risks of allogeneic cell transplants[4]. However, low-level expression, position effects and transcriptional silencing hampered the effectiveness of viral transduction of the human β-globin gene when it was linked to minimal regulatory sequences[5]. Here we show that the use of recombinant lentiviruses enables efficient transfer and faithful integration of the human β-globin gene together with large segments of its locus control region. In long-term recipients of unselected transduced bone marrow cells, tetramers of two murine α-globin and two human β^A-globin molecules account for up to 13% of total haemoglobin in mature red cells of normal mice. In β-thalassaemic heterozygous mice higher percentages are obtained (17% to 24%), which are sufficient to ameliorate anaemia and red cell morphology. Such levels should be of therapeutic benefit in patients with severe defects in haemoglobin production.

Onco-retroviral-mediated transfer in mouse haematopoietic stem cells (HSCs) permits erythroid-specific expression of the human β-globin gene, but expression is low and limited by chromosomal position effects[5]. The same outcome prevails in transgenic mice unless genomic elements encompassing DNase I hypersensitive sites (HS) located 60 kilobases (kb) upstream of the human β-globin gene[6,7], referred to as the locus control region (LCR), are linked to the globin gene[8]. Incorporation of small elements spanning DNase HS2, HS3 and HS4 into viral vectors increases β-globin expression in mouse erythroleukaemia (MEL) cells[9,10]. However, low-level expression, strong position effects and transcriptional inactivation are still observed in bone marrow chimaeras[5,11]. Studies in transgenic mice[12] and deletional analyses[13] support the view that coordinated interaction of several genetic elements including the LCR is required for physiologic β-globin gene expression[12–15]. We therefore thought that incorporation of large elements spanning HS2, HS3 and HS4[16–18] in a vector might enhance β-globin expression beyond the levels previously achieved using arrayed minimal core elements[5,9–11], and thus might diminish position effects and vector silencing. The efficient transduction of large genomic fragments using onco-retroviral vectors has proved to be severely curtailed by splicing and other alterations affecting the stability of the recombinant genomes[9,10,16]. Here we report how these problems may be overcome by using vectors derived from human immunodeficiency virus 1, a retrovirus that has the ability to regulate packaging of unspliced viral genomes[19].

We constructed two recombinant lentiviruses carrying β-globin transcription units (Fig. 1a, b). RNS1 contains a minimal LCR comprising previously tested core elements of HS2, HS3 and HS4 (ref. 9). To create TNS9, large fragments encompassing HS2, HS3 and HS4 were introduced instead of the corresponding core elements. The genomic integrity of each recombinant lentivirus was assessed after transfection and reverse transcription. The dominant RNA species for RNS1 and TNS9 was the full-length transcript (Fig. 1c), suggesting that the intact recombinant genome was available for packaging. This was confirmed by the presence of single, intact proviral structures in transduced cells (Fig. 1d).

To investigate the tissue specificity, stage specificity and expression level of the vector-encoded human β-globin gene, we transduced RNS1 and TNS9 into MEL cells, lymphoid Jurkat cells and myeloid HL-60 cells. To induce β-globin transcription, transduced MEL cell pools were differentiated using hexamethylene bisacetamide (HMBA). Human β-globin (β^A) and mouse β-globin transcripts were measured by quantitative primer extension. After normalization to vector copy number and to endogenous β-globin expression per allele, human β-globin levels were 14.2 ± 4.7% for RNS1 and 71.3 ± 2.3% for TNS9 in pooled MEL cells (Fig. 2a). No human β-globin expression was detected in non-induced MEL, Jurkat and HL-60 cells (Fig. 2a), indicating that human β-globin expression was appropriately regulated in terms of tissue specificity and state of differentiation. We generated a panel of MEL cell clones that carried a single copy of either vector to distinguish whether the increased expression obtained in HMBA-treated MEL cells transduced with TNS9 rather than RNS1 was the result of an increase in β^A

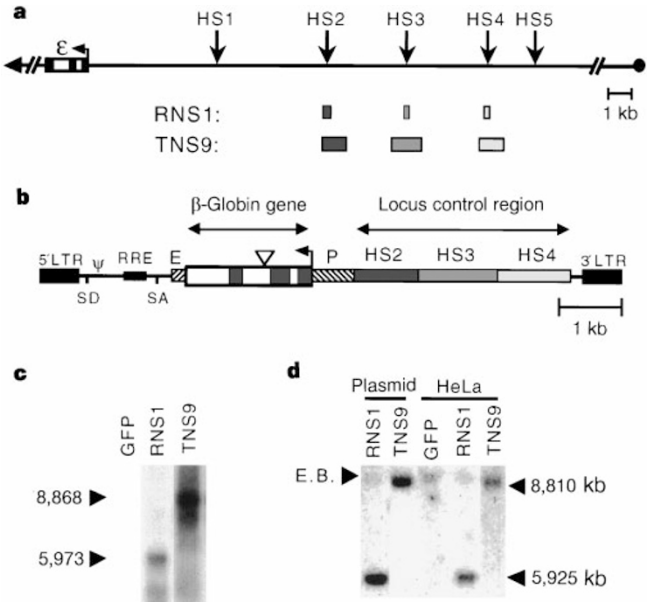

Figure 1 Structure and stability of recombinant lentiviral vectors. **a**, Genomic structure of the human β-globin LCR and mapping of fragments introduced in RNS1 and TNS9. Main DNase I hypersensitive sites (HS) are indicated. RNS1 contains a 1.0-kb LCR consisting of the 423-bp HS2, 280-bp HS3 and 283-bp HS4 core elements previously studied in an MLV based vector[5,9]. TNS9 was generated by replacing the core HS2 element of RNS1 with an 840-bp HS2 fragment, the core HS3 element with a 1,308-bp HS3 fragment, and the core HS4 element with a 1,069-bp HS4 fragment. Boxes represent HS fragments drawn to scale. **b**, Representation of TNS9. Exons and introns of the human β-globin gene (including the IVS2 deletion[9]) are represented by filled and open boxes, respectively. Splice donor (SD), splice acceptor (SA), packaging region (ψ), rev-response element (RRE), human β-globin promoter (P) and 3′ β-globin enhancer (E) are indicated. **c**, Northern blot analysis shows full-length RNA transcripts indicating that the recombinant lentiviral genomes are stable. A lentiviral vector encoding GFP was used as control. **d**, Southern blot analysis on genomic DNA from transduced cells, digested once in each long terminal repeat (LTR), results in a single band corresponding to the expected size for both vectors, indicating that the proviral structure is not rearranged. Plasmid controls are shown in the left panel. The 9.0-kb endogenous murine band (E.B.) is indicated.

expression per cell or of an increase in the fraction of cells expressing human β-globin. Transduced MEL cells were subcloned by limiting dilution immediately after transduction, avoiding any bias towards favourable chromosomal integration sites as produced by drug selection[5]. The proportion of clones expressing human β-globin varied significantly between the two vectors (Fig. 2b). One out of ten RNS1 positive clones yielded measurable human β-globin transcripts, in contrast to 12 out of 12 for TNS9 ($P < 0.01$, Fisher's exact test). Cells bearing TNS9 also expressed higher levels of human β-globin than did those bearing RNS1 ($P < 0.01$, Wilcoxon rank sum test). These findings established that both the level and probability of expression at random integration sites was increased with the TNS9 vector.

To investigate the function of these vectors *in vivo*, we transduced and transplanted murine bone marrow cells without any selection in syngeneic, lethally irradiated recipient mice. The average vector copy number in peripheral blood cells, measured periodically for 24 weeks (Fig. 3a), showed highly efficient gene transfer with both vectors (1.8 ± 0.6 and 0.8 ± 0.6 average vector copies per cell for RNS1 and TNS9, respectively, at week 24). For RNS1, human β-globin transcripts were in the range of 7.0 ± 3.0% after 6 weeks, later decreasing to 2.1% and 3.4% at week 16 and 24, respectively (expressed as fraction of total human + mouse β-globin transcripts, Fig. 3b). In contrast, cells transduced with TNS9 maintained human

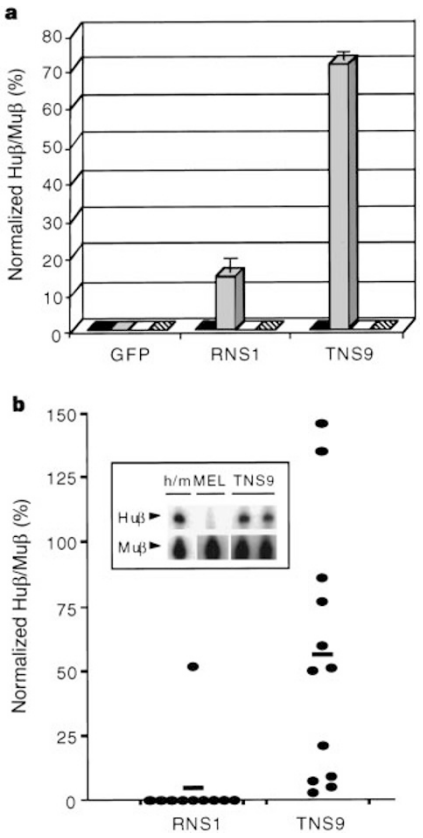

Figure 2 Increased mean β-globin expression in MEL cells and proportion of clones expressing detectable human β-globin with the TNS9 vector. **a**, MEL, Jurkat and HL-60 cells were transduced with RNS1, TNS9 or control GFP recombinant lentivirus. Human β-globin RNA expression in HMBA-induced MEL cells (grey bars) was measured by quantitative primer extension and normalized to mouse β-globin RNA expression per locus. Expression was then normalized to the vector copy number determined by Southern blot. No human β-globin RNA expression was detected in non-induced MEL (black bars), Jurkat (white bars) or HL-60 cells (hatched bars), indicating that globin expression was erythroid- and differentiation-specific. **b**, MEL cell clones carrying an intact single copy of RNS1 or TNS9 were identified and induced to terminal maturation to study variability of expression at individual vector integration sites. Ten RNS1 and twelve TNS9 clones were analysed. The expression of human β-globin relative to mouse β-globin per locus is shown. Black bar represents mean expression levels of each vector. Inset, representative primer extension reactions of two TNS9 transduced MEL cell clones, non-transduced MEL cells and a positive control (equal mix of human and mouse blood RNA, h/m).

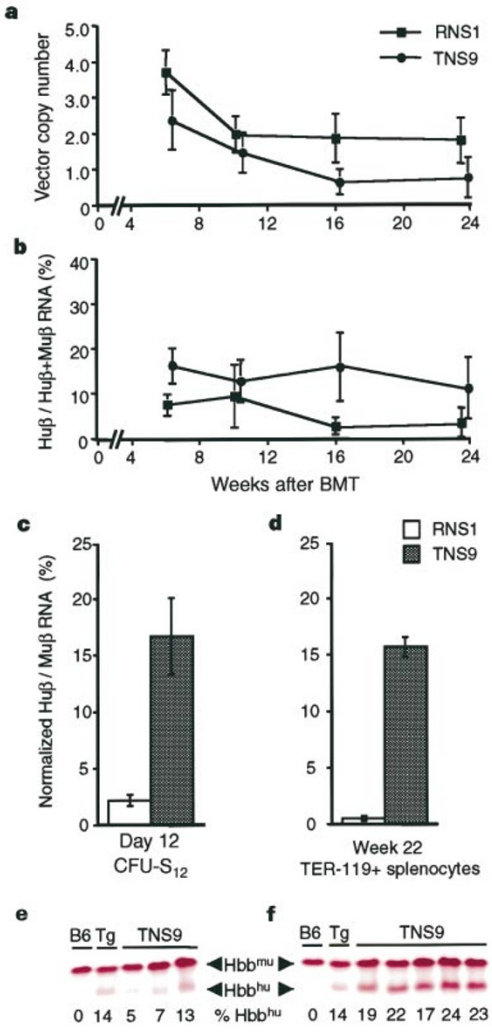

Figure 3 Long-term stability of vector copy number, human β-globin RNA levels and haemoglobin tetramers in peripheral blood of bone marrow chimaeras. Vector copy number and human β-globin RNA transcripts were measured during a 24-week period in mice transplanted with RNS1 ($n = 8$) or TNS9 ($n = 10$) transduced bone marrow. **a**, Vector copy number was assessed by Southern blot analysis of genomic DNA isolated from peripheral blood at weeks 6, 10, 16 and 24. **b**, Human β-globin RNA levels in peripheral blood are expressed relative to total human and mouse β-globin RNA (Huβ/Huβ + Muβ). **c**,**d**, Human β-globin expression per vector copy in spleen cells 12 days (**c**, pooled CFU-S_{12}) and 22 weeks (**d**, TER-119+ cells) after bone marrow transplantation. Human β-globin RNA expression is measured relative to the level of mouse β-globin RNA expression in the splenocytes of bone marrow chimaeras transduced with RNS1 ($n = 6$) or TNS9 ($n = 4$). Mean RNA expression (per vector copy) is normalized to endogenous gene expression. Vector copy number was comparable but no human β-globin RNA was detected in the thymus of the same animals, (data not shown). **e**, Hbb^{hu} (mouse α_2 : human β_2^A levels were analysed 24 weeks after transplantation using cellulose acteate gel electrophoresis. Haemoglobin samples are from a control adult C57BL/6 mouse (B6), the transgenic guman β-globin YAC mouse line A85.68 (Tg; ref. 20), and three TNS9 bone marrow chimaeras. Fraction of Hbb^{hu} relative to total Hbb (Hbb^{hu} / Hbb^{hu} + Hbb^{mu}) is indicated below each sample (see Table 1 in Supplementary Information for detailed analyses). **f**, Hbb^{hu} formation in mice transplanted with β^0 heterozygote ($Hbb^{th3/+}$) bone marrow cells transduced with TNS9 ($n = 5$), and analysed 8 weeks after transplantation.

