ORIGINAL

Dan Lawton (State Bar No. 127342)
Joseph C. Kracht (State Bar No. 228507)
LAWTON LAW FIRM
550 West C Street, Suite 1400
San Diego, CA 92101
(619) 595-1370
(619) 595-1520 (Telefacsimile Number)

Attorneys for Plaintiff AntiCancer, Inc.

UNITED STATES DISTRICT COURT

FOR THE SOUTHERN DISTRICT OF CALIFORNIA

'07 CV 1004 JAH NLS

ANTICANCER, INC., a California corporation,

Plaintiff,

v.

EASTMAN KODAK COMPANY, a New Jersey corporation; and DOES 1-50,

Defendants.

Case No.

COMPLAINT FOR DAMAGES AND PRELIMINARY AND PERMANENT INJUNCTIONS FOR INFRINGEMENT OF U.S. PATENTS NOS. 6,232,523, 6,235,968, 6,251,384, 6,649,159, 6,759,038, AND 6,905,831; DEMAND FOR TRIAL BY JURY AND FOR SPEEDY HEARING

JURISDICTION AND VENUE

1. This action for declaratory judgment and for patent infringement arises under the patent laws of the United States, Title 35 of the United States Code, and under 28 U.S.C. § 2201 and Fed. R. Civ. P. 57.

2. This Court has subject matter jurisdiction under 28 U.S.C. §§ 1331, 1338(a), and 2201.

3. Venue is proper in this judicial district under pertinent law, including, *inter alia*, 28 U.S.C. §§ 1391(b), (c).

THE PARTIES

4. Plaintiff is a corporation organized and existing under the laws of the State of California and having as its principal place of business San Diego, California.

5. Defendant Eastman Kodak Company (hereinafter "Kodak") is a corporation organized and existing under the laws of the State of New Jersey and having as its principal places of business in various locations, including without limitation Rochester, New York.

6. The true names and capacities, whether individual, corporate, associate, representative or otherwise, of DOES 1 through 50, inclusive, are unknown to plaintiff, who therefore sues them by such fictitious names. Plaintiff will seek leave to amend this complaint to show the true names and capacities of said defendants when they are ascertained. Plaintiff is informed and believes, and thereupon alleges, that each of the defendants named as a Doe, along with the named defendants, is responsible in some manner for the occurrences herein alleged, and that plaintiff's injuries herein alleged were legally or proximately caused by said defendants. Wherever it is alleged that any act or omission was also done or committed by any specifically named defendant, or by defendants generally, plaintiff intends thereby to allege, and does allege, that the same act or omission was also done and committed by each and every defendant named as a Doe, and each named defendant, both separately and in concert or conspiracy with the named defendants.

7. At all times mentioned herein, defendants, and each of them, were the agents, servants, co-conspirators, or employees of one another, and the acts and omissions herein alleged were done or suffered by them, acting individually and through or by their alleged capacity, within the scope of their authority. Each of the defendants aided and abetted and rendered substantial assistance in the accomplishment of the acts complained of herein. In taking the actions, as particularized herein, to aid and abet and substantially assist in the commission of the misconduct complained of, each defendant acted with an awareness of his, her or its primary wrongdoing and realized that his, her or its conduct would substantially assist in the accomplishment of that misconduct and was aware of his, her or its overall contribution to, and furtherance of the conspiracy, common enterprise, and common course

of conduct. Defendants' acts of aiding and abetting included, *inter alia*, all of the acts each defendant is alleged to have committed in furtherance of the conspiracy, common enterprise, and common course of conduct complained of herein.

FIRST CLAIM FOR RELIEF

(Infringement of Patent)

(Against All Defendants)

8. Plaintiff realleges and incorporates by reference as though fully set forth preceding paragraphs 1 through 7.

9. United States Patent No. 6,232,523 (the "'523 Patent") issued on May 15, 2001. A true and correct copy of the '523 Patent is attached hereto as Exhibit 1 and incorporated herein by this reference.

10. Plaintiff is the sole owner of the '523 Patent.

11. Plaintiff is informed and believes that Kodak has infringed, and still is infringing, the '523 patent by making, using, selling, offering for sale and/or licensing products and services covered by one or more claims of the '523 Patent without plaintiff's authorization or consent. These products and services include, *inter alia*, Kodak's Image Station imaging systems (fully-integrated systems allowing researchers to use real-time imaging technology, including plaintiff's patented processes, to monitor and record cellular and genetic activity within laboratory animals).

12. Plaintiff is informed and believes that Kodak has infringed the '523 Patent, and will continue to do so unless enjoined by this Court.

13. Plaintiff is informed and believes, and on that basis, alleges that Kodak is aware of the '523 Patent and that its infringement has been willful.

14. Plaintiff is informed and believes that Kodak is actively inducing and/or contributing to infringement of the '523 Patent by others, all of whom are sued herein as DOES 1 through 50. Plaintiff will seek leave to amend this complaint to show the true names and capacities of said defendants when they are ascertained.

15. By reason of the foregoing, plaintiff has suffered damages in an amount to be proven at trial and, in addition, has suffered irreparable loss and injury.

16. The acts of infringement described above are willful, deliberate and in reckless disregard of plaintiff's patent rights.

SECOND CLAIM FOR RELIEF

(Infringement of Patent)

(Against All Defendants)

17. Plaintiff realleges and incorporates by reference as though fully set forth preceding paragraphs 1 through 16.

18. United States Patent No. 6,759,038 (the "'038 Patent") issued on July 6, 2004. A true and correct copy of the '038 Patent is attached hereto as Exhibit 2 and incorporated herein by this reference.

19. Plaintiff is the sole owner of the '038 Patent.

20. Plaintiff is informed and believes that Kodak has infringed, and still is infringing, the '038 patent by making, using, selling, offering for sale and/or licensing products and services covered by one or more claims of the '038 Patent without plaintiff's authorization or consent. These products and services include, *inter alia*, Kodak's Image Station imaging systems (fully-integrated systems allowing researchers to use real-time imaging technology, including plaintiff's patented processes, to monitor and record cellular and genetic activity within laboratory animals).

21. Plaintiff is informed and believes that Kodak has infringed the '038 Patent, and will continue to do so unless enjoined by this Court.

22. Plaintiff is informed and believes, and on that basis, alleges that Kodak is aware of the '038 Patent and that its infringement has been willful.

23. Plaintiff is informed and believes that Kodak is actively inducing and/or contributing to infringement of the '038 Patent by others, all of whom are sued herein as DOES 1 through 50. Plaintiff will seek leave to amend this complaint to show the true names and capacities of said defendants when they are ascertained.

24. By reason of the foregoing, plaintiff has suffered damages in an amount to be proven at trial and, in addition, has suffered irreparable loss and injury.

25. The acts of infringement described above are willful, deliberate and in reckless disregard of plaintiff's patent rights.

THIRD CLAIM FOR RELIEF

(Infringement of Patent)

(Against All Defendants)

26. Plaintiff realleges and incorporates by reference as though fully set forth preceding paragraphs 1 through 25.

27. United States Patent No. 6,235,968 (the "'968 Patent") issued on May 22, 2001. A true and correct copy of the '968 Patent is attached hereto as Exhibit 3 and incorporated herein by this reference.

28. Plaintiff is the sole owner of the '968 Patent.

29. Plaintiff is informed and believes that Kodak has infringed, and still is infringing, the '968 patent by making, using, selling, offering for sale and/or licensing products and services covered by one or more claims of the '968 Patent without plaintiff's authorization or consent. These products and services include, *inter alia*, Kodak's Image Station imaging systems (fully-integrated systems allowing researchers to use real-time imaging technology, including plaintiff's patented processes, to monitor and record cellular and genetic activity within laboratory animals).

30. Plaintiff is informed and believes that Kodak has infringed the '968 Patent, and will continue to do so unless enjoined by this Court.

31. Plaintiff is informed and believes, and on that basis, alleges that Kodak is aware of the '968 Patent and that its infringement has been willful.

32. Plaintiff is informed and believes that Kodak is actively inducing and/or contributing to infringement of the '968 Patent by others, all of whom are sued herein as DOES 1 through 50. Plaintiff will seek leave to amend this complaint to show the true names and capacities of said defendants when they are ascertained.

33. By reason of the foregoing, plaintiff has suffered damages in an amount to be proven at trial and, in addition, has suffered irreparable loss and injury.

34. The acts of infringement described above are willful, deliberate and in reckless disregard of plaintiff's patent rights.

FOURTH CLAIM FOR RELIEF

(Infringement of Patent)

(Against All Defendants)

35. Plaintiff realleges and incorporates by reference as though fully set forth preceding paragraphs 1 through 34.

36. United States Patent No. 6,251,384 (the "'384 Patent") issued on June 26, 2001. A true and correct copy of the '384 Patent is attached hereto as Exhibit 4 and incorporated herein by this reference.

37. Plaintiff is the sole owner of the '384 Patent.

38. Plaintiff is informed and believes that Kodak has infringed, and still is infringing, the '384 patent by making, using, selling, offering for sale and/or licensing products and services covered by one or more claims of the '384 Patent without plaintiff's authorization or consent. These products and services include, *inter alia*, Kodak's Image Station imaging systems (fully-integrated systems allowing researchers to use real-time imaging technology, including plaintiff's patented processes, to monitor and record cellular and genetic activity within laboratory animals).

39. Plaintiff is informed and believes that Kodak has infringed the '384 Patent, and will continue to do so unless enjoined by this Court.

40. Plaintiff is informed and believes, and on that basis, alleges that Kodak is aware of the '384 Patent and that its infringement has been willful.

41. Plaintiff is informed and believes that Kodak is actively inducing and/or contributing to infringement of the '523 Patent by others, all of whom are sued herein as DOES 1 through 50. Plaintiff will seek leave to amend this complaint to show the true names and capacities of said defendants when they are ascertained.

42. By reason of the foregoing, plaintiff has suffered damages in an amount to be proven at trial and, in addition, has suffered irreparable loss and injury.

43. The acts of infringement described above are willful, deliberate and in reckless disregard of plaintiff's patent rights.

FIFTH CLAIM FOR RELIEF

(Infringement of Patent)

(Against All Defendants)

44. Plaintiff realleges and incorporates by reference as though fully set forth preceding paragraphs 1 through 43.

45. United States Patent No. 6,649,159 (the "'159 Patent") issued on November 18, 2003. A true and correct copy of the '159 Patent is attached hereto as Exhibit 5 and incorporated herein by this reference.

46. Plaintiff is the sole owner of the '159 Patent.

47. Plaintiff is informed and believes that Kodak has infringed, and still is infringing, the '159 patent by making, using, selling, offering for sale and/or licensing products and services covered by one or more claims of the '159 Patent without plaintiff's authorization or consent. These products and services include, *inter alia*, Kodak's Image Station imaging systems (fully-integrated systems allowing researchers to use real-time imaging technology, including plaintiff's patented processes, to monitor and record cellular and genetic activity within laboratory animals).

48. Plaintiff is informed and believes that Kodak has infringed the '159 Patent, and will continue to do so unless enjoined by this Court.

49. Plaintiff is informed and believes, and on that basis, alleges that Kodak is aware of the '159 Patent and that its infringement has been willful.

50. Plaintiff is informed and believes that Kodak is actively inducing and/or contributing to infringement of the '159 Patent by others, all of whom are sued herein as DOES 1 through 50. Plaintiff will seek leave to amend this complaint to show the true names and capacities of said defendants when they are ascertained.

51. By reason of the foregoing, plaintiff has suffered damages in an amount to be proven at trial and, in addition, has suffered irreparable loss and injury.

52. The acts of infringement described above are willful, deliberate and in reckless disregard of plaintiff's patent rights.