β-globin transcript levels in the 10–20% range during the same time period. To assess transcriptional activity per vector copy, steady-state RNA transcripts and vector copy number were quantified in pooled CFU-S_{12} and in erythroid TER-119+ spleen cells (Fig. 3c, d). Twelve days after transplantation, human β-globin expression per vector copy was 2.3 ± 0.5% and 16.8 ± 3.9% for RNS1 and TNS9, respectively (normalized to murine β-globin expression per endogenous allele, Fig. 3c). Twenty weeks later, these values were 0.5 ± 0.1% (significantly lower than on day 12, $P = 0.02$) and 15.8 ± 0.9%, respectively (Fig. 3d). These findings established that the larger LCR fragments increased globin expression *in vivo* and, furthermore, suggested that TNS9 is more resistant to transcriptional silencing than is RNS1.

The levels of TNS9-encoded human β-globin RNA measured in peripheral blood suggested that therapeutically relevant levels of human β-globin could be produced. Haemoglobin tetramers incorporating vector-encoded human β^A and endogenous murine α-globin (designated Hbb^{hu}) were quantitated in peripheral blood red cell lysates after cellulose acetate gel fractionation. Hbb^{hu} levels accounting for up to 13% of total haemoglobin were found 24 weeks after transplantation (Fig. 3e, Table 1 in Supplementary Information). In the same assays, transgenic mice bearing one copy of a 230-kb yeast artificial chromosome (YAC) encompassing the entire human β-globin-like gene cluster[20] showed 14% of their total haemoglobin incorporating human β^A (Fig. 3e). No haemoglobin tetramers containing human β^A were measurable in any of the mice bearing RNS1 (Table 1 in Supplementary Information). The proportion of mature peripheral blood red cells expressing human β^A was elevated in most TNS9 bone marrow chimaeras, as shown by dual staining of human β^A and TER-119 (Table 1 in Supplementary Information). In contrast, chimaeras engrafted with RNS1-transduced bone marrow showed highly variable fractions of weakly staining β^A-positive erythrocytes (Table 1 in Supplementary Information). Normalized to the fraction of circulating β^A-positive mature red cells, the levels of haemoglobin containing lentivirus-encoded β^A were on average 64% of those obtained in the YAC transgenic mice (Table 1 in Supplementary Information).

To ascertain that true HSCs were transduced, we carried out secondary transplants using marrow from primary recipients collected 24 weeks after transplantation. The TNS9 and RNS1 vectors were readily detected in all secondary recipients 13 weeks after transplantation. Human β-globin expression was maintained in all recipients of TNS9-transduced marrow (Table 2 in Supplementary Information). The successful transduction of HSCs was confirmed by integration site analyses (Fig. 4).

In view of the high levels of expression, we tested the extent to which the TNS9 vector could correct the phenotype of thalassaemic cells using β^0-thalassaemic heterozygote mice that lack a copy of their b1 and b2 β-globin genes ($Hbb^{th3/+}$)[21]. These heterozygotes have a clinical phenotype similar to human thalassaemia intermedia and exhibit chronic anaemia (haematocrit 28–30%, haemoglobin 8–9 g dl^{-1}) and anomalies in red cell size (anisocytosis) and shape (poikilocytosis). Fifteen weeks after transplantation with unselected TNS9-transduced $Hbb^{th3/+}$ bone marrow, the haematocrit level, red blood cell count, reticulocyte count and haemoglobin level were markedly improved in five out of five recipient mice (Fig. 6). Anisocytosis and poikilocytosis were markedly reduced in the peripheral blood smears of chimaeras bearing the TNS9 vector (Fig. 5c, d). Control mice transplanted with $Hbb^{th3/+}$ bone marrow cells transduced with a vector encoding enhanced green fluorescent protein (eGFP) all remained severely anaemic ($n = 5$, Fig. 6) and maintained their abnormal red cell morphology (Fig. 5a, b). These results establish that levels of circulating haemoglobin obtained with TNS9 were indeed therapeutically relevant.

The combined effect of the high efficiency of gene transfer and the absence of vector rearrangements afforded by the recombinant lentivirus carrying the β-globin gene and LCR configuration adopted in TNS9 yielded levels of human β^A expression in the therapeutic range. The higher expression obtained with TNS9 compared with RNS1 was associated with a higher fraction of permissive integration sites in MEL cells (Fig. 2b) and a higher fraction of human β^A-containing red blood cells in bone marrow chimaeras (Table 1 in Supplementary Information). RNS1, which carries a weaker enhancer, silenced over time whereas TNS9 retained undiminished transcriptional activity over the same time period (Fig. 3c, d), and in secondary transplant recipients (Table 2 in Supplementary Information).

Higher levels of murine α_2:human β_2^A tetramers were obtained in peripheral blood samples from recipients of TNS9-transduced $Hbb^{th3/+}$ bone marrow (21 ± 3% of total haemoglobin, $n = 5$, Fig. 3f) than with $Hbb^{+/+}$ bone marrow (6 ± 4%, $n = 10$, Fig. 3e). The two groups showed comparable peripheral blood vector copy numbers and levels of human β-globin RNA (0.8 ± 0.2 compared with 0.8 ± 0.6, and 16.8 ± 6% compared with 10.8 ± 7%, respectively). This observation is consistent with a competitive advantage of murine β-globin over human β-globin in associating with murine α-globin[22]. In thalassaemic patients, added human β-

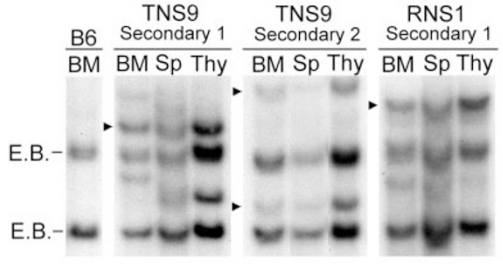

Figure 4 Integration of lentiviral vectors into hematopoietic stem cells. Southern blot analysis was performed on genomic DNA isolated from bone marrow (BM), spleen (Spl) and thymus (Thy) of secondary bone marrow transplant recipients collected 13 weeks after transplant (one representative RNS1, and two representative TNS9 secondary transplant recipients are shown). Arrowheads indicate unique integration bands found in all tissues. Two endogenous bands (E.B.) are found in the genomic DNA of C57BL/6 (B6) mice. See Table 1 in Supplementary Information for further analyses of engraftment and human β-globin expression in secondary transplant recipients.

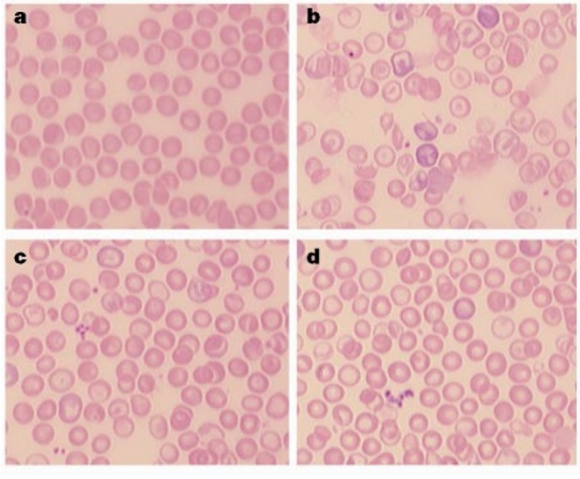

Figure 5 Correction of anisocytosis and poikilocytosis in bone marrow chimaeras reconstituted with TNS9-transduced $Hbb^{th3/+}$ bone marrow cells. **a**, Blood smear of an adult C57BL/6 mouse transplanted with control GFP-transduced C57BL/6 bone marrow. **b**, Blood smear of an adult, $Hbb^{th3/+}$ mouse transplanted with GFP-transduced $Hbb^{th3/+}$ bone marrow. **c**,**d**, Blood smear of two TNS9-transduced $Hbb^{th3/+}$ bone marrow chimaeras. Blood smears were taken 7 weeks after transplantation with transduced bone marrow.

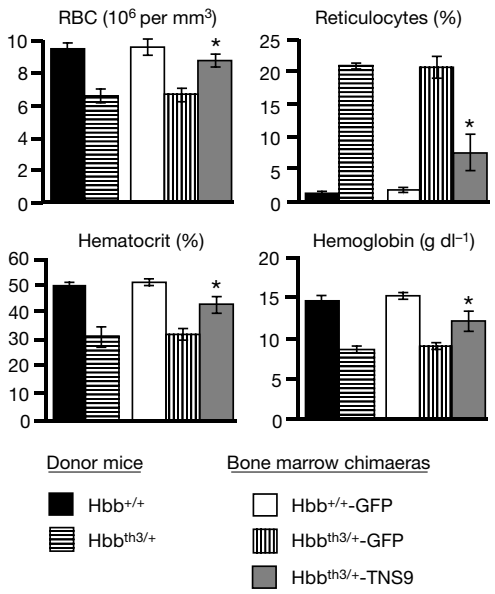

Figure 6 Amelioration of haematological parameters in bone marrow chimaeras reconstituted with TNS9-transduced Hbb$^{th3/+}$ bone marrow cells. Red blood cell (RBC) counts, reticulocyte counts, hematocrit and hemoglobin levels are shown 15 weeks after transplantation (n = 5 mice per group). All measured parameters show significant increases (asterisk) in TNS9-transduced Hbb$^{th3/+}$ as compared with GFP-transduced Hbb$^{th3/+}$ bone marrow chimaeras (P = 0.1, 0.2, 0.1, and 0.4, respectively; Wilcoxon rank sum test).

chain synthesis would improve the α:β chain imbalance and thus increase red cell survival and ameliorate the ineffective erythropoiesis in these patients. In patients with sickle cell disease, transduced β^A chains are expected to have an advantage over the β^Schains produced by both endogenous genes in competing for the available α-chains[23]. Given that patients with S/β-thalassaemia whose HbA represents 10–30% of their total haemoglobin are very mildly affected[1,24], the clinical benefit of such an intervention would be highly significant.

We have shown that recombinant lentiviruses bearing large fragments of the human β-globin gene and its LCR represent an advancement towards the genetic treatment of severe haemoglobinopathies. Lentiviral vectors appear to be well suited for the stable transduction of human CD34$^+$ cells[25] and are expected to be available for therapeutic applications once safety concerns are fully addressed[26]. Furthermore, the principles underlying inclusion of multiple genetic elements within this vector provide a paradigm for any stem cell therapy requiring stable and regulated expression of a tissue-specific transgene. □

Methods

Vector construction and production

The human β-globin gene was subcloned from Mβ6L (ref. 9)into pHR′LacZ (ref. 27; kindly provided by D. Trono) replacing the CMV–LacZ sequence. PHR′eGFP was constructed by replacing LacZ with the eGFP coding sequence (Clonetech). Viral stocks were generated by triple transfection of the recombinant vectors, pCMVΔR8.9 (ref. 27) and pMD.G into 293T cells as described[28]. The pseudotyped virions were concentrated by ultracentrifugation, resuspended and titrated as described[29].

Northern and Southern blot analyses

We transiently transfected 293T cells with 15 μg of the packaging vector pCMVΔR8.9 and 20 μg of RNS1 or TNS9 plasmid. Total RNA was isolated 48 h after transfection with TRIzol (Gibco BRL). Northern blot analyses were done as described[30] with the *Nco*I–*Bam*HI fragment of the human β-globin gene as probe. Cell-free viral supernatant was used to infect HeLa cells in the presence of polybrene (8 μg ml^{-1}). For analysis of integrated vector structure, genomic DNA from cells was isolated, digested with *Sca*I, and studied by Southern blot analysis[9] using a [^{32}P]dCTP-labelled *Nco*I–*Bam*HI fragment of the human β-globin gene as probe. Plasmid controls were also digested with *Sca*I and admixed with 5 μg of *Sca*I-digested HeLa genomic DNA. Quantification of vector copy number in bone marrow chimaeras was performed on genomic DNA digested with *Bam*HI. Analysis of vector integration sites in secondary bone marrow transplant recipients was performed on samples digested with *Nco*I, which cuts only once in the vector.

MEL cell infection, subcloning and differentiation

Cell-free viral supernatant was used to infect C88 MEL cells[9] in the presence of polybrene (8 μg ml^{-1}). Transduced MEL cells were subcloned by limiting dilution, and screened by PCR for transduction[30] using primers that anneal in the human β-globin promoter sequence (βPS, 5′-GTCTAAGTGATGACAGCCGTACCTG-3′) and in HS2 (C2A, 5′-TCAGCCTAGAGT GATGACTCC TATCTG-3′). Vector copy number and integration site analysis was determined by Southern blot analysis[9]. Transduced MEL cells were induced to maturation by 5-day culture in 5 mM *N*,*N*′–hexamethylene bisacetamide (HMBA, Sigma).

Quantification of human β-globin mRNA

Total RNA was extracted from MEL, Jurkat and HL-60 cells, or mouse spleen and blood using TRIzol. Quantitative primer extension assays were done using the Primer Extension System-AMV Reverse Transcriptase kit (Promega) with [^{32}P]dATP end-labelled primers specific for retroviral-derived human β-globin (5′-CAGTAACGGCAGACTTCTCCTC-3′) and mouse β-globin (5′-TGATGTCTGTTTCTGGGGTT GTG-3′), with predicted extension products of 90 bp and 53 bp, respectively. The probes yield products of identical length for β^{maj}, β^{min}, β^s and β^t. Primers were annealed to 4 μg of RNA and reactions were run according to manufacturer's protocols. Radioactive bands were quantitated by phosphorimager analysis (BioRad). RNA isolated from A85.68 mice[20] was used as positive control. After correction for primer labelling, the human to mouse RNA signal was 29 ± 1% per gene copy in repeated experiments ($n > 8$), in agreement with previous findings based on RT–PCR[20]. Values measured in bone marrow chimaeras that were obtained in separate runs were standardized to the value obtained in the A85.68 RNA sample. In Figs 2 and 3c, d, human β-globin expression is expressed per vector copy and normalized to the endogenous transcripts (accounting for two endogenous alleles). In Fig. 3b, human transcripts are reported as the fraction of total β-globin RNA (Huβ / Huβ + Muβ) to reflect absolute contribution of vector-encoded transcripts.

Bone marrow chimaeras

Donor bone marrow was flushed from the femurs of 8- to 16-week-old male C57BL/6 or Hbb$^{th3/+}$ mice (Jackson Laboratories) that had been injected intravenously (i.v.) 6 days earlier with 5-flurouracil (5-FU, Pharmacia; 150 mg kg^{-1} body weight). Bone marrow cells were resuspended in serum-free medium, and supplemented with IL-1α (10 ng ml^{-1}), IL-3 (100 U ml^{-1}), IL-6 (150 U ml^{-1}), Kit ligand (10 ng ml^{-1}) (Genzyme), β-mercaptoethanol (0.5 mM; Sigma), L-glutamine (200 mM), penicillin (100 IU ml^{-1}) and streptomycin (100 μg m^{-1}), and cultured for 18 h. Recipient mice (11- to 14-week-old C57/BL6 or Hbb$^{th3/+}$ mice) were irradiated with 10.5 Gy (split dose 2 × 5.25 Gy) on the day of transplantation. Bone marrow cells were pelleted and resuspended in serum-free medium containing concentrated lentiviral supernatant, and supplemented with polybrene (8 μg ml^{-1}), L-glutamine (200 mM), penicillin (100 IU ml^{-1}) and streptomycin (100 μg ml^{-1}), and cultured for 6 h. Transduced bone marrow cells (1×10^5 or 5×10^5) were then i.v. injected into each of the irradiated female recipients to establish short-term and long-term bone marrow chimaeras, respectively.

In short-term studies, spleens were removed 12 d after transplantation to extract total RNA and genomic DNA. To monitor long-term chimaeras, two or three capillary tubes of blood were collected every 4–6 weeks, from which genomic DNA, total RNA and haemoglobin were extracted. To examine vector function reliably in long-term animals, erythroid cell populations were purified from spleen. Single-cell suspensions were incubated with rat anti-mouse TER-119 monoclonal antibody (PharMingen). Sheep anti-Rat IgG dynabeads (M-450, Dynal Inc.) were added to the antibody-coated spleen cells and purified as recommended by the manufacturer. Vector copy number, integration pattern and chimaerism were determined by Southern blot analysis. The fraction of donor DNA relative to recipient was determined by stripping and reprobing the blot with a [^{32}P]dCTP-labelled probe specific for the Y chromosome and normalizing to an endogenous mouse band. Radioactive bands were quantitated by phosphorimager analysis. Sera from five randomly selected long-term bone marrow chimaeras (30 weeks after transplantation) tested negative for HIV-1 *gag* by RT–PCR using the Amplicor HIV-1 monitor kit (Roche).

Protein analyses

Red cell lysates of freshly collected peripheral blood were analysed by cellulose acetate electrophoresis (pH 8.5, Helena Laboratories). The fraction of Hbb^{hu} relative to total Hbb (Hbb^{hu} / Hbb^{hu} + Hbb^{mu}) was calculated by running four serial dilutions and measuring each band by densitometry in the linear range. To measure the fraction of peripheral blood cells expressing human β^A, smears were stained with PE-conjugated anti-mouse TER-119+ monoclonal antibody and FITC-conjugated monoclonal antibody to human haemoglobin A (EG&G WALLAC) as recommended by the manufacturer. The slides were analysed with an immunofluorescence microscope (Olympus BX60).

Red cell morphology and haematologic studies

Blood smears were stained with Wright-Giemsa. Total haemoglobin, red cell counts and

haematocrits were measured on a CBC analyser (H System, Bayer Corporation). Reticulocytes were counted after staining with New Methylene Blue, Brecher Formula (JT Baker).

Statistics

We used the non-parametric Wilcoxon sum rank test procedure to determine whether human β-globin expression or haematological parameters differed between different treatment groups. Fisher's exact test was used to determine whether two proportions in the population were equal. A low value of *P* is evidence that the two proportions are different.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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Requirement for glycogen synthase kinase-3β in cell survival and NF-κB activation

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Glycogen synthase kinase-3 (GSK-3)-α and -β are closely related protein-serine kinases, which act as inhibitory components of Wnt signalling during embryonic development and cell proliferation in adult tissues[1,2]. Insight into the physiological function of GSK-3 has emerged from genetic analysis in *Drosophila*[3,4], *Dictyostelium*[5] and yeast[6,7]. Here we show that disruption of the murine *GSK-3*β gene results in embryonic lethality caused by severe liver degeneration during mid-gestation, a phenotype consistent with excessive tumour necrosis factor (TNF) toxicity, as observed in mice lacking genes involved in the activation of the transcription factor activation NF-κB. GSK-3β-deficient embryos were rescued by inhibition of TNF using an anti-TNF-α antibody. Fibroblasts from GSK-3β-deficient embryos were hypersensitive to TNF-α and showed reduced NF-κB function. Lithium treatment (which inhibits GSK-3; refs 8, 9) sensitized wild-type fibroblasts to TNF and inhibited transactivation of NF-κB. The early steps leading to NF-κB activation (degradation of I-κB and translocation of NF-κB to the nucleus) were unaffected by the loss of GSK-3β, indicating that NF-κB is regulated by GSK-3β at the level of the transcriptional complex. Thus, GSK-3β facilitates NF-κB function.

In vertebrates, GSK-3 is crucial for the definition of the embryonic axes[10–12]. To investigate the role of GSK-3 in mammalian development, we disrupted the *GSK-3β* gene in murine 129J embryonic stem (ES) cells using a targeting vector in which the exon encoding the ATP-binding loop was deleted (Fig. 1a). Chimaeric mice derived from two independent heterozygous ES clones were back-crossed to C57BL/6J mice, and heterozygous mice were crossed to generate homozygous mutant offspring. The null mutation of *GSK-3β* was confirmed by tail DNA genotyping and Southern (Fig. 1b) and western blot (Fig. 1c) analyses of embryonic fibroblasts derived from mice on day 12.5 of gestation (E12.5). Although *GSK-3β*$^{+/-}$ male and female mice were healthy and fertile, they did not give rise to live *GSK-3β*$^{-/-}$ progeny.

We analysed embryos from timed pregnancies of *GSK-3β*$^{+/-}$ intercrosses. Before E12.5, the mendelian ratio of null embryos was normal and most *GSK-3β*$^{-/-}$ embryos showed no morphological abnormality (not shown). Between E13.5 and E14.5, however, *GSK-3β*$^{-/-}$ embryos were pale and non-viable (Fig. 2a). Histological examination of E13.5 *GSK-3β*$^{+/-}$ and *GSK-3β*$^{-/-}$ embryos showed multifocal haemorrhagic degeneration in the livers of homozygous embryos (Fig. 2b). In these areas, the hepatocytes were pyknotic and karyorrhectic, indicating that they were probably undergoing

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Appendix 1005C

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Therapeutic haemoglobin synthesis in beta-thalassaemic mice expressing lentivirus-encoded human beta-globin

C May [1], S Rivella, J Callegari, G Heller, K M Gaensler, L Luzzatto, M Sadelain

Affiliations

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Abstract

The stable introduction of a functional beta-globin gene in haematopoietic stem cells could be a powerful approach to treat beta-thalassaemia and sickle-cell disease. Genetic approaches aiming to increase normal beta-globin expression in the progeny of autologous haematopoietic stem cells might circumvent the limitations and risks of allogeneic cell transplants. However, low-level expression, position effects and transcriptional silencing hampered the effectiveness of viral transduction of the human beta-globin gene when it was linked to minimal regulatory sequences. Here we show that the use of recombinant lentiviruses enables efficient transfer and faithful integration of the human beta-globin gene together with large segments of its locus control region. In long-term recipients of unselected transduced bone marrow cells, tetramers of two murine alpha-globin and two human betaA-globin molecules account for up to 13% of total haemoglobin in mature red cells of normal mice. In beta-thalassaemic heterozygous mice higher percentages are obtained (17% to 24%), which are sufficient to ameliorate anaemia and red cell morphology. Such levels should be of therapeutic benefit in patients with severe defects in haemoglobin production.

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Globin Gene Therapy: One (Seemingly) Small Vector Change, One Giant Leap in Optimism

David Bodine

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In the July 6, 2000 issue of *Nature,* May *et al.* report the successful generation of a lentiviral vector containing a human β-globin gene that can be used to rescue a mouse model of β-thalassemia (1). This is a significant achievement for the research team lead by Michel Sadelain and brings to an end more than a decade of frustration regarding a critical step in the

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COMMENTARY

Globin Gene Therapy: One (Seemingly) Small Vector Change, One Giant Leap in Optimism

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In the July 6, 2000 issue of *Nature,* May *et al*. report the successful generation of a lentiviral vector containing a human β-globin gene that can be used to rescue a mouse model of β-thalassemia (1). This is a significant achievement for the research team lead by Michel Sadelain and brings to an end more than a decade of frustration regarding a critical step in the development of gene therapy for hemoglobin disorders. Hemoglobinopathies are relatively common disorders, allowing gene therapy to now be proposed for a large number of patients.

In the late 1980s, hemoglobinopathies were considered prime candidates for gene addition therapy. The genes were in hand, the molecular defects were understood, and several important features of globin gene regulation were known. Importantly, analyses of patients indicated that synthesis of 15–20% of the normal amount of β-globin could result in a substantial clinical improvement. Initial studies demonstrated that human β-globin genes could be inserted into oncoretroviral vectors and transduced into the hematopoietic stem cells (HSC) where they were expressed in differentiated red blood cells at low levels (2–4). At the same time, the β-globin locus control region (LCR), a 25-kb sequence containing DNase I hypersensitive sites located ~60 kb upstream of the adult β-globin gene, was shown to be required for high-level expression of β-globin genes in transgenic mice (5). The critical regions of the LCR were rapidly identified, and in this optimistic environment it seemed that the simple addition of small LCR sequences to the globin retroviral vector would allow high-level expression of a transduced globin gene.