SIXTH CLAIM FOR RELIEF

(Infringement of Patent)

(Against All Defendants)

53. Plaintiff realleges and incorporates by reference as though fully set forth preceding paragraphs 1 through 52.

54. United States Patent No. 6,905,831 (the "'831 Patent") issued on June 14, 2005. A true and correct copy of the '831 Patent is attached hereto as Exhibit 6 and incorporated herein by this reference.

55. Plaintiff is the sole owner of the '831 Patent.

56. Plaintiff is informed and believes that Kodak has infringed, and still is infringing, the '831 patent by making, using, selling, offering for sale and/or licensing products and services covered by one or more claims of the '831 Patent without plaintiff's authorization or consent. These products and services include, *inter alia*, Kodak's Image Station imaging systems (fully-integrated systems allowing researchers to use real-time imaging technology, including plaintiff's patented processes, to monitor and record cellular and genetic activity within laboratory animals).

57. Plaintiff is informed and believes that Kodak has infringed the '831 Patent, and will continue to do so unless enjoined by this Court.

58. Plaintiff is informed and believes, and on that basis, alleges that Kodak is aware of the '831 Patent and that its infringement has been willful.

59. Plaintiff is informed and believes that Kodak is actively inducing and/or contributing to infringement of the '831 Patent by others, all of whom are sued herein as DOES 1 through 50. Plaintiff will seek leave to amend this complaint to show the true names and capacities of said defendants when they are ascertained.

60. By reason of the foregoing, plaintiff has suffered damages in an amount to be proven at trial and, in addition, has suffered irreparable loss and injury.

61. The acts of infringement described above are willful, deliberate and in reckless disregard of plaintiff's patent rights.

PRAYER FOR RELIEF

WHEREFORE, Plaintiff AntiCancer prays for relief as follows:

A. That all defendants, and each of them, be adjudged to have infringed the '523, '968, '159, '384, '038, and '831 patent(s) under 35 U.S.C. § 271(a), (b), ©, and (g);

B. That all defendants, and each of them, be adjudged to have willfully infringed the '523, '968, '159, '384, '038, and '831 patent(s) under 35 U.S.C. § 271(a), (b), ©, and (g);

C. That defendants, and each of them, as well as their respective officers, agents, servants, employees and attorneys, and those persons in active concert or participation with them be preliminarily and permanently restrained and enjoined under 35 U.S.C. § 283 from directly or indirectly infringing the '523, '968, '159, '384, '038, and/or '831 patent(s);

D. That the Court award damages to compensate AntiCancer for the defendants' infringement of the '523, '968, '159, '384, '038, and '831 patent(s), as well as enhanced damages pursuant to 35 U.S.C. § 284;

E. That the Court award AntiCancer its attorney's fees pursuant to 35 U.S.C. § 285;

F. That the Court assess pre-judgment and post-judgment interest and costs of suit against defendants, and award such interest and costs to AntiCancer; and

G. That AntiCancer have such other and further relief as this Court may deem just and proper.

Respectfully submitted,

Dated: June 1, 2007 LAWTON LAW FIRM

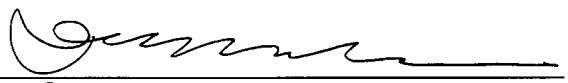
By: _____
Dan Lawton
Attorney for Plaintiff AntiCancer, Inc.

DEMAND FOR TRIAL BY JURY AND FOR SPEEDY HEARING

Plaintiff hereby demands a trial by jury as to all issues triable by jury, specifically including, but not limited to, the infringement of United States Patent Nos. 6,232,523, 6,235,968, 6,251,384, 6,649,159, and 6,759,038. Plaintiff also requests a speedy hearing of its claim for declaratory judgment pursuant to Fed. R. Civ. P. 57.

Respectfully submitted,

Dated: June 1, 2007 LAWTON LAW FIRM

By: 

Dan Lawton
Attorney for Plaintiff AntiCancer, Inc.

EXHIBIT 1

US006232523B1

(12) **United States Patent**
Tan et al.

(10) **Patent No.: US 6,232,523 B1**
(45) **Date of Patent: *May 15, 2001**

(54) **METASTASIS MODELS USING GREEN FLUORESCENT PROTEIN (GFP) AS A MARKER**

(75) Inventors: **Yuying Tan**, San Diego, CA (US); **Takashi Chishima**, Yokohomo (JP)

(73) Assignee: **AntiCancer, Inc.**, San Diego, CA (US)

(*) Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/848,539**

(22) Filed: **Apr. 28, 1997**

(51) **Int. Cl.**[7] **C12N 15/85**; C12N 15/63; A61K 35/00

(52) **U.S. Cl.** **800/10**; 435/325; 435/320.1; 424/93.21

(58) **Field of Search** 424/93.21, 9.6; 435/325, 320.1; 800/2, 10

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,491,084	*	2/1996	Chalfie et al.	435/189
5,650,135		7/1997	Contag et al.	424/9.1
5,650,235	*	7/1997	Contag et al.	424/9.1
5,874,304	*	2/1999	Zolotukhin et al.	435/366

FOREIGN PATENT DOCUMENTS

WO 97/18841	5/1997	(WO) .
WO 97 45550	12/1997	(WO) .
WO 98/49336	11/1998	(WO) .

OTHER PUBLICATIONS

Hoffman, "Orthotopic Metastatic Mouse Models for Anticancer Drug Discovery and Evaluation: A Bridge to the Clinic," *Investigational New Drugs* (1999) 17(4):343–359 (Abstract).

Yang et al., "Widespread Skeletal Metastic Potential of Human Lung Cancer Revealed by Green Fluorescent Protein Expression," *Cancer Research* (1998) 58(19):4217–4221.

Yang et al., "A Fluorescent Orthotopic Bone Metastasis Model of Human Prostate Cancer," *Cancer Research* (1999) 59(4):781–786.

Yang et al., "Green Fluorescent Protein: New Light to Visualize Metastasis and Angiogenesis in Cancer," *Proc SPIE–Int Soc Opt Eng* (1999) 117–124 (Abstract).

Levy, J P et al., "Retroviral Transfer and Expression of a Humanized, Red–Shifted Green Fluorescent Protein Gene Into Human Tumor Cells", *Nature Biotechnology*, vol. 14, No. 5, May 1996, pp 610–614.

Plautz, J. D. et al., "Green fluorescent protein and its derivatives as versatile markers for gene expression in living Drosophila melanogaster, plant and mammalian cells", *GENE*, vol. 173, 1996, pp. 83–87.

Chalfie, M. et al., "Green fluorescent protein as a marker for gene expression", *Science*, vol. 263, Feb. 11, 1994, pp 802–805.

Lin, W. C., et al., "Bacterial lacZ gene as a highly sensitive marker to detect micrometastasis formation during tumor progression", *Cancer Research*, vol. 50, No. 9, May 1, 1990, pp. 2808–2817.

Lin, W. C., et al., "Altered establishement/clearance mechanisms during experimental micrometastasis with live and/or disabled bacterial lacZ–tagged tumor cells", *Invasion Metastasis*, vol. 12, May 1992–Aug. 1992.

Chishisma, T. et al., "Metastatic patterns of lung cancer visualized live and in process by green fluorescence protein expression", *Clinical and Experimental Metastasis*, vol. 15, No. 5, Sep. 1997, pp. 547–552.

Chishima, T. et al., "Visualization of the metastatic process by green fluorescent protein expression", *Anticancer Research*, vol. 17, No. 4a, Jul. 1997, pp. 2377–2384.

Chishima, T. et al., "Cancer Invasion and micrometastasis visualized in live tissue by green fluorescent protein expression", *Cancer Research*, vol. 57, No. 10, May 15, 1997, pp 2042–2047.

Chishima, T. et al., "Governing step of metastasis visualized in vitro", *Proceedings of the National Academy of Sciences of USA*, vol. 94, No. 21, Oct. 1997, pp. 11573–11576.

Hyer, M. L. et al., "Adenvirus–mediated gene transfer of GFP (green fluorescent protein) to mouse and human prostate cancer cells", *Cancer Gene Therapy*, vol. 4, No. 6, Nov. 1997, pp. s29–s30.

Li, Y. et al., "Use of a Green Fluorescent Protein in Studies of Apoptosis of Transfected Cells", *Biotechnologies*, vol. 23, No. 6, Dec. 1997, pp. 1026–1029.

Astoul, P. et al., *Anticancer Res.* (1994) 14:85–92.

Astoul, P., *J. Cell Biochem.* (1994) 56:9–15.

Cody, C. W. et al., *Biochemistry* (1993) 32:1212–1218.

Cormack, B. et al., *Gene* (1996) 173:33–38.

Crameri, A. et al., *Nature Biotechnol.* (1996) 14:315–319.

Delagrave, S. et al., *Biotechnology* (1995) 13:151–154.

Fu, X. et al., *Anticancer Res.* (1993) 13:283–286.

(List continued on next page.)

Primary Examiner—Karen M. Hauda
Assistant Examiner—AnneMarie S. Beckerleg
(74) *Attorney, Agent, or Firm*—Morrison & Foerster LLP

(57) **ABSTRACT**

A method to follow the progression of metastasis of a primary tumor, which method comprises removing fresh organ tissues from a vertebrate subject which has been modified to contain tumor cells that express GFP and observing the excised tissues for the presence of fluorescence is disclosed. Vertebrate subjects which contain GFP producing tumors are useful models to study the mechanism of metastasis.

7 Claims, No Drawings

OTHER PUBLICATIONS

Heim, R. et al., *Nature* (1995) 373:663–664.
Hoffman, R.M., *Cancer Cells* (1991) 3:86–92.
Kaufman, R.J. et al., *Nucleic Acids Res.* (1991) 19:4485–4490.
Khokha, R. et al., *Cancer Metastasis Rev.* (1995) 14:279–301.
Koop, S. et al., *Cancer Research* (1995) 55:2520–2523.
Leighton, J., *Cancer Res.* (1957) 17:929–941.
Leighton, J., *Cancer Res.* (1960) 20:575–597.
Levy, J.P. et al., *Nature Biotechnol.* (1996) 14:610–614.
Lin, W.C. et al., *Cancer Research* (1990) 50:2808–2817.
Lin, W.C. et al., *Invasion and Metastasis* (1992) 12:197–209.
Margolis, L.B. et al., *In Vitro Cell Div. Biol.* (1995) 31:221–226.
Morin, J. et al., *J. Cell. Physiol.* (1972) 77:313–318.
Prasher, D.C. et al., *Gene* (1992) 111:229–233.
Yokoe, H. et al., *Nature Biotechnol.* (1996) 14:1252–1256.
Zolotukhin, S. et al., *J. Virol.* (1996) 70:4646–4654.
Romer et al. (1995) APMIS, vol. 103, 582–587.*
Astoul et al. (1994) J. Cell. Biochem. , vol. 56, 1–15.*
Kaufman et al. (1995) Annu. Rev. Immunol., vol. 13, 339–367.*
Vieweg et al.(1995) Cancer Investigation, 13 (2), 193–201.*
Kaufman et al. (1991) Nucleic Acids. Res., vol. 19 (16) 4485–4490.*
Chishima et al. (Mar. 1997) Proc. Am. Assoc. Canc. Res., vol. 38, p. 489 #3276.*
Aboody–Guterman et al. (1996) Society for Neuroscience Abstracts, vol. 22, 949.*
Romer et al. (1995) APMIS, vol. 103, 582–587.*
Levy et al. (1996) Nature Biotech., vol. 14, 610–614.*
Astoul et al. (1994) J. Cell. Biochem., vol. 56, 9–15.*
Margolis et al. (1995) In Vitro Cell. Dev. Biol., vol. 31, 221–226.*
Kaufman et al. (1995) Annu. Rev. Immunol., vol. 13, 339–367.*

* cited by examiner

METASTASIS MODELS USING GREEN FLUORESCENT PROTEIN (GFP) AS A MARKER

TECHNICAL FIELD

The invention relates to the study of tumor progression. Specifically, it concerns model systems for studying the metastasis of tumors in vertebrate systems.

BACKGROUND ART

It has long been recognized that the ability of tumor tissues to metastasize constitutes a major portion of the life-threatening aspects of malignancy. Metastasis is the growth of secondary tumors at sites different from the primary tumor. Thus, despite surgical removal of the primary tumor, it may not be possible to arrest the progress of this condition. An understanding of the mechanism whereby metastasis occurs will be crucial to the development of protocols whereby the growth of secondary tumors can be controlled. In order to understand the mechanism of metastasis, it will be necessary to provide a model which permits identification of small numbers of tumor cells against a background of many host cells so that secondary tumor emboli and micrometastases can be observed over the course of real time.

Others have demonstrated extravasation and initial seeding steps in tumor metastasis in vitro using externally fluorescently labeled tumor cells. Khokha, R. et al., *Cancer Metastasis Rev* (1995) 14:279–301; Koop, S. et al., *Cancer Res* (1995) 55:2520–2523. Further, Margolis, L. B. et al., *In Vitro Cell Dev Biol* (1995) 31:221–226 was able to visualize the migration of externally fluorescently labeled lung tumor cells in host mouse lung in histoculture. In all cases, however, long-term observation was not possible due to the limitation of exogenous fluorescent labels. Retroviral transfer of a green fluorescent protein (GFP) gene has been shown to result in stable transfectants of human cancer cells in vitro. Levy, J. P. et al., *Nature Biotechnol* (1996) 14:610–614.

Attempts have been made to provide such a model using the β-galactosidase gene as a marker (Lin, W. C. et al., *Cancer Res* (1990) 50:2808–2817; Lin, W. C. et al., *Invasion and Metastasis* (1992) 12:197–209). However, this marker has not proved satisfactory, as fresh or processed tissue cannot be used. The present invention provides a marker which permits visualization of tumor invasion and micrometastasis formation in viable fresh tissue.

DISCLOSURE OF THE INVENTION

The invention provides models which permit the intimate study of formation of metastases from primary tumors in a realistic and real-time setting. By using green fluorescent protein (GFP) as a stable and readily visualized marker, the progression of such metastasis can be modeled and the mechanism elucidated.

Thus, in one aspect, the invention is directed to a method to follow the progression of metastasis of a primary tumor, which method comprises removing fresh organ tissues from a vertebrate subject which has been modified to contain tumor cells that express GFP and observing the excised tissues for the presence of fluorescence.

In another aspect, the invention is directed to a vertebrate subject which has been modified to contain tumor cells expressing GFP.

In still other aspects, the invention is directed to tumor cells modified to produce GFP under control of nonretroviral control elements, to tissues containing metastatic tumors that produce GFP, and to histocultures of tissues which contain such metastasized tumors.

MODES OF CARRYING OUT THE INVENTION

The invention provides model systems for the study of the mechanism of metastasis of tumors generally. Advantage is taken of the visible marker green fluorescence protein (GFP) to label the tumor cells so that their migration and colonization in tissues distal to the tumor can be followed as the migration and colonization progresses.

In general, the model involves modifying a vertebrate, preferably a mammal, so as to contain tumor tissue, wherein the tumor cells have, themselves, been modified to contain an expression system for GFP. Tumors can be formed in such vertebrate systems by administering the transformed cells containing the GFP expression system and permitting these transformed cells to form tumors. Typically such administration is subcutaneous and the tumors are formed as solid masses. The tumors thus formed can be implanted in any suitable host tissue and allowed to progress, metastasize and develop.

Suitable procedures for growing the initial tumor, thus, involve transcutaneous injection of the tumor cells, such as CHO cells, HeLa cells, carcinoma and sarcoma cell lines, and well established cell lines such as the human lung adenocarcinoma line anip 973, and others that may become available in the art. The administered cells will have been modified to contain an expression system for GFP. After administration, solid tumors generally develop, typically at the site of subcutaneous injection. These tumors, which are themselves fluorescent, can then be removed and used for implantation in the model vertebrate.

Techniques for implantation of the solid tumors, now labeled with GFP, into vertebrates include direct implantation by surgical orthotopic implantation (SOI) at the desired site, typically the site from which the tumor cells were derived. Suitable sites include lung, liver, pancreas, stomach, breast, ovary, prostate, bone marrow, brain, and other tissues susceptible to malignancy. Once the solid tumors have been implanted, the vertebrate becomes a model system for studying metastasis. The tumor is thus allowed to progress and develop and the vertebrate is monitored for appearance of the GFP labeled cells at sites distal from the original implantation site. The monitoring can occur either on the whole vertebrate by opening the animal and observing the organs directly with a fluorescent microscope, or the tissues may be excised and examined microscopically. As GFP is visible to the naked eye, no development systems to stain the tissue samples are required. The tissue samples are simply properly processed as fresh samples in slices of suitable size, typically 1 mm thick, and placed under a microscope for examination. Even colonies of less than 10 cells are thus visible. A variety of microscopic visualization techniques is known in the art and any appropriate method can be used.

In addition, the development of the tumor can be studied in vitro in histological culture. Suitable systems for such study include solid supported cultures such as those maintained on collagen gels and the like.

Suitable vertebrate subjects for use as models are preferably mammalian subjects, most preferably convenient laboratory animals such as rabbits, rats, mice, and the like. For closer analogy to human subjects, primates could also be used. Particularly useful are subjects that are particularly susceptible to tumor development, such as subjects with

impaired immune systems, typically nude mice or SCID mice. Any appropriate vertebrate subject can be used, the choice being dictated mainly by convenience and similarity to the system of ultimate interest.

The label used to follow the metastasis is green fluorescent protein (GFP). The gene encoding this protein has been cloned from the bioluminescent jellyfish *Aequorea victoria* (Morin, J. et al., *J Cell Physiol* (1972) 77:313–318). The availability of the gene has made it possible to use GFP as a marker for gene expression. GFP itself is a 283 amino acid protein with a molecular weight of 27 kD. It requires no additional proteins from its native source nor does it require substrates or cofactors available only in its native source in order to fluoresce. (Prasher, D. C. et al., *Gene* (1992) 111:229–233; Yang, F. et al., *Nature Biotechnol* (1996) 14:1252–1256; Cody, C. W. et al., *Biochemistry* (1993) 32:1212–1218.) Mutants of the GFP gene have been found useful to enhance expression and to modify excitation and fluorescence. GFP-S65T (wherein serine at 65 is replaced with threonine) is particularly useful in the invention method and has a single excitation peak at 490 nm. (Heim, R. et al., *Nature* (1995) 373:663–664). Other mutants have also been disclosed by Delagrade, S. et al., *Biotechnology* (1995) 13:151–154; Cormack, B. et al., *Gene* (1996) 173:33–38 and Cramer, A. et al. *Nature Biotechnol* (1996) 14:315–319. Any suitable and convenient form of the GFP gene can be used to modify the tumor cells useful in the models of the invention. The particular humanized hGFP-S65T clone is used in the examples set forth below for illustration.

Similarly, any suitable expression system operable in the tumor cells to be implanted may be used. A number of vectors are commercially available that will effect expression in tumor cells of various types. The nature of the vector may vary with the nature of the tumor and the vertebrate in which it finds its origin. However, it is preferred to utilize vectors which do not use retroviral or other viral promoters which may complicate the nature of the model.

In order to transfect and modify the transformed cells which are effective in generating tumors, any suitable transfection method may be used, such as liposomes, calcium phosphate precipitation, electroporation and use of a gene gun. Lipofection is preferred.

These examples are intended to illustrate but not to limit the invention.

EXAMPLE 1

Preparation of Tumor Cells that Produce GFP

The humanized hGFP-S65T clone described by Zolotukhin, S. et al., *J Virol* (1996) 70:4646–4654 was used as the green fluorescent protein coding sequence. This codon-optimized gene was purchased from CLONTECH Laboratories, Inc. (Palo Alto, Calif.) and ligated into the dicistronic expression vector (pED-mtx[1]) obtained from Genetics Institute, Cambridge, Mass. and described in Kaufman, R. J. et al., *Nucleic Acids Res* (1991) 19:4485–4490. hGFP-S65T was digested with HindIII and blunted; the entire hGFP coding region was excised with XbaI and then unidirectionally subcloned into pED-mtx[1] which had been digested with PstI, blunted and then further digested with XbaI.

CHO-K1 cells were cultured in DMEM containing 10% fetal calf serum, 2 mM L-glutamine and 100 μM nonessential amino acids. Near confluent cells were incubated with a precipitated mixture of LipofectAMINE™ reagent (GIBCO) and saturating amounts of plasmids for six hours and then replenished with fresh medium. The cells were harvested by trypsin/EDTA 48 hours later and subcultured at 1:15 into selective medium containing 1.5 μM methotrexate (MTX). Cells with stably integrated plasmids were selected in MTX-containing medium and isolated with cloning cylinders (Bel-Art Products, Pequannock, N.J.) by EDTA. After amplification and transfer, Clone-38 was selected because of its high-intensity GFP fluorescence and stability.

In a similar manner, Anip 973 cells, a human lung cancer cell line obtained from Harbin Medical University, China, were cultured as described above for CHO-K1 cells except using RPMI1640 (GIBCO) in place of DMEM. Transfection, selection and amplification and transfer were conducted as described above. Clone 26 was chosen because of its high-intensity GFP fluorescence and stability.

EXAMPLE 2

Mouse Model Using Modified CHO Cells

Clone 38, which was stable at 1.5 μM MTX and which proliferated at the same rate as the parental CHO-K1 cells as ascertained by comparing doubling times, was used in this model.

Three six-week old Balb/C nu/nu female mice were injected subcutaneously with a single dose of 10^7 Clone-38 cells that had been harvested by trypsinization and washed three times with cold serum-containing medium and then kept on ice. The cells were injected in a total volume of 0.4 ml within 40 minutes of harvesting and the nude mice sacrificed three weeks after injection. All of the mice had a subcutaneous tumor ranging in diameter from 13.0 mm to 18.5 mm (mean=15.2 mm±2.9 mm). The tumor tissue was strongly fluorescent. It was shown by extracting GFP from cultured Clone-38 cells in comparison to Clone-38 cells prepared from the tumor that the levels of production of GFP were the same in both.

To construct the model, tumor fragments (1 mm^3) derived from the nude mouse subcutaneous Clone-38 tumor grown as described above, were implanted by surgical or surgical orthotopic implantation (SOI) on the ovarian serosa in six nude mice as described by Fu, X. et al., *Anticancer Res* (1993) 13:283–286, incorporated herein by reference. Briefly, the mice were anesthetized by isofluran inhalation and an incision was made through the left lower abdominal pararectal line and peritoneum to expose the left ovary and part of the serosal membrane, which was scraped with a forceps. Four 1 mm^3 tumor pieces were fixed on the scraped site with an 8-0 nylon suture and the ovary then returned to the peritoneal cavity. The abdominal wall and skin were closed with 6-0 silk sutures.

Four weeks later, the mice were sacrificed and lung and various other organs were removed. The fresh samples were sliced at approximately 1 mm thickness and observed directly under fluorescent and confocal microscopy. Samples were also processed for histological examination for fluorescence and conventional staining. Frozen sections were prepared wherein the slides were rinsed with phosphate buffer saline and fixed for 10 minutes at 4° C.; 10% formaldehyde plus 0.2% glutaraldehyde and PBS were added and the slides were then washed with PBS. The fixed tissue was stained with hematoxylin and eosin using standard techniques.