Unfortunately, the next decade was spent demonstrating that retroviral vectors containing globin genes and LCR sequences were not able to introduce unrearranged copies of the transgene to target cells at high frequency (6). A variety of approaches were taken to solve this problem by a group of dedicated investigators. Removal of part of the second intervening sequence (IVS2) of the β-globin gene improved the stability of LCR/globin retrovirus vectors (7–9). Specific contexts of globin genes and LCR fragments also contributed to more stable retrovirus vectors (7). Leboulch *et al*. hypothesized that processing of the genomic LCR/globin retroviral RNA at cryptic sites was responsible for internal deletions of the globin gene. Mutagenesis of many of these sites improved the stability of LCR/globin retrovirus vectors (10). These improvements were sufficient to introduce unrearranged LCR/globin cassettes into mouse HSC, only to reveal a second problem. As had been observed in transgenic mice, the expression of the transferred globin genes with small LCR sequences varied depending on the position of integration in the target cell genome (position effects) (7, 11, 12). In addition, silencing of LCR/globin transgenes expressed from retrovirus vectors was frequently observed (7). Although frustrating to those observing the field, much of what was learned from these studies led to the recent success.

The development and characterization of lentivirus vectors for gene transfer (13) offered the break that the field needed, and Sadelain and co-workers took advantage of the opportunity. Sadelain recognized that one of the functions of the HIV rev protein, which is expressed during the packaging of lentivirus vectors, was to suppress splicing of the viral RNA genome. Sadelain hypothesized that the presence of rev would prevent splicing of the LCR/globin cassette. Furthermore, lentivirus vectors allow the packaging of larger constructs than oncoretroviral vectors. Sadelain hypothesized that larger LCR fragments would be more resistant to position effects as they appeared to be in transgenic mice. Finally, Sadelain noted that silencing of expression from lentivirus vectors over time was much less frequent than with comparable oncoretrovirus vectors. The research team led by Chad May linked a β-globin gene with the IVS2 sequence deletion to larger fragments of the LCR and inserted this construct into a lentivirus vector. VSV-G pseudotyped lentivirus vectors were generated and used to transduce HSC from both normal mice and mice heterozygous for the thalassemia-inducing knockout of the β-globin genes. After transduction, the HSC were transplanted into recipient mice whose endogenous marrow had been destroyed by radiation.

"These findings have made gene therapy for thalassemia a realistic goal that will be aggressively pursued."

The results described in the *Nature* paper are nothing short of outstanding. In mice repopulated with normal cells exposed to the virus, human β-globin mRNA was present at levels that were nearly 15% of the level of the endogenous mouse β-globin mRNA after adjustment for the ratio of marked to unmarked cells. This value was consistent from mouse to mouse, suggesting that no sig-

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nificant position effects were encountered and that few of the vectors were silenced. Hemoglobin tetramers consisting of two mouse α-globin chains and two human β-globin chains were easily detectable in red blood cells. In β-thalassemic mice repopulated with autologous cells exposed to the vector, the results were even more striking. Due to the reduced number of mouse β-globin chains per cell, mouse α2/human β2 tetramers represented about 25% of the hemoglobin molecules. This additional hemoglobin caused significant increases in the number of red blood cells and the amount of hemoglobin per red blood cell in the treated animals. While not entirely cured, the mouse model of β-thalassemia was clearly and significantly improved by gene therapy.

The findings by May *et al.* (1) have given a badly needed shot in the arm to globin gene therapy research and should energize the field for years to come. These findings have made gene therapy for thalassemia a realistic goal that will be aggressively pursued. As discussed in the manuscript, several problems remain to be addressed. *In vivo* selection for corrected red blood cells is not likely to be as powerful as the selection for corrected lymphocytes demonstrated in the recent successful cure of X-linked SCID (14). Therefore, for gene therapy to have a realistic chance at improving a human hemoglobinopathy, gene transfer into human HSC must become more efficient than in previous human stem cell marking trials. One idea is to use a dominant selectable marker gene such as dihydrofolate reductase in the vector and *in vivo* selection after repopulation with methotrexate (15). This concept has been successfully demonstrated in mouse models and could be combined into the Sadelain vector design. Second, the degree and method of bone marrow ablation needed to give globin gene marked stem cells a competitive advantage over their endogenous counterparts must be determined. The mouse models may be excellent vehicles to determine how much marrow ablation and how many corrected cells are required for an effective cure. Finally, it would be even better if even more β-like globin could be expressed in red blood cells. For example, if a γ-globin gene could be expressed to approximately 25% of the level of the sickle β-globin mRNA, gene therapy could be extended to sickle cell disease.

Through the dedication of Michel Sadelain and his research team, optimism about globin gene therapy has returned. It is sincerely hoped that frustration of the past 12 years will be replaced with success during the next decade.

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Appendix 1006A

Correction of this defect in the bronchial epithelial cells by CFTR gene transfer may correct the biochemical transport defect and, hence, the lung disease. Clinical trials so far have generated encouraging data but highlighted the need for more efficient, non-toxic vectors. We have developed a novel integrin-targeted synthetic vector based on integrin binding cationic peptides (I), DNA (D) and cationic liposomes (L) to form the IDL vector complex, and here we describe in vitro evaluation of the potential of this vector system for gene therapy of CF. The IDL vectors used in this report contained a peptide that targeted α 5β1 integrins. This integrin is expressed abundantly on the bronchial epithelial cell lines used in these experiments. Complexes were made at a range of charge ratios and the transfection activity of targeted complexes reached a plateau at 3:1 (N:P). Subconfluent normal and CF epithelial cell lines were transfected with the green fluorescent protein (GFP) reporter gene and transfection efficiencies of 40% to 50% were achieved. Cells were then cultured in confluent monolayers to more closely model airway structures and, as expected, the transfection efficiency of confluent cells was reduced, to approximately 5%. Pre-incubation of confluent cells with EGTA, a calcium-chelating agent that disrupts tight junctions, restored the transfection efficiency to 20% to 30 %. There was no effect of EGTA treatment on the transfection efficiency of subconfluent cells and there was no evidence that either treatment with EGTA or the transfection process itself increases the rate of mitosis in confluent monolayers. The IDL vector, therefore, transfects confluent, slow or non-dividing cells, with much higher efficiency when treated with EGTA. This treatment restores the transfection efficiency of confluent cells to about half of that seen in subconfluent, rapidly dividing cells. In this study we have demonstrated the efficiency of the IDL vector system for transfection of bronchial epithelial cells and the influence of receptor availability and rate of mitosis on transfection efficiency. The increased transfection efficiency of EGTA treated confluent cells compared to untreated confluent cells is due most likely to the breakdown of tight junctions and increased availability of receptors for the IDL vector exposed on lateral membranes, enhancing binding and uptake. The alternative hypothesis that the nuclear envelope in slowly dividing cells prevents access of plasmid DNA to the nuclear machinery is not supported by these results. These data represent a significant step towards the development of the IDL vector for gene therapy of CF.

677. The Effect of Integrin-targeting Oligoplex and Oligolipoplex Vectors on Cellular Viability

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The elucidation of novel ligands for cell targeting of both viral and non-viral gene transfer vectors is an important approach to increase both vector efficiency and safety. The desired end point of cell targeting and gene delivery in most cases is the expression of the transported gene. An undesirable consequence however, is damage to the cell or more seriously, cellular toxicity which may arise through gene delivery. We are developing non-viral delivery systems based on novel integrin targeting ligands and have previously demonstrated gene delivery mediated by a variety of integrin subtypes using the oligolysine vector $[K]_{16}$RGD. This vector allows gene transfer to cells presenting the fibronectin (α5β1 and α3β1) and vitronectin (αVβ1 and αVβ3) integrin receptors. It is effective not only when used in an oligoplex but also enhances lipofection (with a variety of liposomes) while retaining a certain degree of targeting specificity when incorporated into an oligolipoplex. We also recently elucidated a minimal binding motif of the extracellular matrix protein tenascin (PLAEIDGIELTY) which binds to the α9β1 integrin, known to be present on airway epithelial cells. Oligolysine vectors incorporating this peptide sequence were shown to deliver DNA specifically to cells expressing this integrin. As a consequence of the relatively short duration of expression achieved by plasmid based vectors, few studies have investigated the repercussions that gene delivery may have on cell vitality. Cells are generally harvested 48 hours post-transfection and destroyed during the subsequent analysis of transgene expression. Of particular significance to gene transfer studies using integrin-targeting vectors is the recent identification of integrin-αVβ3 activated apoptosis and also integrin-independent RGD-peptide induction of apoptosis by direct caspase-3 activation. We report here that the successful transfer and expression of DNA mediated by integrin-targeting vectors does not have an apoptotic effect on a variety of cell lines and has no adverse effect on cellular viability and subsequent growth parameters. Several cell lines were transfected using RGD and PLAEIDGIELTY oligopeptides complexed with plasmid DNA either alone or in combination with liposomes. Transfected cells were subsequently analysed for transgene expression or maintained in culture to investigate their viability. The apoptotic state of transfected cells was determined by several means including detection of annexin-V and analysis of the integrity of mitochondria. The disruption of the mitochondrial membrane and translocation of annexin-V to the cell surface are indicative of apoptosis. The level of cellular mortality and growth parameters were also investigated for several weeks following transfection. Transfected cells showed no sign of undergoing apoptosis or other adverse effects compared to other transfected control cells. This is likely to be due to the relatively low concentrations of integrin targeting ligands used for gene transfer compared to the concentrations which activate apoptosis. In contrast, cells exposed to high concentrations of GRGDSP peptide, staurosporine, or other apoptotic agents did undergo apoptosis. In conclusion, the viabilty of cells following transfection by integrin targeting peptides is unaffected. Integrin targeting ligands, therefore, remain viable candidates for the further development of non-viral vectors and the retargeting of viruses.

STEM CELL AND BLOOD—PRECLINICAL AND CLINICAL APPLICATIONS

678. Therapeutic Liver Reconstitution by Adult Pancreatic and Bone Marrow Hepatic Stem Cells

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In embryonic development the liver and pancreas both develop from a common premordium in the ventral foregut. Hepatocytes, bile ducts, pancreatic ducts, exocrine and endocrine pancreatic cells are all derived from this endodermal precursor. This raises the possibility that adult animals might contain cells of similar differentiation potential in pancreas and liver. Recently, the presence of hepatic precursors (stem cells) has also been suggested in adult bone marrow. Although previous work has shown the existence of pancreas and bone marrow derived cells expressing hepatocyte markers, it remained unknown whether the cells were fully functional and therefore therapeutically useful.

In order to determine whether pancreas and bone marrow of adult mouse contain cells which can give rise to fully functional hepatocytes, we transplanted suspensions of pancreatic

or bone marrow cells into syngeneic recipients deficient in fumarylacetoacetate hydrolase (FAH-) and manifesting tyrosinemia. In the murine FAH- model, transplanted wild-type hepatocytes can repopulate mutant liver. The donor cells were from old (>6 months) wild-type mice transgenic for E. coli lacZ. In independent experiments of pancreatic cell transplantation, 6/109 mutant mice were fully rescued by transplanted pancreatic cells and had normal liver function. Thirteen additional mice showed histological evidence of donor derived hepatocytes in the liver. Of the 109 recipient mice, 44 mice were transplanted with cells enriched for pancreatic duct cells. Of these only 4/44 had donor derived hepatocytes in the liver. In addition, we also cultured pancreatic duct cells in tissue culture. In 17 FAH- mice transplanted with cultured duct cells, no donor derived hepatocytes were observed. Published studies suggested that pancreatic duct cells may contain pancreatic liver precursors. Our current results are not consistent with this hypothesis.

For bone marrow transplantation, whole body irradiation of 1100 cGy was given to recipient mice and one million cells were transplanted. In a total of 24 bone marrow recipients, four mice (~17%) were rescued by transplanted bone marrow cells and showed >50% of hepatocyte repopulation by donor cells.

In summary, our results provide a proof of concept that both pancreas and bone marrow of adult mice contain hepatocyte precursors (stem cells) capable of significant therapeutic liver reconstitution. Our results also suggest the pancreatic derived liver stem cells are not the pancreatic duct cells. We are currently investigating whether the bone marrow and pancreas derived liver stem cells represent the same cell population.

679. Adenoviral Delivery of LIM Mineralization Protein-1 Successfully Induces Bone Formation *In Vivo*

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LIM Mineralization Protein-1 (LMP-1) is a novel intracellular protein that induces bone formation in vitro and in vivo. The purpose of this study was to determine the feasibility and optimal dose of adenoviral delivery of the LMP-1 cDNA to form bone in a rabbit spine fusion model. A replication deficient (E1,E3) recombinant human serotype 5 adenovirus was constructed with the LMP-1 driven by a CMV promoter. The optimal dose of AdLMP-1 to induce bone differentiation in rat calvarial osteoblast cultures was found to be at a multiplicity of infection (MOI) of 0.25 pfu of virus per cell. In vivo experiments were performed to determine if the optimal in vitro dose could promote lumbar spine fusion in skeletally mature New Zealand white rabbits. In a pilot study, 3 mL of bone marrow was aspirated from the distal femur; the buffy coat was isolated and transduced for 10 min with AdV. Single level bilateral posterolateral lumbar spine arthrodesis was performed and carrier matrix (rabbit devitalized bone matrix or collagen sponge) was implanted with each side containing 8-15x10^6 buffy coat cells transduced with either AdLMP-1 (MOI=0.4 or 0.04) or Adβgal (MOI=0.4). After 5 weeks, all 8 spine fusion sites that received AdLMP-1 (MOI=0.4) contained solid, continuous spine fusion masses. In contrast, sites receiving Adβgal or the lower dose of AdLMP-1 did not form solid fusion mass bone. A pivotal study was then performed (n=20) with several enhancements to the technique: 1) peripheral venous blood was used as a cell source instead of marrow; 2) the carrier matrix was switched to a collagen/ceramic composite sponge to prevent premature resorption; and, 3) the control cells received empty adenovirus rather than the βgal transgene. All 10 rabbits that received AdLMP-1 had solid spine fusions as determined by manual palpation, x-ray, CT scan, and biomechanical testing. In the 10 rabbits that received cells transduced with the empty adenovirus, little or no bone was formed. Histology confirmed that the bone formed was normal primary trabeculae with active osteoblasts and marrow elements. Adenovirus delivery of transgenes in vivo has been limited by immune response to the virus, toxicity from high doses, long transduction times, and inconsistent results. We showed consistent bone induction in a challenging spine fusion model. Intraoperative ex vivo gene transduction (10 min) of venous blood cells is easier than other protocols that require overnight transduction, cell selection, or cell expansion in culture. The dose of virus (MOI=0.4, 5 X 10^8 viral particles) was substantially lower than in most other gene therapy applications. The low dose required may be due to the fact that LMP-1 is an intracellular molecule and may induce potent signal amplification cascades. These data suggest that local gene therapy using adenovirus to deliver the LMP-1 cDNA is both feasible and effective and that the low virus dose required may minimize the negative effects of the immune response to the adenoviral vector.

680. Primary Neural Progenitor Cells: The Effects of FIV-mediated Transduction on Differentiation *In Vitro* and *In Vivo*

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Multipotent progenitor cells hold promise as vehicles for gene delivery of secreted proteins and for cell replacement in neurodegenerative disease. Expression of a secreted protein by these cells in vivo would be enhanced by its expression from a viral vector, however the effects of viral-mediated gene delivery on progenitor cell populations are unknown. Multipotent neuronal progenitor cells were cultured from embryonic day 15 - 18 mouse brain in EGF-containing serum-free media. Within 10 days, nestin-positive cells formed neurospheres, which were maintained over multiple passages. Neurospheres were differentiated with 1% serum and IGF-1 or B27 supplement on polyornithine substrate. Under both conditions, neurons, astrocytes and oligodendrocytes were differentiated. However, in B27, astrocyte migration was less marked, with neurons migrating beyond the astrocyte boundary. To investigate how feline immunodeficiency virus (FIV) based vectors affect the differentiation properties, neurospheres were infected with FIVeGFP or FIVß-gal (MOI approximately 10) prior to differentiation. Expression of the transgene was apparent within 24 hours, and maintained to at least twenty days (last time point tested) in vitro after transduction. Importantly,differentiation of transduced neurospheres in B27/1% serum produced neurons, astrocytes and oligodendrocytes that were similar in number to non-infected neurospheres. Approximately 60% of the cells within the sphere expressed ß-galactosidase. To test the capacity of undifferentiated FIVßgal-transduced neurospheres to survive and differentiate in vivo, they were transplanted into the striatum of C57Bl/6 mice. At ten days post-transplantation, ß-galactosidase positive cells were found in the striatum, the needle tract and migrating through the corpus callosum. Our results suggest that FIV-based vectors can infect progenitor cell populations in vitro, and that infection does not inhibit the ability of these cells to differentiate into multiple cell types. These results hold promise for the use of genetically-manipulated stem cells for CNS therapies.

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681. Enhanced Human Beta-globin Gene Expression Using Retroviral Vectors Containing Multiple Hypersensitive Sites but Persistence of Heterocellular Effects Limiting Expression

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By transplanting preselected GFP+ cells it is possible to achieve complete hematopoietic reconstitution of mice from HSC transduced with complex MSCV retroviral vectors carrying the GFP selectable marker and a genomic human ß-globin gene with its proximal promoter and hypersensitive site-2 (HS2) or hypersensitive sites-2, 3 and 4 (HS234). With this strategy, human ß-globin gene expression is obtained in peripheral red blood cells in all recipients for both vectors. These levels are higher for HS234-containing vectors (8.1% ± 1.3% human ß-globin mRNA vs 3.1% ± 1.3% for the HS2 vector relative to endogenous mouse ß-major globin) but remain subtherapeutic. For the HS2 vector, this in part appears to be due to integration site-specific effects that impact on both the absolute levels of expression and proportion of transgene expressing red blood cells (PNAS, in press). To determine if the HS234 vector is still subject to these effects, we analyzed highly erythroid primary day 12 spleen colonies derived from HS234 vector-infected bone marrow cells. FACS analysis revealed expression of both ß-globin and GFP in all cells in approximately half of the colonies tested. In the other colonies, while there was again concordance between ß-globin- and GFP-expressing cells, the proportion of transgene expressing cells was variable ranging from 19% to 69%. To assess whether these heterocellular expression patterns also occurred following transduction of HSC, mice initially transplanted with GFP+ HSC following infection with the HS234 vector were sacrificed at late times post reconstitution (5 and 9 months). Recovered bone marrow cells were then transplanted into secondary recipients to derive day 12 spleen colonies which could be analyzed at the clonal level. Proviral integration analysis allowed the identification of multiple colonies representing the clonal progeny of 9 unique HSC transduced with the HS234 vector. Strikingly these clonal progeny manifested clone-specific patterns of expression both in terms of proportion of ß-globin- and GFP-expressing cells and absolute level of expression. Approximately half of the clones yielded pancellular erythroid expression in spleen colonies; whereas the remaining clones were associated with heterocellular expression (mean 22% ± 3% GFP+ erythroid cells, range 0-60%). These findings based on GFP expression were confirmed by analysis of simultaneous Ter119, ß-globin and GFP expression in a subset of colonies. Together these data suggest that further vector modifications to overcome position effect variegationand achieve pancellular expression will be critical to reach therapeutic levels of expression.

682. High-level Erythroid-specific Gene Expression in Primary Human and Murine Hematopoietic Cells with Lentiviral Vectors

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Self-inactivating (SIN) lentiviral vectors integrate into non-dividing hematopoietic stem cells and eliminate transcription from the viral LTR promoter/enhancer without loss of viral titers(Zufferey, et al., Miyoshi, et al., 1998). This makes them safer vectors that are ideal for lineage-specific gene expression. The aim of the present study was to generate lentiviral vectors with high level erythroid-specific expression for gene therapy for hemoglobinopathies. We adopted a modular approach, generating 18 SIN-lentiviral vectors in which 4 different promoters [α-spectrin, ankyrin, β-globin and the derepressed ζ-globin (Huang, et al. 1998) promoters] were tested in combination with 4 erythroid enhancers [α-globin HS-40 (Huang, et al. 1998.), intron 8 from eALAS (I8)(Surinya, et al.1998), GATA-1 autoregulatory element (Grande, et al., 1999) and HS2 from the β-globin LCR (Leboulch et al., 1995)] with or without woodchuck hepatitis virus post regulatory element (WPRE), using the green fluorescent protein (GFP) as a reporter gene. The vectors were first screened in MEL and K562 erythroid cell lines, Jurkat T-cells and 293 non-hematopoïetic cells at equivalent MOI. While all cell lines transduced by the control CMV lentiviral vector expressed GFP, expression from vectors containing any of the erythroid elements was observed exclusively in K562 and MEL cells. Expression from the spectrin promoter was less than half that of the ankyrin promoter. The expression was greatly enhanced in the presence of two erythroid enhancers in tandem, i.e. the HS-40 and I8 or the HS-40 and GATA-1 enhancers and was greater than that from the HS2/β-promoter. These vectors were stably transmitted, as determined by genomic southern blot analyses of transduced cells. The presence of WPRE increased expression by two-three fold. We then transduced primary human bone marrow derived CD34+ cells in vitro. In four independent experiments, we identified two vectors, THKGW and I8HKGW, both containing the ankyrin promoter and either of the two enhancer pairs (GATA-1 and HS-40 or I8 and HS-40), and the WPRE, that expressed at levels higher than that seen from the HS2/βpromoter vector (containing WPRE) in the erythroid progeny of CD34+ cells, either in bulk unilineage erythroid cultures (Malik, et al. 1998) or at a clonal level in individual BFU-e. These two vectors were then tested in-vivo in mice. Sorted Sca1(+)/Lineage(-) mouse hematopoietic stem cells were transduced with THKGW and I8HKGW and reinjected into lethally irradiated recipient mice. In fully reconstituted mice 10 weeks post-transplant, we observed a high frequency of expression from both vectors in peripheral blood RBC and erythroid cells in bone marrow and spleen (upto 69%), compared with very low expression from the same vectors in myeloid and lymphoid lineages in bone marrow and blood, and in thymus(0-5%). Progress in gene therapy for hemoglobinopathies using onco-retroviral vectors has been difficult due to low titer vectors, vector instability and poor expression of β globin using the β globin regulatory elements. Our results show that modular use of erythroid-specific enhancers and promoters and inclusion of the WPRE in SIN-lentiviral vectors can result in stably transmitted, high level erythroid-specific expression cassettes for globin gene therapy.

683. Lentiviral-mediated Transfer of the Human *β*-Globin Gene and Large Locus Control Region Elements Permit Sustained Production of Therapeutic Levels of *β*-Globin in Long-term Bone Marrow Chimeras

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The stable introduction of a functional globin gene in autologous hematopoietic stem cells is a potentially powerful approach to treat ß-thalassemia and sickle cell anemia. The challenge facing this approach is to stably express high levels of the human ß- or γ-globin genes in an erythroid-specific, regulated, and sustained fashion. Until now, low level expression, position effects and transcriptional silencing have hampered the effectiveness of viral transduction of the human ß-globin gene linked to minimal regulatory sequences. We demonstrate that the use of recombinant lentiviruses enables efficient transfer and faithful integration of the human ß-globin gene together with large segments (3.2 kb) of its locus control region (LCR). Incorporation of large LCR segments encompassing hypersensitive sites 2, 3, and 4 in a vector, termed TNS9, results in a significant increase in the level of ß-globin expression relative to a vector harboring smaller previously studied LCR elements (1.0 kb), termed RNS1. Studies comparing TNS9 and RNS1 in HMBA-induced MEL cell pool populations and in single vector copy MEL cell clones

indicate both a higher mean level of human ß-globin expression by TNS9 (5-fold) and a higher fraction of positive clones (12/12 TNS9 vs. 1/10 RNS1). In long-term in vivo studies in recipients of unselected TNS9 transduced bone marrow cells (n=10), production of lentivirus–encoded ß-globin is substantially augmented, owing to an increase in both the level of globin expression (~16% of that of mouse ß-globin per gene copy, stable over a 20 week period), as shown by RNA analysis, and the fraction of red cells expressing human $ß^A$ (46 ± 29%), as demonstrated by immunostaining at 22 weeks post-transplant. Remarkably, and for the first time, we find murine α_2 : human $ß^A{}_2$ tetramers account for up to 13% of total hemoglobin in mature red cells in normal long–term bone marrow chimeras (24 weeks post transplant). Our findings establish that the large LCR fragments incorporated into the TNS9 lentiviral vector increased the probability and level of globin expression *in vitro* and *in vivo*. Furthermore, the durable expression of TNS9 suggests that this vector maybe resistant to transcriptional silencing. The levels of expression we obtained in normal mice suggested that therapeutically relevant levels of human ß-globin could be produced with vectors such as TNS9. We are currently undertaking *in vivo* studies in ß-thalassemic heterozygous mice.