Light and fluorescence microscopy were carried out using a Nikon microscope equipped with a Xenon lamp power supply and a GFP filter set (Chromotechnology Corp., Brattleboro, Vt.). Confocal microscopy was with an MRC-

600 Confocal Imaging System (Bio-Rad) mounted on a Nikon microscope with an argon laser.

The mice, at sacrifice, had tumors in the ovaries ranging in diameter from 18.7 mm–25.3 mm (mean 21.9±3.1 mm). The fresh organ tissues examined under fluorescence microscopy with no treatment of the tissues showed seeding of the tumor throughout the peritoneal cavity, including the colon (6/6 mice), cecum (5/6), small intestine (4/6), spleen (1/6), and peritoneal wall (6/6). Numerous micrometastases were detected in the lungs of all mice and multiple micrometastases were also detected on the liver (1/6), kidney (1/6), contralateral ovary (3/6), adrenal gland (2/6), para-aortic lymph node (5/6) and pleural membrane (5/6). Single-cell micrometastases could not be detected by the standard histological techniques described above and even the multiple cell colonies were difficult to detect using them. As the colonies developed, the density of tumor cells decreased markedly in the center.

In an additional experiment, 5×10^6 Clone-38 cells were injected into a nude mouse through the tail vein and the mouse sacrificed after two minutes. Fresh visceral organs were analyzed by fluorescence microscopy and showed the presence of fluorescent cells in peritoneal wall vessels which formed emboli in the capillaries of the lung, liver, kidney, spleen, ovary, adrenal gland, thyroid gland and brain.

Thus, using these techniques, progression of micrometastasis can be observed as seeded cells develop into colonies within the relevant target organs. Further, screening for micrometastasis can be done easily and quickly in all systemic organs.

EXAMPLE 3

Murine Model Using Human Lung Cancer Cells

The procedures are generally those set forth in Example 2 except that Clone-26 cells as prepared in Example 1 were used instead of Clone-38 CHO cells.

As in Example 2, tumors were grown in six-week-old Balb/C nu/nu male mice injected subcutaneously with a single 0.4 ml dose of 10^7 Clone-26 cells within 40 minutes of harvesting by trypsinization and washing three times with cold serum-containing medium. The cells were kept on ice prior to injection. The animals were sacrificed when the tumors had reached approximately 1.2 cm diameters. The 1.2 cm tumors formed after about 5 weeks.

The tumor pieces, 1 mm^3, were implanted by SOI into the left visceral pleura of 8 mice as described by Astoul, P. et al., *Anticancer Research* (1994) 14:85–92; Astoul, P. *J Cell Biochem* (1994) 56:9–15, both incorporated herein by reference. Briefly, the mice were anesthetized by isofluoran inhalation an a small 1 cm transverse incision made on the left lateral chest, via the fourth intercostal space, resulting in total lung collapse. Five tumor pieces were sewn together with a 7-0 nylon surgical suture and fixed by making one knot. The lung was taken up by forceps and the tumor sewn into the lower part of the lung with one suture, after which the lung was returned to the chest cavity and the muscles and skin closed with a single layer of 6-0 silk sutures. The lung was reinflated by withdrawing air from the chest cavity with a 23-gauge needle.

Four of the mice were sacrificed at 4 weeks and another 4 at 8 weeks. Pleural tumors for the 4-week group ranged from 244.40 mm^3–522.88 mm^3; those from the 8 week group from 1279.08 mm^3–2714.40 mm^3. This represented mean volumes of 371 mm^3 and 1799 mm^3. Specimens of tissue were sliced at 1 mm thickness and observed directly under fluorescent microscopy using a Nikon microscope equipped with a Xenon lamp power supply and a Leica stereo fluorescence microscope equipped with a mercury lamp power supply and GFP filter sets. All of the animals showed chest wall invasion and local and regional spread of the tumor, but in the 8-week mice, all tumors involved the mediastinum and contralateral pleural cavity as well as metastases on the visceral and parietal pleura. Pulmonary hilum lymph nodes were involved in 3 of 4 mice of the 4-week group and all of the mice in the 8-week group. Cervical node involvement was detected in one of the mice of the 8-week group, but no other metastases were observed. The animals were also observed directly before the tissues were excised. The margin of the invading tumor in normal lung tissue could be detected by GFP fluorescence and a small vessel could be seen developing at the margin of the tumor.

In an additional experiment, 8 nude mice were injected in the tail vein with a single dose of 1×10^7 Clone-26 cells that had been harvested by trypsinization and washed 3 times with cold serum-containing medium. The injection contained a total volume of 0.8 ml within 40 min. of harvesting. Again, 4 mice were sacrificed at 4 weeks and another 4 at 8 weeks and tissue specimens were obtained and studied by microscopy as described above. Numerous micrometastatic colonies were detected in whole lung tissue in both groups ranging from 5.2 μm to 32.5 μm in the 4-week group and 5.5 μm–178.3 μm in the 8-week group. The colonies from the 8-week group did not appear further developed as compared with those from the 4-week group. Numerous small colonies ranging in number to less than 10 cells were detected at the lung surface in both groups and brain metastases were detected in 1 mouse of the 4-week group and 2 from the 8-week group. One mouse in the 8-week group had systemic metastases in the brain, the submandibular gland, the whole lung, the pancreas, the bilateral adrenal glands, the peritoneum and the pulmonary hilum lymph nodes.

In an additional experiment, similar to that set forth in the previous paragraph, the mice injected in a tail vein with 10^7 Clone-26 cells were sacrificed at 4, 8 and 12 weeks and the tissues examined as described. Most of the colonies and mice sacrificed at 8 weeks were not obviously further developed compared with those sacrificed at 4 weeks, but numerous small quantities ranging in number down to less than 10 cells and ranging in size from 5.5 μm–110 μm were detected at the lung surface. At 12 weeks, there were many small metastatic colonies which appeared dormant, although other colonies grew extensively by this time, reaching a size up to 1100 μm, suggesting a heterogeneity of dormant and active tumor colonies in the lung.

EXAMPLE 4

Growth of Clone-26 Tumor Cells in Histoculture

Six-week old SCID/SCID mice were injected intravenously with a single dose of 7.5×10^7 Clone-26 cells which had been harvested by trypsinization and washed 3 times with cold serum-containing medium and kept on ice as described above. The cells were injected in a total volume of 0.5 ml within 40 minutes of harvesting. After 3 weeks, numerous micrometastatic colonies were detected in whole lung tissue up to approximately 550 μm. After 5 weeks, the mice were sacrificed and the Clone-26 seeded mouse lungs were removed and histocultured on spun gels using the histoculture methods developed by Leighton, J. *Cancer Res* (1957) 17:929–941; Leighton, J. et al., *Cancer Res* (1960) 20:575–597; Hoffman, R. M. *Cancer Cells* (1991) 3:86–92.

Tumor colonies spread rapidly in the lung tissue over time and after 1 week the tumor cells started to invade and colonize supporting collagen sponge-gel. After 2 weeks, tumor cells formed satellite colonies in the sponge-gel distant from the primary colonies in the lung tissue, thus growing faster in histoculture than in SCID mice. Tumor colonies could grow in histoculture for more than 1 month.

What is claimed is:

1. A stably transformed tumor cell line which has been transfected with an expression vector containing a first nucleotide sequence encoding green fluorescent protein (GFP) or mutants thereof, and a second nucleotide sequence encoding a selection marker, both said first and said second nucleotide sequences being under control of a viral promoter, wherein said vector is derived from pED-mtx, and wherein said cell line stably effects high level expression of said GFP in the absence of a selection agent and maintains said high level expression of said GFP when said cell line proliferates through multiple passages of said cell line.

2. The cell line of claim **1** which is resistant to 1.5 μM methotrexate.

3. The cell line of claim **1**, which forms a solid tumor.

4. A method to prepare a mammal which harbors a tumor expressing GFP which method comprises administering to a mammal an amount of cells of the cell line of claim **1** sufficient to effect production of a tumor in said mammal and wherein said mammal is genetically immunocompromised or syngeneic with said tumor.

5. A method to prepare a mammal which harbors a tumor expressing GFP which method comprises implanting into a mammal the cell line of claim **3** which comprise a solid tumor by surgical orthotopic implantation and wherein said mammal is genetically immunocompromised or syngeneic with said tumor.

6. A non-human mammal which has been modified to contain a tumor expressing GFP prepared by the method of claim **4**.

7. A non-human mammal which has been modified to contain a tumor expressing GFP prepared by the method of claim **5**.

* * * * *

EXHIBIT 3

US006235968B1

(12) **United States Patent**
Tan et al.

(10) **Patent No.: US 6,235,968 B1**
(45) **Date of Patent: May 22, 2001**

(54) **METASTASIS MODELS USING GREEN FLUORESCENT PROTEIN (GFP) AS A MARKER**

(75) Inventors: **Yuying Tan**, San Diego, CA (US); **Takashi Chishima**, Yokohamo (JP)

(73) Assignee: **AntiCancer, Inc.**, San Diego, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/067,734**

(22) Filed: **Apr. 28, 1998**

Related U.S. Application Data

(63) Continuation-in-part of application No. 09/049,544, filed on Mar. 27, 1998, which is a continuation-in-part of application No. 08/848,539, filed on Apr. 28, 1997.

(51) **Int. Cl.**[7] **A61K 48/00**; A61K 35/00; C12N 15/63; C12N 15/85

(52) **U.S. Cl.** **800/10**; 800/9; 514/44; 424/93.21; 604/5.2; 435/325; 435/320.1

(58) **Field of Search** 514/44; 424/93.21; 604/52; 800/9, 10; 435/325, 320.1

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,650,135 7/1997 Contag et al. 424/9.1

FOREIGN PATENT DOCUMENTS

WO 97/18841 5/1997 (WO) .
WO 97 45550 12/1997 (WO) .
WO 98/49336 11/1998 (WO) .

OTHER PUBLICATIONS

Kaufman et al. (1995) Annu. Rev. Immunol., vol. 13, 339–367.*
Miller et al. (1995) FASEB, vol. 9, 190–199.*
Hoffman, "Orthotopic Metastatic Mouse Models for Anticancer Drug Discovery and Evaluation: A Bridge to the Clinic," *Investigational New Drugs* (1999) 17(4):343–359 (Abstract).
Yang et al., "Widespread Skeletal Metastatic Potential of Human Lung Cancer Revealed by Green Fluorescent Protein Expression," *Cancer Research* (1998) 58(19):4217–4221.
Yang et al., "A Fluorescent Orthotopic Bone Metastasis Model of Human Prostate Cancer," *Cancer Research* (1999) 59(4):781–786.
Yang et al., "Green Fluorescent Protein: New Light to Visualize Metastasis and Angiogenesis in Cancer," *Proc SPIE–Int Soc Opt Eng* (1999) 117–124 (Abstract).
Levy, J P et al., "Retroviral Transfer and Expression of a Humanized, Red–Shifted Green Fluorescent Protein Gene Into Human Tumor Cells", *Nature Biotechnology*, vol. 14, No. 5, May 1996, pp 610–614.
Plautz, J. D. et al., "Green fluorescent protein and its derivatives as versatile markers for gene expression in living *Drosophila melanogaster*, plant and mammalian cells", *GENE*, vol. 173, 1996, pp. 83–87.

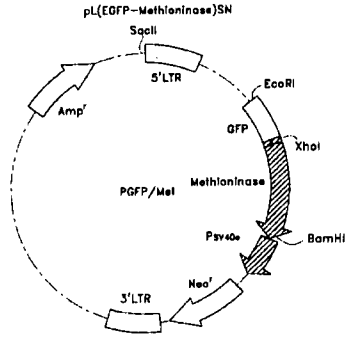
(List continued on next page.)