684. Stable *In Vivo* Expression of Human Glucose-6-phosphate Deydrogenase (hG6PD) after Retroviral-mediated Transfer into Murine and Human Hematopoietic Stem Cells (HSC)

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G6PD is an X linked housekeeping gene which encodes a NADP-linked dehydrogenase essential in defense against oxidative stress. Clinical manifestations of G6PD deficiency, a disease highly prevalent in human populations, are mostly limited to acute hemolytic anemia upon exposure to oxidative agents. However, a subset of patients suffers from a severe chronic hemolytic anemia for which there is no radical treatment. We have constructed a set of VSV-G pseudotyped retroviral vectors harboring hG6PD driven by different LTRs: the Moloney Murine Leukemia Virus LTR (MM-G6PD); the Myeloproliferative Sarcoma Virus LTR (MPSV-G6PD); a hybrid LTR in which most of the MMLV-LTR is replaced by the hG6PD promoter (GRU5-G6PD). Using high titer preparations of these vectors and short transduction protocols we have transduced murine and human HSC. Transduced mouse HSC were transplanted into syngeneic lethally irradiated recipients. 72% of 46 transplanted mice expressed hG6PD for at least 3 months; 18 mice showed stable and life-long expression up to 20 months since BMT. HSC from 10 primary recipients (4-12 months since BMT) were transplanted into 32 secondary recipients: 28 of them expressed hG6PD for at least 2 months and 61% of these showed life-long expression up to 16 months since BMT. Two secondary recipients were used for tertiary BMT: out of 6 recipients, 5 expressed hG6PD. hG6PD expression was detected in all hematopoietic tissues from both primary and secondary recipients that showed life-long expression. In peripheral red blood cells of these mice the average total G6PD activity was twice that of untransplanted controls: this level of expression is much above the level that would prevent chronic hemolysis. Next, we have transduced human hematopoietic cells, depleted of cells expressing lineage differentiation markers, with the MPSV-G6PD. After 6 weeks in long-term culture hG6PD expression was obtained in HSC from normal donors and from G6PD deficient patients. The transduced cells were injected into 5 sub-lethally irradiated NOD/SCID mice. After 8 weeks the MPSV-G6PD provirus was detected in 18% of human hematopoietic colonies obtained from these mice. The presence of G6PD mRNA originating from the integrated provirus was detected by RT-PCR in the BM and in pools of hematopoietic colonies. In addition, enzymatically active human G6PD of proviral origin was detected in pools of hematopoietic colonies. Thus, on the strength of the most stringent pre-clinical assay currently available, we have transduced *bona fide* totipotent HSC and we have obtained therapeutically effective G6PD levels. We have now submitted to the appropriate institutional regulatory bodies a protocol for the retroviral–mediated correction of severe G6PD deficiency in human patients without myelo-ablation. This is based on the fact that the levels of G6PD we have observed ought to be sufficient to achieve selection of corrected cells: similar to that occurring naturally in females heterozygous for G6PD deficiency, who are genetics mosaics as a result of X-chromosome inactivation, and who often have normal G6PD activity in their blood.

685. Development of Preclinical Protocol for *In Vivo* Selection in Fanconi Anemia Gene Therapy

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Fanconi's Anemia (FA) is an autosomal recessive disorder characterized by birth defects, increased incidence of malignancy and progressive bone marrow failure. Bone marrow transplantation is therapeutic and therefore FA is a candidate disease for hematopoietic gene therapy. The frequent finding of somatic mosaicism in blood of FA patients has raised the question, whether wild-type bone marrow has a selective growth advantage. Since, cyclophosphamide (CPA) is already used in human FA patients for preparation during bone marrow transplantation, it could potentially be used for enhancement of human FA gene therapy. Preliminary experiments determined that the LD_{50} of this compound in FACKO mutant mice was five-fold lower than for wild type mice (80-100 mg/kg vs. 560-580 mg/kg). Therefore, FACKO mice were transplanted with 1 x 10^6 wild-type marrow cells without prior radio-ablation and were then given a single dose of 10-140 mg/kg of CPA. In contrast to untreated mice, CPA-treated mice showed a significant percentage of wild-type DNA in peripheral blood (~ 20% at the lower doses to >80% at the higher doses after 8 weeks). Also, a combination of significantly low dose of CPA and radio-ablation (10 mg/Kg CPA + 1 Gy) showed about 15% wild-type DNA in peripheral blood of transplanted FACKO mice in 8 weeks, which seems to be increasing further. Serial transplantation showed that the selection occurred at the level of hematopoietic stem cells (HSCs). Most of the groups have been followed for up to a year and the graft is stable. We next used a VSVG pseudo-typed MMLV vector expressing the human FANCC cDNA to transduce FACKO mutant marrow cells. Similar to the results of obtained with transplanted wild-type cells, a single dose of CPA produced significant selection of retrovirally corrected HSCs. We conclude that cyclophosphamide or a combination of cyclophosphamide and radio-ablation could be used to enhance stem cell selection in gene therapy of Fanconi anemia. We have also generated the FAAKO mice and currently, we are in the process of carrying out the similar in vivo selection procedures in these mice. Our preliminary data on FAAKO mice show that LD_{50} of CPA in these mice is about the same (100 – 140 mg/Kg) as in FACKO mice.

686. Discontinuation of PEG-ADA in an ADA^-SCID Patient Undergoing PBL Gene Therapy

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Gene therapy in adenosine deaminase- deficient (ADA^-) severe combined immunodeficiency (SCID) demonstrated the safety and feasibility of retroviral-mediated gene transfer into peripheral blood and hematopoietic progenitor cells. Since our first report of gene

therapy in two patients with delayed onset of disease, 6 additional patients with different ADA mutations and clinical phenotypes have been enrolled in our protocol. The results of our study, as well as of other clinical trials, have provided evidence of the long-term survival and function of transduced cells, with improvements in the immune functions. However, the therapeutic effect of gene therapy remains difficult to evaluate because patients are maintained on enzyme replacement therapy with PEG-ADA. In order to evaluate the efficacy of gene therapy with peripheral blood lymphocytes (PBL), we have recently undertaken a program of PEG-ADA tapering down and discontinuation in a patient who showed high proportion of transduced T cells which were polyclonal, functional and displayed long-term high expression of vector-derived ADA. The PEG-ADA dosage was gradually reduced from 50 to 6.2 U/Kg/week within 4 months. During this period, the patient received $1.7x10^9$ transduced PBLs in 7 doses. Based on the good clinical status, and maintenance of previous immune parameters at the end of "tapering-down", PEG-ADA was discontinued in June 1999. Following PEG-ADA discontinuation, transduced lymphocytes became the large majority of PBLs, as assessed by quantitative PCR analysis. This increase was paralleled by one log raise in intracellular PBL ADA activity, reaching the levels of her heterozygous, healthy, parents. Lymphocytes counts declined in the first month and then stabilized to the average values observed during PEG-ADA treatment at full dosage. In spite of increased ADA activity in PBL, deoxyadenosine nucleotides (dAXP) in red blood cells rose from <10 to 550-700 nmol/ml at 3-5 months after PEG-ADA activity in plasma became undetectable. TCR repertoire in freshly isolated PBL analysed by PCR remained intact. Proliferative responses to anti-CD3 mAb, anti-CD3+anti-CD28 mAbs, and PHA progressively increased to normal levels. The proportion of CD3+ T cells and NK cells was maintained in the normal range, whereas the number of peripheral blood B cells was reduced. The patient received monthly IVIg infusions to maintain Ig levels above 800 mg/dl. In summary, results of 6 months follow up from PEG-ADA discontinuation showed that transduced lymphocytes have a selective advantage of growth in vivo in a non-detoxified environment, are polyclonal, express adequate levels of vector-derived ADA and are functional at levels significantly higher than those observed during PEG-ADA treatment. Presently the patient is at home, off PEG-ADA, in good clinical conditions, with normal growth and body weight. It remains to be determined whether the transduced lymphocytes are able to ensure long-term adequate immune functions, including the capacity to mount primary immune responses. In conclusion, although the future clinical relevance of the increased levels of dAXP remains to be evaluated, these preliminary results show the feasibility and clinical efficacy of gene therapy as a therapeutic approach for ADA$^-$SCID patients who responded to PEG-ADA with an increase in circulating lymphocytes.

687. Stimulation of T-Lymphopoiesis in Thymectomized Bone Marrow Transplant (BMT) Recipients Treated with Stromal Cells Retrovirally Transduced with the IL-7 Gene

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Adult patients undergoing BMT typically have thymic damage or involution which inhibits new T lymphopoiesis. Hence, methods to stimulate the generation of new T lymphocytes may be limited by the loss of adult thymic function. Our previous analyses of transplanted mice showed that post-BMT thymopoiesis is impaired by radiation-induced loss of IL-7 producing stromal cells. We have also previously shown that injection of either IL-7 post-BMT or co-transplantation of marrow stroma cells transduced with the IL-7 gene corrects the thymic defects seen in young (6-12 week old) recipient mice. As an extreme model of the loss of thymic function, we have transplanted thymectomized mice to test the hypothesis that co-transplantation of IL-7 engineered stromal cells would permit T cell generation at extrathymic sites in the absence of thymic function. To test our hypothesis, we transduced C57/B6 marrow stromal cells with the retroviral vector JZEN hIL-7/tk neo containing the human IL-7 cDNA and the neo^R gene, or with the LN vector which only contains the neo^R gene. The IL-7 transduced stromal cells (IL-7 stroma) produced approximately 2 logs more IL-7 than non-transduced or LN stroma. Recipient C57/B6 (CD45.2) mice were thymectomized at 4 weeks of age and then received BMT 4-6 weeks later. Following 1400 cGy radiation in two fractions, the mice were co-transplanted with T-cell depleted marrow cells from congenic B6/SJL (CD45.1) donors and either the IL-7 or LN stroma. At day 28 post-BMT, the recipients were analyzed for sites where the stroma and thymocyte-like cells were present. Using EGFP co-labeling, IL-7 stroma was detected in the spleen, marrow and mesenteric lymph nodes (MLN) of the recipient mice. However, the MLN were the only sites where T lymphopoiesis occurred. In two-thirds of IL-7 stroma recipients, the MLN contained Thy-1+ cells expressing terminal deoxynucleotidyl transferase (Tdt), consistent with TCR repertoire formation in immature T progenitors. The T lymphoid progenitors expressed pre-TCRα, indicative of normal β-selection. Cells with a CD4+ CD8+ (double-positive, DP) phenotype were present in the MLN. The DP thymocyte-like cells expressed TCRα/β and a subset expressed CD69, as is normally seen during positive thymic selection. Analyses of single-positive (SP) cells showed that the CD8 was comprised of α/β heterodimers, not CD8α/α homodimers. Evidence of T lymphopoiesis was seen in occasional recipients of LN stroma, and never of the magnitude observed in the IL-7 stroma group. Mature donor-derived CD4+ and CD8+ T lymphocytes were present in the spleens of the IL-7 stroma recipients. Sixty percent of the IL-7 stroma recipients evaluated for antigen-specific function responded to immunization with sheep red blood cells (SRBC). IL-7 stroma recipients have survived for up to one year without evidence of infection, autoimmunity or lymphoproliferation. The results indicate that engineering of stromal cells by IL-7 gene therapy can stimulate the development of functional T lymphocytes, even in the absence of a thymus. IL-7 gene therapy may be useful in the stimulation of T lymphopoiesis in adults who are immunodeficient as a result of BMT, chemotherapy, HIV infection or aging.

Lysosomal Storage Diseases, CF, and Other Inherited Diseases

688. Correction of Cellular Pathology and Behavioral Deficits in Adult β-Glucuronidase-Deficient Mice after FIV Vector-Mediated Gene Transfer to Brain

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ß-glucuronidase deficiency, like other mucopolysaccharidoses, results in progressive lysosomal accumulation of glycosaminoglycans, resulting in cell, tissue and organ dysfunction, including mental deterioration. In the present study, the ß-glucuronidase-deficient mouse was used as a model to examine the ability of a

Appendix 1006B

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Stem Cell and Blood—Preclinical and Clinical Applications

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678. Therapeutic Liver Reconstitution by Adult Pancreatic and Bone Marrow Hepatic Stem Cells

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678. Therapeutic Liver Reconstitution by Adult Pancreatic and Bone Marrow Hepatic Stem Cells

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In embryonic development the liver and pancreas both develop from a common premordium in the ventral foregut. Hepatocytes, bile ducts, pancreatic ducts, exocrine and endocrine pancreatic cells are all derived from this endodermal precursor. This raises the possibility that adult animals might contain cells of similar differentiation potential in pancreas and liver. Recently, the presence of hepatic precursors (stem cells) has also been suggested in adult bone marrow. Although previous work has shown the existence of pancreas and bone marrow derived cells expressing hepatocyte markers, it remained unknown whether the cells were fully functional and therefore therapeutically useful.

In order to determine whether pancreas and bone marrow of adult mouse contain cells which can give rise to fully functional hepatocytes, we transplanted suspensions of pancreatic or bone v cells into syngeneic recipients deficient in fumarylacetoacetate hydrolase (I an

manifesting tyrosinemia. In the murine FAH- model, transplanted wild-type hepatocytes can repopulate mutant liver. The donor cells were from old (>6 months) wild-type mice transgenic for E. coli lacZ. In independent experiments of pancreatic cell transplantation, 6/109 mutant mice were fully rescued by transplanted pancreatic cells and had normal liver function. Thirteen additional mice showed histological evidence of donor derived hepatocytes in the liver. Of the 109 recipient mice, 44 mice were transplanted with cells enriched for pancreatic duct cells. Of these only 4/44 had donor derived hepatocytes in the liver. In addition, we also cultured pancreatic duct cells in tissue culture. In 17 FAH- mice transplanted with cultured duct cells, no donor derived hepatocytes were observed. Published studies suggested that pancreatic duct cells may contain pancreatic liver precursors. Our current results are not consistent with this hypothesis.

For bone marrow transplantation, whole body irradiation of 1100 cGy was given to recipient mice and one million cells were transplanted. In a total of 24 bone marrow recipients, four mice (~17%) were rescued by transplanted bone marrow cells and showed > 50% of hepatocyte repopulation by donor cells.

In summary, our results provide a proof of concept that both pancreas and bone marrow of adult mice contain hepatocyte precursors (stem cells) capable of significant therapeutic liver reconstitution. Our results also suggest the pancreatic derived liver stem cells are not the pancreatic duct cells. We are currently investigating whether the bone marrow and pancreas derived liver stem cells represent the same cell population.

679. Adenoviral Delivery of LIM Mineralization Protein-1 Successfully Induces Bone Formation *In Vivo*

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LIM Mineralization Protein-1 (LMP-1) is a novel intracellular protein that induces bone formation in vitro and in vivo. The purpose of this study was to determine the feasibility and optimal dose of adenoviral delivery of the LMP-1 cDNA to form bone in a rabbit spine fusion model. A replication deficient (E1,E3) recombinant human serotype 5 adenovirus was constructed with the LMP-1 driven by a CMV promoter. The optimal dose of AdLMP-1 to induce bone differentiation in rat calvarial osteoblast cultures was found to be at a multiplicity of infection (MOI) of 0.25 pfu of virus per cell. In vivo experiments were performed to determine if the optimal in vitro dose could promote lumbar spine fusion in skeletally mature New Zealand white rabbits. In a pilot study, 3 mL of bone marrow was aspirated from the distal femur; the buffy coat was isolated and transduced for 10 min with AdV. Single level bilateral posterolateral lumbar spine arthrodesis was performed and carrier matrix (rabbit devitalized bone matrix or collagen sponge) was implanted with each side containing 8-15×10^6 buffy coat cells transduced with either AdLMP-1 (MOI=0.4 or 0.04) or Adβgal (MOI=0.4). After 5 weeks, all 8 spine fusion sites that received AdLMP-1 (MOI=0.4) contained solid, continuous spine fusion masses. In contrast, sites receiving Adβgal or the lower dose of AdLMP-1 did not form solid fusion mass bone. A pivotal study was then performed (n=20) with several enhancements to the technique: 1) peripheral venous blood was used as a cell source instead of marrow; 2) the carrier matrix was switched to a collagen/ceramic composite sponge to prevent premature resorption; and, 3) the control cells received empty adenovirus rather than the βgal transgene. All 10 rabbits that received AdLMP-1 had solid spine fusions as determined by manual palpation, x-ray, CT scan, and biomechanical testing. In the 10 rabbits that received cells transduced with the empty adenovirus, little or no bone was formed. Histology confirmed that the bone formed was normal primary trabeculae with active osteoblasts and marrow elements. Adenovirus delivery of transgenes in vivo has been limited by immune response to the virus, toxicity from high doses, long transduction times, and inconsistent results. We showed consistent bone induction in a challenging spine fusion model. Intraoperative ex vivo gene transduction (10 min) of venous blood cells is easier than other protocols that require overnight transduction, cell selection, or cell expansion in culture. The dose of virus (MOI=0.4, 5 X 10^8 viral particles) was substantially lower than in most other gene therapy applications. The low dose required may be due to the fact that LMP-1 is an intracellular molecule and may induce potent signal amplification cascades. These data suggest that local gene therapy using adenovirus to deliver the LMP-1 cDNA is both feasible and effective and that the low virus dose required may minimize the negative effects of the immune response to the adenoviral vector.

680. Primary Neural Progenitor Cells: The Effects of FIV-mediated Transduction on ...entiation *In Vitro* and *In Vivo*

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Multipotent progenitor cells hold promise as vehicles for gene delivery of secreted proteins and for cell replacement in neurodegenerative disease. Expression of a secreted protein by these cells in vivo would be enhanced by its expression from a viral vector, however the effects of viral-mediated gene delivery on progenitor cell populations are unknown. Multipotent neuronal progenitor cells were cultured from embryonic day 15 – 18 mouse brain in EGF-containing serum-free media. Within 10 days, nestin-positive cells formed neurospheres, which were maintained over multiple passages. Neurospheres were differentiated with 1% serum and IGF-1 or B27 supplement on polyornithine substrate. Under both conditions, neurons, astrocytes and oligodendrocytes were differentiated. However, in B27, astrocyte migration was less marked, with neurons migrating beyond the astrocyte boundary. To investigate how feline immunodeficiency virus (FIV) based vectors affect the differentiation properties, neurospheres were infected with FIVeGFP or FIVβ-gal (MOI approximately 10) prior to differentiation. Expression of the transgene was apparent within 24 hours, and maintained to at least twenty days (last time point tested) in vitro after transduction. Importantly, differentiation of transduced neurospheres in B27/1% serum produced neurons, astrocytes and oligodendrocytes that were similar in number to non-infected neurospheres. Approximately 60% of the cells within the sphere expressed β-galactosidase. To test the capacity of undifferentiated FIVβgal-transduced neurospheres to survive and differentiate in vivo, they were transplanted into the striatum of C57Bl/6 mice. At ten days post-transplantation, β-galactosidase positive cells were found in the striatum, the needle tract and migrating through the corpus callosum. Our results suggest that FIV-based vectors can infect progenitor cell populations in vitro, and that infection does not inhibit the ability of these cells to differentiate into multiple cell types. These results hold promise for the use of genetically-manipulated stem cells for CNS therapies.

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Multiple Hypersensitive Sites but Persistence of Heterocellular Effects Limiting Expression

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By transplanting preselected GFP+ cells it is possible to achieve complete hematopoietic reconstitution of mice from HSC transduced with complex MSCV retroviral vectors carrying the GFP selectable marker and a genomic human β-globin gene with its proximal promoter and hypersensitive site-2 (HS2) or hypersensitive sites-2, 3 and 4 (HS234). With this strategy, human β-globin gene expression is obtained in peripheral red blood cells in all recipients for both vectors. These levels are higher for HS234-containing vectors (8.1% ± 1.3% human β-globin mRNA vs 3.1% ± 1.3% for the HS2 vector relative to endogenous mouse β-major globin) but remain subtherapeutic. For the HS2 vector, this in part appears to be due to integration site-specific effects that impact on both the absolute levels of expression and proportion of transgene expressing red blood cells (PNAS, in press). To determine if the HS234 vector is still subject to these effects, we analyzed highly erythroid primary day 12 spleen colonies derived from HS234 vector-infected bone marrow cells. FACS analysis revealed expression of both β-globin and GFP in all cells in approximately half of the colonies tested. In the other colonies, while there was again concordance between β-globin- and GFP-expressing cells, the proportion of transgene expressing cells was variable ranging from 19% to 69%. To assess whether these heterocellular expression patterns also occurred following transduction of HSC, mice initially transplanted with GFP+ HSC following infection with the HS234 vector were sacrificed at late times post reconstitution (5 and 9 months). Recovered bone marrow cells were then transplanted into secondary recipients to derive day 12 spleen colonies which could be analyzed at the clonal level. Proviral integration analysis allowed the identification of multiple colonies representing the clonal progeny of 9 unique HSC transduced with the HS234 vector. Strikingly these clonal [illegible]y manifested clone-specific patterns of expression both in terms of proportion of β-globin- [illegible]FP-expressing cells and absolute level of expression. Approximately half of t[illegible]

yielded pancellular erythroid expression in spleen colonies; whereas the remaining clones were associated with heterocellular expression (mean 22% ± 3% GFP+ erythroid cells, range 0-60%). These findings based on GFP expression were confirmed by analysis of simultaneous Ter119, β-globin and GFP expression in a subset of colonies. Together these data suggest that further vector modifications to overcome position effect variegationand achieve pancellular expression will be critical to reach therapeutic levels of expression.

682. High-level Erythroid-specific Gene Expression in Primary Human and Murine Hematopoietic Cells with Lentiviral Vectors

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Self-inactivating (SIN) lentiviral vectors integrate into non-dividing hematopoietic stem cells and eliminate transcription from the viral LTR promoter/enhancer without loss of viral titers(Zufferey, et al., Miyoshi, et al., 1998). This makes them safer vectors that are ideal for lineage-specific gene expression. The aim of the present study was to generate lentiviral vectors with high level erythroid-specific expression for gene therapy for hemoglobinopathies. We adopted a modular approach, generating 18 SIN-lentiviral vectors in which 4 different promoters [α-spectrin, ankyrin, β-globin and the dere-pressed ζ-globin (Huang, et al. 1998) promoters] were tested in combination with 4 erythroid enhancers [α-globin HS-40 (Huang, et al. 1998.), intron 8 from eALAS (I8)(Surinya, et al.1998), GATA-1 autoregulatory element (Grande, et al., 1999) and HS2 from the β-globin LCR (Leboulch et al., 1995)] with or without woodchuck hepatitis virus post regulatory element (WPRE), using the green fluorescent protein (GFP) as a reporter gene. The vectors were first screened in MEL and K562 erythroid cell lines, Jurkat T-cells and 293 non-hematopoïetic cells at equivalent MOI. While all cell lines transduced by the control CMV lentiviral vector expressed GFP, expression from vectors containing any of the erythroid elements was observed exclusively in K562 and MEL cells. Expression from the spectrin promoter was less than half that of the ankyrin promoter. The expression was greatly enhanced in the presence of two erythroid enhancers in tandem, i.e. the HS-40 and I8 or the HS-40 and GATA-1 enhancers and was greater than that from the HS2/β-promoter. These vectors were stably transmitted, as determined by genomic southern blot analyses of transduced cells. The presence of WPRE increased expression by two-three fold. We then transduced primary human bone marrow derived CD34+ cells in vitro. In four independent experiments, we identified two vectors, THKGW and HKGW, both containing the ankyrin promoter and either of the two enhancer pairs (GATA-1 HS-40 or I8 and HS-40), and the WPRE, that expressed at levels higher than t

the HS2/βpromoter vector (containing WPRE) in the erythroid progeny of CD34+ cells, either in bulk unilineage erythroid cultures (Malik, et al. 1998) or at a clonal level in individual BFU-e. These two vectors were then tested in-vivo in mice. Sorted Sca1(+)/Lineage(-) mouse hematopoietic stem cells were transduced with THKGW and I8HKGW and reinjected into lethally irradiated recipient mice. In fully reconstituted mice 10 weeks post-transplant, we observed a high frequency of expression from both vectors in peripheral blood RBC and erythroid cells in bone marrow and spleen (upto 69%), compared with very low expression from the same vectors in myeloid and lymphoid lineages in bone marrow and blood, and in thymus(0-5%). Progress in gene therapy for hemoglobinopathies using onco-retroviral vectors has been difficult due to low titer vectors, vector instability and poor expression of β globin using the β globin regulatory elements. Our results show that modular use of erythroid-specific enhancers and promoters and inclusion of the WPRE in SIN-lentiviral vectors can result in stably transmitted, high level erythroid-specific expression cassettes for globin gene therapy.