Primary Examiner—Karen M. Hauda
Assistant Examiner—Anne Marie S. Beckerleg
(74) *Attorney, Agent, or Firm*—Morrison & Foerster LLP

(57) **ABSTRACT**

A method to follow the progression of metastasis of a primary tumor, which method comprises removing fresh organ tissues from a vertebrate subject which has been modified to contain tumor cells that express GFP and observing the excised tissues for the presence of fluorescence is disclosed. The fluorescence can also be monitored by observing the tissues in situ. Vertebrate subjects which contain GFP producing tumors are useful models to study the mechanism of metastasis. In addition, subjects already harboring tumors can be treated so as to modify the endogenous tumors to contain GFP. This permits clinical applications. Finally, by injecting a contrast dye into a subject harboring a GFP-labeled tumor, angiogenesis in the tumor can be observed directly.

8 Claims, 2 Drawing Sheets



OTHER PUBLICATIONS

Chalfie, M. et al., "Green fluorescent protein as a marker for gene expression", *Science*, vol. 263, Feb. 11, 1994, pp 802–805.
Lin, W. C., et al., "Bacterial lacZ gene as a highly sensitive marker to detect micrometastasis formation during tumor progression", *Cancer Research*, vol. 50, No. 9, May 1, 1990, pp. 2808–2817.
Lin, W. C., et al., "Altered establishment/clearance mechanisms during experimental micrometastasis with live and/or disabled bacterial lacZ–tagged tumor cells", *Invasion Metastasis*, vol. 12, May 1992–Aug. 1992.
Chishisma, T. et al., "Metastatic patterns of lung cancer visualized live and in process by green fluorescence protein expression", *Clinical and Experimental Metastasis*, vol. 15, No. 5, Sep. 1997, pp. 547–552.
Chishima, T. et al., "Visualization of the metastatic process by green fluorescent protein expression", *Anticancer Research*, vol. 17, No. 4a, Jul. 1997, pp. 2377–2384.
Chishima, T. et al., "Cancer Invastion and micrometastasis visualized in live tissue by green fluorescent protein expression", *Cancer Research*, vol. 57, No. 10, May 15, 1997, pp 2042–2047.
Chishima, T. et al., "Governing step of metastasis visualized in vitro", *Proceedings of the National Academy of Sciences of USA*, vol. 94, No. 21, Oct. 1997, pp. 11573–11576.
Hyer, M. L. et al., "Adenovirus–mediated gene transfer of GFP (Green fluorescent protein) to mouse and human prostate cancer cells", *Cancer Gene Therapy*, vol. 4, No. 6, Nov. 1997, pp. s29–s30.
Li, Y. et al., "Use of a Green Fluorescent Protein in Studies of Apoptosis of Transfected Cells", *Biotechnologies*, vol. 23, No. 6, Dec. 1997, pp. 1026–1029.
Chishima et al. (1997) Proc. Am. Assoc. Canc. Res., vol. 38, p. 489 #3276—Dated Mar. 1997.
Aboody–Guterman et al. (1996) Society for Neuroscience Abstracts, vol. 22, 949—1998.
Romer et al. (1995) APMIS, vol. 103,582–587.
Levy et al (1996) Nature Biotech., vol. 14, 610–614.
Astoul et al. (1994) J. Cell, Biochem., vol. 56, 9–15.
Margolis et al. (1995) Annu. Rev. Immunol., vol. 13, 339–367.
Astoul, P. et al., *Anticancer Res.* (1994) 14:85–92.
Astoul, P., *J. Cell Biochem.* (1994) 56:9–15.
Cody, C.W. et al., *Biochemistry* (1993) 32:1212–1218.
Cormack, B. et al., *Gene* (1996) 173:33–38.
Crameri, A. et al., *Nature Biotechnol.* (1996) 14:315–319.
Delagrave, S. et al., *Biotechnology* (1995) 13:151–154.
Fu, X. et al., *Anticancer Res.* (1993) 13:283–286.
Heim, R. et al., *Nature* (1995) 373:663–664.
Hoffman, R.M., *Cancer Cells* (1991) 3:86–92.
Kaufman, R.J. et al., *Nucleic Acids Res.* (1991) 19:4485–4490.
Khokha, R. et al., *Cancer Metastasis Rev.* (1995) 14:279–301.
Koop, S. et al., *Cancer Research* (1995) 55:2520–2523.
Leighton, J., *Cancer Res.* (1957) 17:929–941.
Leighton, J., *Cancer Res.* (1960) 20:575–597.
Levy, J.P. et al., *Nature Biotechnol.* (1996) 14:610–614.
Lin, W.C. et al., *Cancer Research* (1990) 50:2808–2817.
Lin, W.C. et al., *Invasion and Metastasis* (1992) 12:197–209.
Margolis, L.B. et al., *In Vitro Cell Dev. Biol.* (1995) 31:221–26.
Morin, J. et al., *J. Cell. Physiol.* (1972) 77:313–318.
Prasher, D.C. et al., *Gene* (1992) 111:229–233.
Yokoe, H. et al., *Nature Biotechnol.* (1996) 14:1252–1256.
Zolotukhin, S. et al., *J. Virol.* (1996) 70:4646–4654.

* cited by examiner

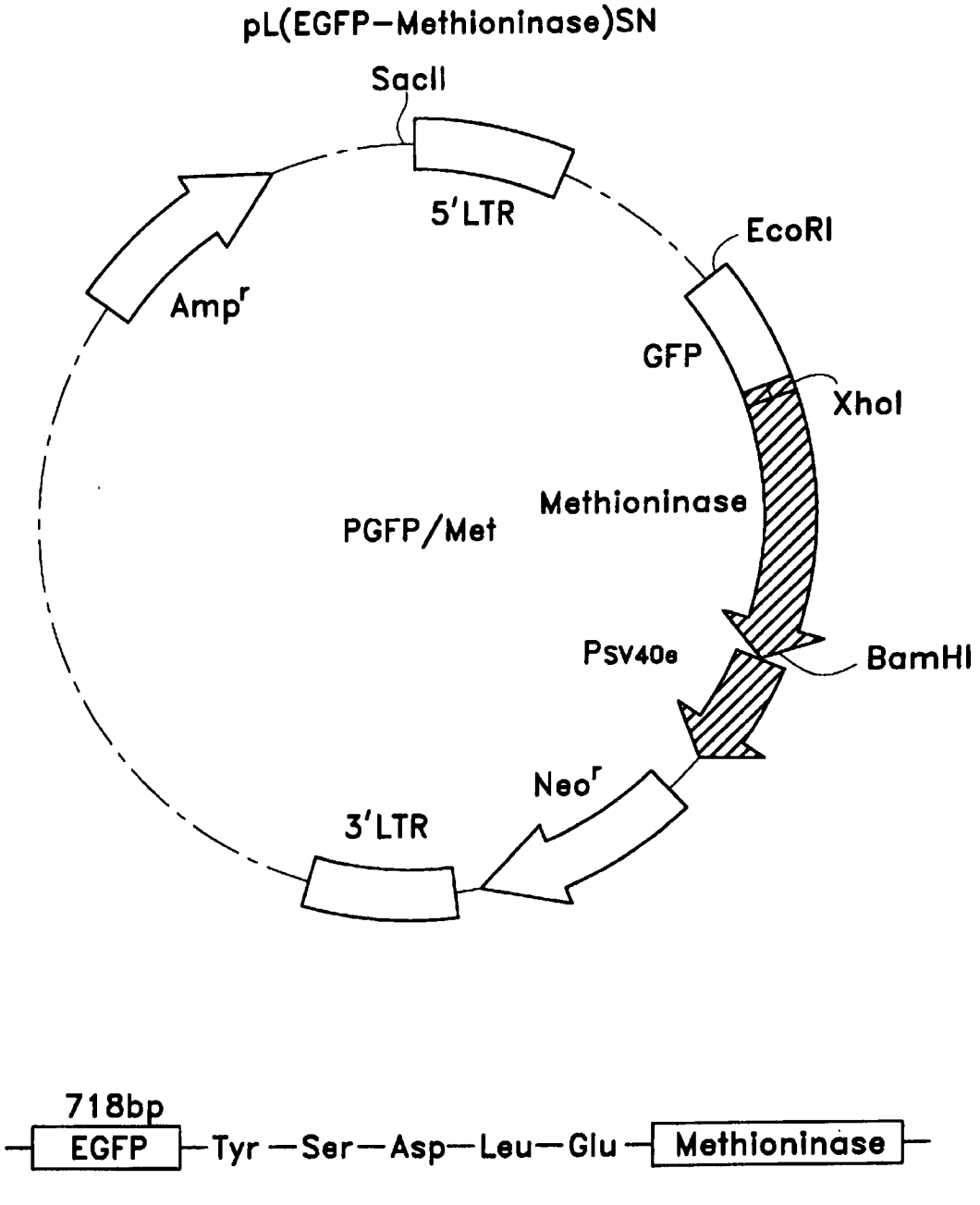


Fig. 1a

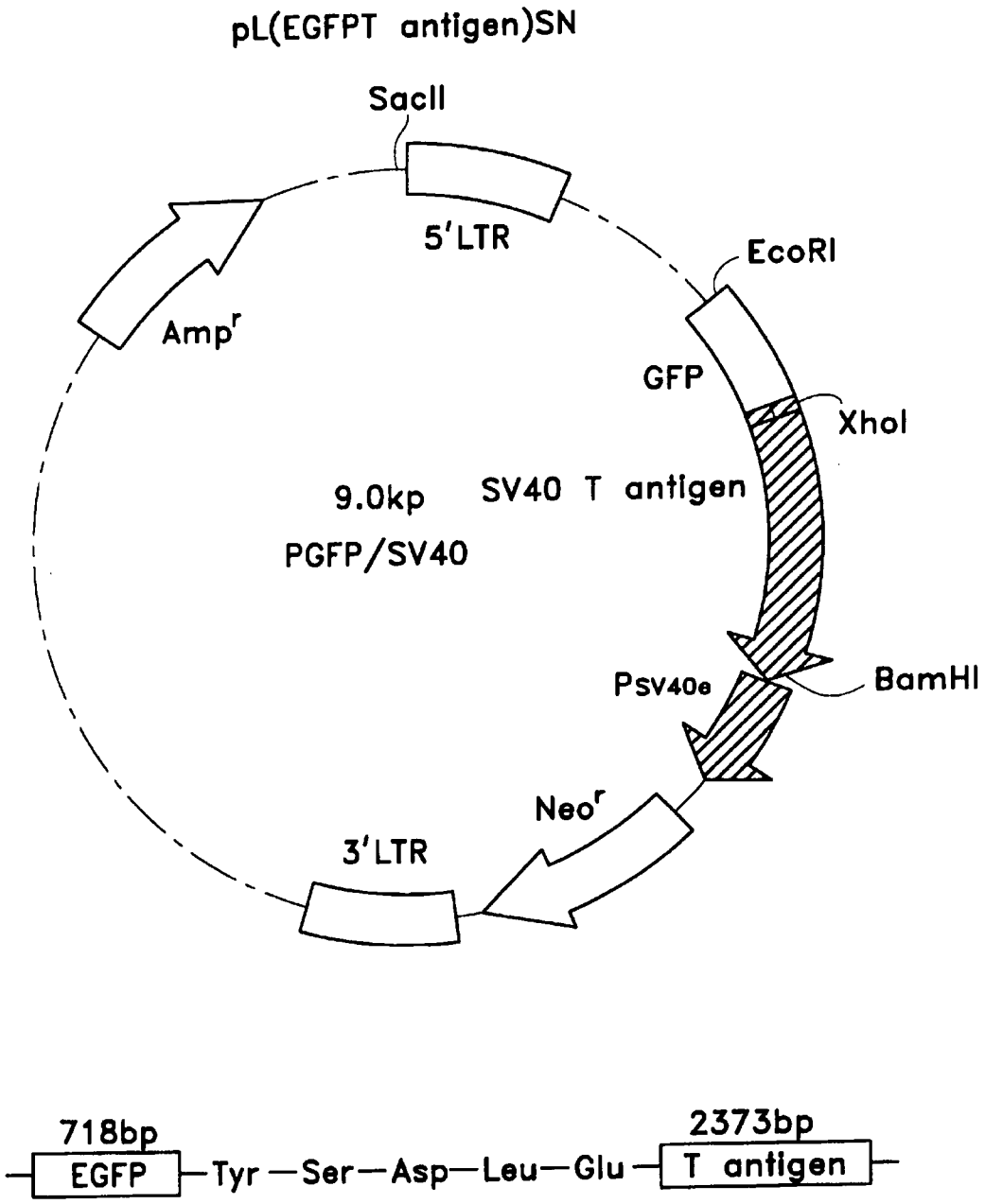

pL(EGFPT antigen)SN
SacII
5'LTR
EcoRI
Amp^r
GFP
XhoI
9.0kp
SV40 T antigen
PGFP/SV40
Psv40e
BamHI
Neo^r
3'LTR
718bp
EGFP
Tyr
Ser
Asp
Leu
Glu
2373bp
T antigen

Fig. 1b

METASTASIS MODELS USING GREEN FLUORESCENT PROTEIN (GFP) AS A MARKER

This application is a continuation-in-part of U.S. Ser. No. 09/049,544 filed Mar. 27, 1998 which is a continuation-in-part of U.S. Ser. No. 08/848,539 filed Apr. 28, 1997, the contents of which are incorporated herein by reference.

TECHNICAL FIELD

The invention relates to the study of tumor progression. Specifically, it concerns model systems for studying the metastasis of tumors in vertebrate systems.

Background Art

It has long been recognized that the ability of tumor tissues to metastasize constitutes a major portion of the life-threatening aspects of malignancy. Metastasis is the growth of secondary tumors at sites different from the primary tumor. Thus, despite surgical removal of the primary tumor, it may not be possible to arrest the progress of this condition. An understanding of the mechanism whereby metastasis occurs will be crucial to the development of protocols whereby the growth of secondary tumors can be controlled. In order to understand the mechanism of metastasis, it will be necessary to provide a model which permits identification of small numbers of tumor cells against a background of many host cells so that secondary tumor emboli and micrometastases can be observed over the course of real time.

Others have demonstrated extravasation and initial seeding steps in tumor metastasis in vitro using externally fluorescently labeled tumor cells. Khokha, R. et al., Cancer Metastasis Rev (1995) 14:279–301; Koop, S. et al, Cancer Res (1995) 55:2520–2523. Further, Margolis, L.B. et al., In Vitro Cell Dev Biol (1995) 31:221–226 was able to visualize the migration of externally fluorescently labeled lung tumor cells in host mouse lung in histoculture. In all cases, however, long-term observation was not possible due to the limitation of exogenous fluorescent labels. Retroviral transfer of a green fluorescent protein (GFP) gene has been shown to result in stable transfectants of human cancer cells in vitro (Levy, J. P. et al, Nature Biotechnol (1996) 14:610–614), as well as of hematopoietic cells (Grignani, F. et al. Cancer Res (1998) 58:14–19 and by Cheng, L. et al. Gene Therapy (1997) 4:1013–1022).

Attempts have been made to provide such a model using the β-galactosidase gene as a marker (Lin, W. C. et al, Cancer Res (1990) 50:2808–2817; Lin, W.C. et al., Invasion and Metastasis (1992) 12:197–209). However, this marker has not proved satisfactory, as fresh or processed tissue cannot be used. The present invention provides a marker which permits visualization of tumor invasion and micrometastasis formation in viable fresh tissue. In addition, by providing suitable contrast media, the method of the invention can be adapted to visualize angiogenesis in established and growing tumors. The methods of the invention can be applied not only to models of tumor growth and metastasis, but, through the use of retroviral vectors, can be employed to obtain clinical data in human subjects bearing tumors.

The present invention utilizes green fluorescent protein (GFP) as a marker. Heterologous expression of this protein, principally to monitor expression of fused DNA, was disclosed in U.S. Pat. No. 5,491,084. This document describes the expression of GFP in *E. coli* and *C. elegans* and postulates that cells in general can be modified to express GFP. Such expression, according to this document, permits not only a method to monitor expression of fused DNA, but also a means of monitoring protein localization within the cell.

The aspect of the invention which provides a metastatic model has been reported and described in a series of publications. Chishima, T. et al. Cancer Research (1997) 57:2042–2047 describe the construction of a dicistronic vector containing the gene for humanized green fluorescent protein (GFP) and dihydrofolate reductase (DHFR). This vector was transfected into CHO-K1 cells to obtain clone-38. Clone-38 showed stable GFP expression which was maintained in the presence of methotrexate (MTX). Clone-38 cells were injected into mice to obtain tumor fragments which were then implanted by surgical orthotopic implantation (SOI) on the ovarian serosa in nude mice. Metastasis could be followed in this model.

Chishima, T. et al. Proc Natl Acad Sci USA (1997) 94:11573–11576 describe the preparation of clone-26 by transfection of Anip 973 human lung adenocarcinoma cells with the codon optimized hGFP-S65T clone obtained from Clontech. Clone-26 was injected intravenously into nude mice and the resulting tumors were followed in histoculture.

Chishima, T. et al. Clin Exp Metastasis (1997) 15:547–552 and Chishima, T. et al. Anticancer Res (1997) 17:2377–2384 describe similar work with clone-26 wherein the cells were inoculated subcutaneously into nude mice resulting in a visualizable tumor which was then implanted into the visceral pleura of nude mice by SOI. Metastases were observed in this model as well.

Chishima, T. et al. In Vitro Cell Dev Biol (1997) 33:745–747 describe histoculture of clone-26 and visualization of growth using the fluorescence emitted by GFP.

The contents of the foregoing publications are incorporated herein by reference.

In addition to the foregoing, Cheng, L., et al., Gene Therapy (1997) 4:1013–1022, describe the modification of hematopoietic stem cells using the GFP gene under control of a retroviral promoter. Although the authors state that human stem cells are transfected with this system only with difficulty, by using an enhanced form of the GFP, satisfactory brightness could be achieved.

In addition, Grignani, F., et al., Cancer Res (1998) 58:14–19, report the use of a hybrid EBV/retroviral vector expressing GFP to effect high-efficiency gene transfer into human hematopoietic progenitor cells.

Vectors containing various modified forms of GFP to provide various colors are marketed by Clontech. The Clontech vectors intended for mammalian cell expression place the GFP under control of the cytomegalovirus (CMV) promoter.

Disclosure of the Invention

The invention provides models which permit the intimate study of formation of metastases from primary tumors in a realistic and real-time setting. By using green fluorescent protein (GFP) as a stable and readily visualized marker, the progression of such metastasis can be modeled and the mechanism elucidated.

Thus, in one aspect, the invention is directed to a method to follow the progression of metastasis of a primary tumor, which method comprises removing fresh organ tissues from a vertebrate subject which has been modified to contain tumor cells that express GFP and observing the excised tissues for the presence of fluorescence.

In another aspect, the invention is directed to a vertebrate subject which has been modified to contain tumor cells expressing GFP.

In these aspects, the vertebrate subject may constitute a model system, such as an immunocompromised mouse wherein tumor cells or a tumor, modified to express green fluorescent protein has been introduced into the subject. Alternatively, the subject may be a human or other vertebrate which natively contains the tumor, but wherein the tumor has been subjected to viral infection or to transfection with a retroviral vector so as to produce said GFP.

In still other aspects, the invention is directed to tumor cells modified to produce GFP under control of heterologous control elements, to cells that are immortalized to provide stable cell lines as well as comprising visible amounts of GFP, to tissues containing metastatic tumors that produce GFP, and to histocultures of tissues which contain such metastasized tumors.

The invention also includes a method to observe and follow angiogenesis in solid tumors which method comprises (usually) exposing and observing said tumors. The tumors will have been modified to express GFP, and the subject will have been administered a contrast dye to permit this observation.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1*a* and 1*b* show the construction of expression vectors useful in the invention.

MODES OF CARRYING OUT THE INVENTION

The invention provides model systems for the study of the mechanism of metastasis of tumors generally, as well as to study angiogenesis in solid tumors. Advantage is taken of the visible marker green fluorescence protein (GFP) to label the tumor cells so that their migration and colonization in tissues distal to the tumor can be followed as the migration and colonization progresses. Further, by administering to the subject a contrast dye, such as rhodamine, the growth of blood vessels in solid tumors which have been labeled with GFP can also be observed.

The label used in the various aspects of the invention is green fluorescent protein (GFP). The native gene encoding this protein has been cloned from the bioluminescent jellyfish Aequorea victoria (Morin, J. et al., J Cell Physiol (1972) 77:313–318). The availability of the gene has made it possible to use GFP as a marker for gene expression. GFP itself is a 283 amino acid protein with a molecular weight of 27 kD. It requires no additional proteins from its native source nor does it require substrates or cofactors available only in its native source in order to fluoresce. (Prasher, D. C. et al., Gene (1992) 111:229–233; Yang, F. et al, Nature Biotechnol (1996) 14:1252–1256; Cody, C. W. et al., Biochemistry (1993) 32:1212–1218.) Mutants of the GFP gene have been found useful to enhance expression and to modify excitation and fluorescence. GFP-S65T (wherein serine at 65 is replaced with threonine) is particularly useful in the invention method and has a single excitation peak at 490 nm. (Heim, R. et al., Nature (1995) 373:663–664); U.S. Pat. No. 5,625,048. Other mutants have also been disclosed by Delagrade, S. et al., Biotechnology (1995) 13:151–154; Cormack, B. et al, Gene (1996) 173:33–38 and Cramer, A. et al. Nature Biotechnol (1996) 14:315–319. Additional mutants are also disclosed in U.S. Pat. No. 5,625,048. By suitable modification, the spectum of light emitted by the GFP can be altered. Thus, although the term "GFP" is used in the present application, the proteins included within this

definition are not necessarily green in appearance. Various forms of GFP exhibit colors other than green and these, too, are included within the definition of "GFP" and are useful in the methods and materials of the invention. In addition, it is noted that green fluorescent proteins falling within the definition of "GFP" herein have been isolated from other organisms, such as the sea pansy, Renilla reriformis. Any suitable and convenient form of the GFP gene can be used to modify the tumor cells useful in the models of the invention, and for retroviral transformation of endogenous tumors. The particular humanized hGFP-S65T clone is used in the examples set forth below for illustration.

Techniques for labeling cells in general using GFP are disclosed in U.S. Pat. No. 5,491,084 (supra).

In one application, the method of the invention provides a model system for studying the effects of various therapeutic candidate protocols and substances on metastatic growth of tumors.

In general, the model involves modifying a vertebrate, preferably a mammal, so as to contain tumor tissue, wherein the tumor cells have, themselves, been modified to contain an expression system for GFP. The tumor cells may arise from cell lines of the invention wherein tumor cells have been modified to contain expression systems for GFP and SV40 T-antigen. Tumors can be formed in such vertebrate systems by administering the transformed cells containing the GFP expression system and permitting these transformed cells to form tumors. Typically such administration is subcutaneous and the tumors are formed as solid masses. The tumors thus formed can be implanted in any suitable host tissue and allowed to progress, metastasize and develop.

Suitable procedures for growing the initial tumor, thus, involve transcutaneous injection of the tumor cells producing GFP, such as CHO cells, HeLa cells, carcinoma and sarcoma cell lines, well established cell lines such as the human lung adenocarcinoma line Anip 973, as well as GFP-containing human breast cancer lines MDA-MB468 and MDA-MB435; human prostate cancer lines PC3 and DU-145, human glioblastoma line 324, mouse melanoma B16 and others that may become available in the art, including the immortalized cells of the invention. The administered cells will have been modified to contain an expression system for GFP. After administration, solid tumors generally develop, typically at the site of subcutaneous injection. These tumors, which are themselves fluorescent, can then be removed and used for implantation in the model vertebrate.