683. Lentiviral-mediated Transfer of the Human *β*-Globin Gene and Large Locus Control Region Elements Permit Sustained Production of Therapeutic Levels of *β*-Globin in Long-term Bone Marrow Chimeras

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The stable introduction of a functional globin gene in autologous hematopoietic stem cells is a potentially powerful approach to treat β-thalassemia and sickle cell anemia. The challenge facing this approach is to stably express high levels of the human β- or γ-globin genes in an erythroid-specific, regulated, and sustained fashion. Until now, low level expression, position effects and transcriptional silencing have hampered the effectiveness of viral transduction of the human β-globin gene linked to minimal regulatory sequences. We demonstrate that the use of recombinant lentiviruses enables efficient transfer and faithful integration of the human β-globin gene together with large segments (3.2 kb) of its locus control region (LCR). Incorporation of large LCR segments encompassing hypersensitive sites 2, 3, and 4 in a vector, termed TNS9, results in a significant increase in the level of β-globin expression relative to a vector harboring smaller previously studied LCR elements (1.0 kb), termed RNS1. Studies comparing TNS9 and in HMBA-induced MEL cell pool populations and in single vector copy MEL c ne e both a higher mean level of human β-globin expression by TNS9 (5-fold) a igh

fraction of positive clones (12/12 TNS9 vs. 1/10 RNS1). In long-term in vivo studies in recipients of unselected TNS9 transduced bone marrow cells (n=10), production of lentivirus–encoded β-globin is substantially augmented, owing to an increase in both the level of globin expression (~16% of that of mouse β-globin per gene copy, stable over a 20 week period), as shown by RNA analysis, and the fraction of red cells expressing human β^A (46 ± 29%), as demonstrated by immunostaining at 22 weeks post-transplant. Remarkably, and for the first time, we find murine α_2 : human $\beta^A{}_2$ tetramers account for up to 13% of total hemoglobin in mature red cells in normal long–term bone marrow chimeras (24 weeks post transplant). Our findings establish that the large LCR fragments incorporated into the TNS9 lentiviral vector increased the probability and level of globin expression *in vitro* and *in vivo.* Furthermore, the durable expression of TNS9 suggests that this vector maybe resistant to transcriptional silencing. The levels of expression we obtained in normal mice suggested that therapeutically relevant levels of human β-globin could be produced with vectors such as TNS9. We are currently undertaking *in vivo* studies in β-thalassemic heterozygous mice.

684. Stable *In Vivo* Expression of Human Glucose-6-phosphate Deydrogenase (hG6PD) after Retroviral-mediated Transfer into Murine and Human Hematopoietic Stem Cells (HSC)

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G6PD is an X linked housekeeping gene which encodes a NADP-linked dehydrogenase essential in defense against oxidative stress. Clinical manifestations of G6PD deficiency, a disease highly prevalent in human populations, are mostly limited to acute hemolytic anemia upon exposure to oxidative agents. However, a subset of patients suffers from a severe chronic hemolytic anemia for which there is no radical treatment. We have constructed a set of VSV-G pseudotyped retroviral vectors harboring hG6PD driven by different LTRs: the Moloney Murine Leukemia Virus LTR (MM-G6PD); the Myeloproliferative Sarcoma Virus LTR (MPSV-G6PD); a hybrid LTR in which most of the MMLV-LTR is replaced by the hG6PD promoter (GRU5-G6PD). Using high titer preparations of these vectors and short transduction protocols we have transduced murine and human HSC. Transduced mouse HSC were transplanted into syngeneic lethally irradiated recipients. 72% of 46 transplanted mice expressed hG6PD for at least 3 [illegible]s; 18 mice showed stable and life-long expression up to 20 months since BM [illegible] f

10 primary recipients (4-12 months since BMT) were transplanted into 32 secondary recipients: 28 of them expressed hG6PD for at least 2 months and 61% of these showed life-long expression up to 16 months since BMT. Two secondary recipients were used for tertiary BMT: out of 6 recipients, 5 expressed hG6PD. hG6PD expression was detected in all hematopoietic tissues from both primary and secondary recipients that showed life-long expression. In peripheral red blood cells of these mice the average total G6PD activity was twice that of untransplanted controls: this level of expression is much above the level that would prevent chronic hemolysis. Next, we have transduced human hematopoietic cells, depleted of cells expressing lineage differentiation markers, with the MPSV-G6PD. After 6 weeks in long-term culture hG6PD expression was obtained in HSC from normal donors and from G6PD deficient patients. The transduced cells were injected into 5 sub-lethally irradiated NOD/SCID mice. After 8 weeks the MPSV-G6PD provirus was detected in 18% of human hematopoietic colonies obtained from these mice. The presence of G6PD mRNA originating from the integrated provirus was detected by RT-PCR in the BM and in pools of hematopoietic colonies. In addition, enzymatically active human G6PD of proviral origin was detected in pools of hematopoietic colonies. Thus, on the strength of the most stringent pre-clinical assay currently available, we have transduced *bona fide* totipotent HSC and we have obtained therapeutically effective G6PD levels. We have now submitted to the appropriate institutional regulatory bodies a protocol for the retroviral–mediated correction of severe G6PD deficiency in human patients without myelo-ablation. This is based on the fact that the levels of G6PD we have observed ought to be sufficient to achieve selection of corrected cells: similar to that occurring naturally in females heterozygous for G6PD deficiency, who are genetics mosaics as a result of X-chromosome inactivation, and who often have normal G6PD activity in their blood.

685. Development of Preclinical Protocol for *In Vivo* Selection in Fanconi Anemia Gene Therapy

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Fanconi's Anemia (FA) is an autosomal recessive disorder characterized by birth defects, sed incidence of malignancy and progressive bone marrow failure. Bone mar antation is therapeutic and therefore FA is a candidate disease for hematopc ıer

therapy. The frequent finding of somatic mosaicism in blood of FA patients has raised the question, whether wild-type bone marrow has a selective growth advantage. Since, cyclophosphamide (CPA) is already used in human FA patients for preparation during bone marrow transplantation, it could potentially be used for enhancement of human FA gene therapy. Preliminary experiments determined that the LD_{50} of this compound in FACKO mutant mice was five-fold lower than for wild type mice (80-100 mg/kg vs. 560-580 mg/kg). Therefore, FACKO mice were transplanted with 1×10^6 wild-type marrow cells without prior radio-ablation and were then given a single dose of 10-140 mg/kg of CPA. In contrast to untreated mice, CPA-treated mice showed a significant percentage of wild-type DNA in peripheral blood (~20% at the lower doses to > 80% at the higher doses after 8 weeks). Also, a combination of significantly low dose of CPA and radio-ablation (10 mg/Kg CPA + 1 Gy) showed about 15% wild-type DNA in peripheral blood of transplanted FACKO mice in 8 weeks, which seems to be increasing further. Serial transplantation showed that the selection occurred at the level of hematopoietic stem cells (HSCs). Most of the groups have been followed for up to a year and the graft is stable. We next used a VSVG pseudo-typed MMLV vector expressing the human FANCC cDNA to transduce FACKO mutant marrow cells. Similar to the results of obtained with transplanted wild-type cells, a single dose of CPA produced significant selection of retrovirally corrected HSCs. We conclude that cyclophosphamide or a combination of cyclophosphamide and radio-ablation could be used to enhance stem cell selection in gene therapy of Fanconi anemia. We have also generated the FAAKO mice and currently, we are in the process of carrying out the similar in vivo selection procedures in these mice. Our preliminary data on FAAKO mice show that LD_{50} of CPA in these mice is about the same (100 – 140 mg/Kg) as in FACKO mice.

686. Discontinuation of PEG-ADA in an ADA$^-$SCID Patient Undergoing PBL Gene Therapy

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herapy in adenosine deaminase- deficient (ADA$^-$) severe combined immuno nc

(SCID) demonstrated the safety and feasibility of retroviral-mediated gene transfer into peripheral blood and hematopoietic progenitor cells. Since our first report of gene therapy in two patients with delayed onset of disease, 6 additional patients with different ADA mutations and clinical phenotypes have been enrolled in our protocol. The results of our study, as well as of other clinical trials, have provided evidence of the long-term survival and function of transduced cells, with improvements in the immune functions. However, the therapeutic effect of gene therapy remains difficult to evaluate because patients are maintained on enzyme replacement therapy with PEG-ADA. In order to evaluate the efficacy of gene therapy with peripheral blood lymphocytes (PBL), we have recently undertaken a program of PEG-ADA tapering down and discontinuation in a patient who showed high proportion of transduced T cells which were polyclonal, functional and displayed long-term high expression of vector-derived ADA. The PEG-ADA dosage was gradually reduced from 50 to 6.2 U/Kg/week within 4 months. During this period, the patient received 1.7×10^9 transduced PBLs in 7 doses. Based on the good clinical status, and maintenance of previous immune parameters at the end of “tapering-down”, PEG-ADA was discontinued in June 1999. Following PEG-ADA discontinuation, transduced lymphocytes became the large majority of PBLs, as assessed by quantitative PCR analysis. This increase was paralleled by one log raise in intracellular PBL ADA activity, reaching the levels of her heterozygous, healthy, parents. Lymphocytes counts declined in the first month and then stabilized to the average values observed during PEG-ADA treatment at full dosage. In spite of increased ADA activity in PBL, deoxyadenosine nucleotides (dAXP) in red blood cells rose from <10 to 550-700 nmol/ml at 3-5 months after PEG-ADA activity in plasma became undetectable. TCR repertoire in freshly isolated PBL analysed by PCR remained intact. Proliferative responses to anti-CD3 mAb, anti-CD3+anti-CD28 mAbs, and PHA progressively increased to normal levels. The proportion of CD3+ T cells and NK cells was maintained in the normal range, whereas the number of peripheral blood B cells was reduced. The patient received monthly IVIg infusions to maintain Ig levels above 800 mg/dl. In summary, results of 6 months follow up from PEG-ADA discontinuation showed that transduced lymphocytes have a selective advantage of growth in vivo in a non-detoxified environment, are polyclonal, express adequate levels of vector-derived ADA and are functional at levels significantly higher than those observed during PEG-ADA treatment. Presently the patient is at home, off PEG-ADA, in good clinical conditions, with normal growth and body weight. It remains to be determined whether the transduced lymphocytes are able to ensure long-term adequate immune functions, including the capacity to mount primary immune responses. In conclusion, although the future clinical relevance of the increased levels of remains to be evaluated, these preliminary results show the feasibility and clinical efficacy e therapy as a therapeutic approach for ADA SCID patients who responded t

with an increase in circulating lymphocytes.

687. Stimulation of T-Lymphopoiesis in Thymectomized Bone Marrow Transplant (BMT) Recipients Treated with Stromal Cells Retrovirally Transduced with the IL-7 Gene

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Adult patients undergoing BMT typically have thymic damage or involution which inhibits new T lymphopoiesis. Hence, methods to stimulate the generation of new T lymphocytes may be limited by the loss of adult thymic function. Our previous analyses of transplanted mice showed that post-BMT thymopoiesis is impaired by radiation-induced loss of IL-7 producing stromal cells. We have also previously shown that injection of either IL-7 post-BMT or co-transplantation of marrow stroma cells transduced with the IL-7 gene corrects the thymic defects seen in young (6-12 week old) recipient mice. As an extreme model of the loss of thymic function, we have transplanted thymectomized mice to test the hypothesis that co-transplantation of IL-7 engineered stromal cells would permit T cell generation at extrathymic sites in the absence of thymic function. To test our hypothesis, we transduced C57/B6 marrow stromal cells with the retroviral vector JZEN hIL-7/tk neo containing the human IL-7 cDNA and the neo^R gene, or with the LN vector which only contains the neo^R gene. The IL-7 transduced stromal cells (IL-7 stroma) produced approximately 2 logs more IL-7 than non-transduced or LN stroma. Recipient C57/B6 (CD45.2) mice were thymectomized at 4 weeks of age and then received BMT 4-6 weeks later. Following 1400 cGy radiation in two fractions, the mice were co-transplanted with T-cell depleted marrow cells from congenic B6/SJL (CD45.1) donors and either the IL-7 or LN stroma. At day 28 post-BMT, the recipients were analyzed for sites where the stroma and thymocyte-like cells were present. Using EGFP co-labeling, IL-7 stroma was detected in the spleen, marrow and mesenteric lymph nodes (MLN) of the recipient mice. However, the MLN were the only sites where T lymphopoiesis occurred. In two-thirds of IL-7 stroma recipients, the MLN contained Thy-1 + cells expressing terminal deoxynucleotidyl transferase (Tdt), consistent with TCR repertoire formation in immature T progenitors. The T lymphoid progenitors expressed pre-TCRα, indicative of normal β-selection. Cells with a CD4 + CD8+ (double-positive, DP) phenotype were present in the MLN. The DP thymocyte-like cells expressed TCRa/β and a subset expressed CD69, as is normally seen during positive thymic selection. Analyses of single-positive (SP) cells showed that the CD8 was comprised of α/β heterodimers, not CD8a/a imers. Evidence of T lymphopoiesis was seen in occasional recipients of LN a,

never of the magnitude observed in the IL-7 stroma group. Mature donor-derived CD4+ and CD8+ T lymphocytes were present in the spleens of the IL-7 stroma recipients. Sixty percent of the IL-7 stroma recipients evaluated for antigen-specific function responded to immunization with sheep red blood cells (SRBC). IL-7 stroma recipients have survived for up to one year without evidence of infection, autoimmunity or lymphoproliferation. The results indicate that engineering of stromal cells by IL-7 gene therapy can stimulate the development of functional T lymphocytes, even in the absence of a thymus. IL-7 gene therapy may be useful in the stimulation of T lymphopoiesis in adults who are immunodeficient as a result of BMT, chemotherapy, HIV infection or aging.

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