Techniques for implantation of the solid tumors, now labeled with GFP, into vertebrates include direct implantation by surgical orthotopic implantation (SOI) at the desired site, typically the site from which the tumor cells were derived. Suitable sites include lung, liver, pancreas, stomach, breast, ovary, prostate, bone marrow, brain, and other tissues susceptible to malignancy. Once the solid tumors have been implanted, the vertebrate becomes a model system for studying metastasis. The tumor is thus allowed to progress and develop and the vertebrate is monitored for appearance of the GFP labeled cells at sites distal from the original implantation site. The monitoring can occur either on the whole vertebrate by opening the animal and observing the organs directly with a fluorescent microscope, or the tissues may be excised and examined microscopically. In some cases the tumors are sufficiently bright that opening the animal is unnecessary—they can be seen directly through the skin. In any case, as GFP is visible to the naked eye, no development systems to stain the tissue

samples are required. Tissue samples are simply properly processed as fresh samples in slices of suitable size, typically 1 mm thick, and placed under a microscope for examination. Even colonies of less than 10 cells are thus visible. A variety of microscopic visualization techniques is known in the art and any appropriate method can be used.

In addition, the development of the tumor can be studied in vitro in histological culture. Suitable systems for such study include solid supported cultures such as those maintained on collagen gels and the like.

Suitable vertebrate subjects for use as models are preferably mammalian subjects, most preferably convenient laboratory animals such as rabbits, rats, mice, and the like. For closer analogy to human subjects, primates could also be used. Particularly useful are subjects that are particularly susceptible to tumor development, such as subjects with impaired immune systems, typically nude mice or SCID mice. Any appropriate vertebrate subject can be used, the choice being dictated mainly by convenience and similarity to the system of ultimate interest.

Any suitable expression system operable in the tumor cells to be implanted may be used. A number of vectors are commercially available that will effect expression in tumor cells of various types. The nature of the vector may vary with the nature of the tumor and the vertebrate in which it finds its origin. However, when GFP is used to visualize metastasis in a model system, it is preferred to utilize vectors which do not use retroviral or other viral promoters which may complicate the nature of the model.

In order to provide cell lines that are helpful in establishing tumors for these model systems, it is also advantageous to employ expression vectors which provide the cells with the SV40 T-antigen. The presence of this antigen ensures immortality of the culture. Thus, particularly useful in the invention are vectors which comprise expression systems that result in the production both of GFP and SV40 T-antigen.

In order to transfect and modify the transformed cells which are effective in generating tumors, any suitable transfection method may be used, such as liposomes, calcium phosphate precipitation, electroporation and use of a gene gun. Lipofection is preferred.

In contrast, when the method of the invention is used to visualize metastasis in tumors that natively occur in a subject such as a human cancer patient, vectors that employ retroviral or other viral promoters are preferred. The use of such vectors permits the insertion of an expression system for GFP into the already existent tumor. In addition, the expression system may contain nucleotide sequence encoding other useful proteins such as therapeutic proteins which permit simultaneous diagnosis of metastasis and treatment. Among such suitable proteins are included methioninase (see, for example, PCT/US93/11311 and PCT/US96/09935). Such proteins may be produced either as fusions with the GFP, or independently either using a dicistronic expression system or independent expression systems, one for the therapeutic protein and the other for the GFP.

Retroviral based expression systems for GFP have already been described by Grignani, F. et al. Cancer Res (1998) 58:14–19 and by Cheng, L. et al. Gene Therapy (1997) 4:1013–1022. In these reports, the retroviral expression system itself was used to transfect hematopoietic progenitor cells or packaging cells were employed to provide virus-containing supernatants which can be used directly for infection of the mammalian cells. Thus, in the method of the invention, the tumor contained in the vertebrate subject is typically infected with virus which has been modified and packaged to contain the expression system for GFP. In situ infection with virus results in the ability of the tumor to produce GFP and, in effect, label itself.

Various retroviral systems useful in producing proteins in mammalian cells are known in the art. Examples include commercially available vector and packaging systems such as those sold by Clontech, San Diego, California, including their Retro-X vectors pLNCX and pLXSN which permit expression of GFP under a variety of promoters by insertion into the multiple cloning site. These vectors contain ψ* (the extended viral packaging signal) and antibiotic resistance genes for selection. A number of these systems have been developed for use in gene therapy, including vectors which provide a multiple cloning site sandwiched between 5' and 3' LTR derived from retroviral sources, and thus would be useful in labeling the tumors of human patients.

Thus, retroviral based vectors such as those set forth in FIGS. 1*a*–1*b* can be transfected into packaging cells and transferred directly to targeted cancer cells or supernatants from the packaging cells can be used to infect tumor cells with the retrovirus. Preferred combinations of retrovirus and packaging cells include the GFP-retrovirus vector pLEIN in PT-67 packaging cells. Co-culture of the packaging cells with colon cancer cells results in transfer of the GFP-retrovirus to the cancer cells.

Using histoculture techniques, and supernatants from PT-67 packaging cells generating GFP-pLEIN virus, the successful modification of a human cancer tissue to display the fluorescence associated with GFP has been demonstrated. For use in vivo, the virus is administered, preferably locally to the tumor, which can be observed within hours after injection either of packaging cells or of the viral containing supernatants. The malignant cells can be identified by their green color, sometimes sufficiently bright so that the tumors can be seen through the skin.

In addition to direct observation of tumor metastasis and growth either in a model system or in a vertebrate, typically mammalian and more typically a human subject which is already afflicted by a tumor, the methods of the invention can be adapted to observe angiogenesis in solid tumors. The tumor is itself labeled with GFP as described above. The subject is then administered a contrast dye, typically by injection, preferably intravenous injection, which allows blood vessels in the tumor to be observed. Suitable dyes include rodamine and other contrast dyes. Any dye which forms a contrasting color with the green color of the GFP can be used. Preferably, the dye is coupled to an inert polymer such as polyethylene glycol to increase the length of time the dye will remain in the blood vessel. A sufficient amount of dye is provided to permit ready visualization; the amount of dye required will depend on the choice of dye, the location of the tumor, the nature of the background GFP, and the method used for observation. Within a few minutes, vessels growing into the solid tumors in such areas as the mesentery, colon wall, and omentum can be observed. Observations can be continued over substantial periods; for example, angiogenesis after several hours is still observed by using this method.

The following examples are intended to illustrate but not to limit the invention.

EXAMPLE 1

Preparation of Tumor Cells that Produce GFP

The humanized hGFP-S65T clone described by Zolotukhin, S. et al., J Virol (1996) 70:464–4654 was used

as the green fluorescent protein coding sequence. This codon-optimized gene was purchased from Clontech Laboratories, Inc. (Palo Alto, Calif.) and ligated into the dicistronic expression vector (pED-mtx[1]) obtained from Genetics Institute, Cambridge, Mass. and described in Kaufinan, R. J. et al., Nucleic Acids Res (1991) 19:4485–4490. hGFP-S65T was digested with HindIII and blunted; the entire hGFP coding region was excised with XbaI and then unidirectionally subcloned into pED-mtx[1] which had been digested with PstI, blunted and then further digested with XbaI.

CHO-K1 cells were cultured in DMEM containing 10% fetal calf serum, 2 mM L-glutamine and 100 μM nonessential amino acids. Near confluent cells were incubated with a precipitated mixture of LipofectAMINE™ reagent (GIBCO) and saturating amounts of plasmids for six hours and then replenished with fresh medium. The cells were harvested by trypsin/EDTA 48 hours later and subcultured at 1:15 into selective medium containing 1.5 μM methotrexate (MTX). Cells with stably integrated plasmids were selected in MTX-containing medium and isolated with cloning cylinders (Bel-Art Products, Pequannock, N.J.) by EDTA. After amplification and transfer, Clone-38 was selected because of its high-intensity GFP fluorescence and stability.

In a similar manner, Anip 973 cells, a human lung cancer cell line obtained from Harbin Medical University, China, were cultured as described above for CHO-K1 cells except using RPMI1640 (GIBCO) in place of DMEM. Transfection, selection and amplification and transfer were conducted as described above. Clone-26 was chosen because of its high-intensity GFP fluorescence and stability.

EXAMPLE 2

Mouse Model Using Modified CHO Cells

Clone-38, which was stable at 1.5 μM MTX and which proliferated at the same rate as the parental CHO-K1 cells as ascertained by comparing doubling times, was used in this model.

Three six-week old Balb/C nu/nu female mice were injected subcutaneously with a single dose of 10^7 Clone-38 cells that had been harvested by trypsinization and washed three times with cold serum-containing medium and then kept on ice. The cells were injected in a total volume of 0.4 ml within 40 minutes of harvesting and the nude mice sacrificed three weeks after injection. All of the mice had a subcutaneous tumor ranging in diameter from 13.0 mm to 18.5 mm (mean=15.2 mm ±2.9 mm). The tumor tissue was strongly fluorescent. It was shown by extracting GFP from cultured Clone-38 cells in comparison to Clone-38 cells prepared from the tumor that the levels of production of GFP were the same in both.

To construct the model, tumor fragments (1 mm^3) derived from the nude mouse subcutaneous Clone-38 tumor grown as described above, were implanted by surgical or surgical orthotopic implantation (SOI) on the ovarian serosa in six nude mice as described by Fu, X. et al., Anticancer Res (1993) 13:283–286, incorporated herein by reference. Briefly, the mice were anesthetized by isofluran inhalation and an incision was made through the left lower abdominal pararectal line and peritoneum to expose the left ovary and part of the serosal membrane, which was scraped with a forceps. Four 1 mm^3 tumor pieces were fixed on the scraped site with an 8-0 nylon suture and the ovary then returned to the peritoneal cavity. The abdominal wall and skin were closed with 6-0 silk sutures.

Four weeks later, the mice were sacrificed and lung and various other organs were removed. The fresh samples were sliced at approximately 1 mm thickness and observed directly under fluorescent and confocal microscopy. Samples were also processed for histological examination for fluorescence and conventional staining. Frozen sections were prepared wherein the slides were rinsed with phosphate buffer saline and fixed for 10 minutes at 4° C.; 10% formaldehyde plus 0.2% glutaraldehyde and PBS were added and the slides were then washed with PBS. The fixed tissue was stained with hematoxylin and eosin using standard techniques.

Light and fluorescence microscopy were carried out using a Nikon microscope equipped with a Xenon lamp power supply and a GFP filter set (Chromotechnology Corp., Brattleboro, VT). Confocal microscopy was with an MRC-600 Confocal Imaging System (Bio-Rad) mounted on a Nikon microscope with an argon laser.

The mice, at sacrifice, had tumors in the ovaries ranging in diameter from 18.7 mm-25.3 mm (mean 21.9±3.1 mm). The fresh organ tissues examined under fluorescence microscopy with no treatment of the tissues showed seeding of the tumor throughout the peritoneal cavity, including the colon (6/6 mice), cecum (5/6), small intestine (4/6), spleen (1/6), and peritoneal wall (6/6). Numerous micrometastases were detected in the lungs of all mice and multiple micrometastases were also detected on the liver (1/6), kidney (1/6), contralateal ovary (3/6), adrenal gland (2/6), para-aortic lymph node (5/6) and pleural membrane (5/6). Single-cell micrometastases could not be detected by the standard histological techniques described above and even the multiple cell colonies were difficult to detect using them. As the colonies developed, the density of tumor cells decreased markedly in the center.

In an additional experiment, 5×10^6 Clone-38 cells were injected into a nude mouse through the tail vein and the mouse sacrificed after two minutes. Fresh visceral organs were analyzed by fluorescence microscopy and showed the presence of fluorescent cells in peritoneal wall vessels which formed emboli in the capillaries of the lung, liver, kidney, spleen, ovary, adrenal gland, thyroid gland and brain.

Thus, using these techniques, progression of micrometastasis can be observed as seeded cells develop into colonies within the relevant target organs. Further, screening for micrometastases can be done easily and quickly in all systemic organs.

EXAMPLE 3

Murine Model Using Human Lung Cancer Cells

The procedures are generally those set forth in Example 2 except that Clone-26 cells as prepared in Example 1 were used instead of Clone-38 CHO cells.

A. As in Example 2, tumors were grown in six-week-old Balb/C nu/nu male mice injected subcutaneously with a single 0.4 ml dose of 10^7 Clone-26 cells within 40 minutes of harvesting by trypsinization and washing three times with cold serum-containing medium. The cells were kept on ice prior to injection. The animals were sacrificed when the tumors had reached approximately 1.2 cm diameters. The 1.2 cm tumors formed after about 5 weeks.

B. The tumor pieces, 1 mm^3, were implanted by SOI into the left visceral pleura of 8 mice as described by Astoul, P. et al., Anticancer Research (1994) 14:85–92; Astoul, P. J Cell Biochem (1994) 56:9–15, both incorporated herein by reference. Briefly, the mice were anesthetized by isofluoran

inhalation an a small 1 cm transverse incision made on the left lateral chest, via the fourth intercostal space, resulting in total lung collapse. Five tumor pieces were sewn together with a 7-0 nylon surgical suture and fixed by making one knot. The lung was taken up by forceps and the tumor sewn into the lower part of the lung with one suture, after which the lung was returned to the chest cavity and the muscles and skin closed with a single layer of 6-0 silk sutures. The lung was reinflated by withdrawing air from the chest cavity with a 23-gauge needle.

C. Four of the mice were sacrificed at 4 weeks and another 4 at 8 weeks. Pleural tumors for the 4-week group ranged from 244.40 mm^3-522.88 mm^3; those from the 8 week group from 1279.08 nmm^3-2714.40 mm^3. This represented mean volumes of 371 mm^3 and 1799 mm^3. Specimens of tissue were sliced at 1 mm thickness and observed directly under fluorescent microscopy using a Nikon microscope equipped with a Xenon lamp power supply and a Leica stereo fluorescence microscope equipped with a mercury lamp power supply and GFP filter sets. All of the animals showed chest wall invasion and local and regional spread of the tumor, but in the 8-week mice, all tumors involved the mediastinum and contralateral pleural cavity as well as metastases on the visceral and parietal pleura. Pulmonary hilum lymph nodes were involved in 3 of 4 mice of the 4-week group and all of the mice in the 8-week group. Cervical node involvement was detected in one of the mice of the 8-week group, but no other metastases were observed. The animals were also observed directly before the tissues were excised. The margin of the invading tumor in normal lung tissue could be detected by GFP fluorescence and a small vessel could be seen developing at the margin of the tumor.

D. In an additional experiment, 8 nude mice were injected in the tail vein with a single dose of 1×10^7 Clone-26 cells that had been harvested by trypsinization and washed 3 times with cold serum-containing medium. The injection contained a total volume of 0.8 ml within 40 min. of harvesting. Again, 4 mice were sacrificed at 4 weeks and another 4 at 8 weeks and tissue specimens were obtained and studied by microscopy as described above. Numerous micrometastatic colonies were detected in whole lung tissue in both groups ranging from 5.2 μm to 32.5 μm in the 4-week group and 5.5 μm–178.3 μm in the 8-week group. The colonies from the 8-week group did not appear further developed as compared with those from the 4-week group. Numerous small colonies ranging in number to less than 10 cells were detected at the lung surface in both groups and brain metastases were detected in 1 mouse of the 4-week group and 2 from the 8-week group. One mouse in the 8-week group had systemic metastases in the brain, the submandibular gland, the whole lung, the pancreas, the bilateral adrenal glands, the peritoneum and the pulmonary hilum lymph nodes.

E. In an additional experiment, similar to that set forth in the previous paragraph, the mice injected in a tail vein with 10^7 Clone-26 cells were sacrificed at 4, 8 and 12 weeks and the tissues examined as described. Most of the colonies and mice sacrificed at 8 weeks were not obviously further developed compared with those sacrificed at 4 weeks, but numerous small quantities ranging in number down to less than 10 cells and ranging in size from 5.5 μm–110 μm were detected at the lung surface. At 12 weeks, there were many small metastatic colonies which appeared dormant, although other colonies grew extensively by this time, reaching a size up to 1100 μm, suggesting a heterogeneity of dormant and active tumor colonies in the lung.

EXAMPLE 4

Growth of Clone-26 Tumor Cells in Histoculture

Six-week old SCID/SCID mice were injected intravenously with a single dose of 7.5×10^7 Clone-26 cells which had been harvested by trypsinization and washed 3 times with cold serum-containing medium and kept on ice as described above. The cells were injected in a total volume of 0.5 ml within 40 minutes of harvesting. After 3 weeks, numerous micrometastic colonies were detected in whole lung tissue up to approximately 550 μm. After 5 weeks, the mice were sacrificed and the Clone-26 seeded mouse lungs were removed and histocultured on spun gels using the histoculture methods developed by Leighton, J. Cancer Res (1957) 17:929–941; Leighton, J. et al., Cancer Res (1960) 20:575–597; Hoffman, R.M. Cancer Cells (1991) 3:86–92. Tumor colonies spread rapidly in the lung tissue over time and after 1 week the tumor cells started to invade and colonize supporting collagen sponge-gel. After 2 weeks, tumor cells formed satellite colonies in the sponge-gel distant from the primary colonies in the lung tissue, thus growing faster in histoculture than in SCID mice. Tumor colonies could grow in histoculture for more than 1 month.

EXAMPLE 5

Construction of a Retroviral Expression Vector for GFP and Preparation of Labeled Tumor Cell Lines

FIGS. 1*a* and 1*b* show the construction of expression vectors for GFP under control of the SV40 promoter. The constructs employ commercially available pEGFP series vectors available from Clontech. Both bacterial and mammalian expression vectors are available which permit production of additional proteins, as well as GFP, either as fusions or in dicistronic systems. FIG. 1*a* shows the construction of an expression vector, pGFP/Met, for a fusion of GFP with methioninase; FIG. 1*b* shows the construction of a vector pGFP/SV40 for production of a fusion protein of GFP with the SV40 T-antigen.

Commercial vectors containing the GFP coding sequence of the desired spectral characteristics using the pLEIN system described in Example 6 were transfected into cell lines originating from tumors, such as human breast cancer, human prostate cancer, human glioblastoma and mouse melanoma. In this manner, human breast cancer cell lines MF-7, MDA-MB468 and MDA-MB435, human prostate cancer cell lines PC3 and DU145, human glioblastoma cell line 324, human lung cancer cells Anip-73 and H460, human colon cancer cells lines Colo-205, HCT-15 and WiDr, human gastric cancer cell line NVGC-4, human kidney cancer cell line SN12C, human tongue cancer cell line SCC-25, human melanomas LOX and SK-mel-5, labeled Chinese hamster ovary cells from cell line CHO-K1 and mouse melanoma cell line B16 labeled with green fluorescent protein were established.

The SV40 T-antigen protein is useful to immortalize cultured cells so as to establish permanent cell lines. Accordingly, the vector pGFP/SV40 is transfected into a series of tumor cell cultures to provide fluorescent immortalized cell lines.

EXAMPLE 6

In Vivo Labeling of Established Tumors

Unlabeled tumors derived from the human lung cancer cell line Anip973 were established in mice using the procedure set forth in Example 3, paragraphs A and B, but substituting unlabeled Anip973 cells for clone 26. The mice were then injected with 1×10^7 packaging cells containing the retroviral vector GFP-retrovirus pLEIN contained in PT67 cells. This virus packaging system is available from

Clontech, San Diego, Calif. pLEIN contains an insert of the coding sequence for EGFP, a red-shifted variant of wild-type GFP that has been optimized for brighter fluorescence and higher expression in mammalian cells. It has an excitation maximum of 488 nm and an position 64 from Phe to Leu and at position 65 from Ser to Thr. It is described by Comack, B. et al. Gene (1996) 173:31–38. There are more than 190 silent base changes to maximize human codon usage preferences as described by Haas, J. et al. Curr Biol (1996) 6:315–324. Thus, pLEIN contains the above-described GFP coding sequence inserted into the multiple cloning site of pLXMN to obtain a dicistronic expression system which permits coordinated translation of the GFP and neomycin resistance. Three days after injection of the cells into the peritoneal cavity of the mice, the tumor cells could be seen in the seminal vesicles under bright-field microscopy and under fluorescent microscopy.

EXAMPLE 7

Observation of Angiogenesis

A suspension containing 1×10^7 clone-38 cells, described in Example 1, were injected into the peritoneal cavity of a mouse. Five days later, the mouse was injected in the tail with rhodamine and the mouse was then put under anesthesia and the abdominal cavity opened sufficiently to visualize the tumor. Recovery from this surgery is straightforward. In some cases, abdominal opening is unnecessary as the intraperitoneal tumors can be visualized through intact skin. Tumors were visible in the abdominal cavity and angiogenesis was apparent as identified by the rhodamine fluorescence. Similar results were found in tumors growing in the omentum in the wall of the small intestine, and in the mesentery.

In an analogous experiment, a suspension containing 1×10^7 cells of clone-26, described in Example 1, were injected into the peritoneal cavity of a mouse. After one day, tumors appeared in the mesentery and in the colon wall. These were observed by anesthetizing the mouse and a minimal opening of the abdomen. Observations on day 3 of a similarly treated mouse showed tumors in the wall of the small intestine and in the omentum as well as in the colon wall and mesentery. On day 5, a similarly treated mouse was injected in the tail with 100 μl of 2×10^{-3} M rhodamine and a few vessels could be seen in the tumor growing in the mesentery. After day 60, numerous vessels were seen in the tumor growing in the colon wall.

What is claimed is:

1. A method to observe angiogenesis in at least one solid tumor contained in a first mammalian subject, which method comprises optionally exposing at least one tissue containing said tumor of said subject and observing the blood vessels in said tumor, wherein said blood vessels contain a fluorescent contrast dye and said tumor expresses green fluorescent protein (GFP) whereby the presence of angiogenesis is observable by the contrast dye in the blood vessels seen against the GFP of the tumor,

wherein said subject has been admistered said contrast dye by intravenous injection;

wherein said solid tumor that expresses GFP has been obtained by a method which comprises providing a tumor cell line comprising an expression system for said GFP;

injecting a second mammalian subject which is genetically immunocompromised or syngeneic with said tumor cell line subcutaneously with said cell line to convert said cell line to a subcutaneous tumor; and

surgically implanting said subcutaneous tumor into said first mammalian subject which is genetically immunocompromised or syngeneic with said tumor cell line in a location orhotopic to said tumor cell line; or

wherein said tumor is endogenous to said first mammalian subject and has been modified to express GFP by a method which comprises infecting said endogenous tumor contained in said first mammalian subject with a retroviral vector derived from pLEIN comprising an expression system for said GFP by injecting said vector locally to said tumor.

2. The method of claim **1** wherein said contrast dye is rhodamine.

3. The method of claim **1** wherein said GFP is hGFP-S65T.

4. The method of claim **1** wherein said tumor is a lung tumor or an ovarian tumor.

5. The method of claim **1** wherein said first mammalian subject is a rabbit, rat or mouse.

6. The method of claim **1** wherein said first mammalian subject is human.

7. A non-human mammalian subject which contains a solid tumor expressing GFP and which has, contained in its blood vessels, a fluorescent contrast dye administered by intravenous injection.

8. A non-human mammalian subject which contains a solid tumor expressing GFP and which has, contained in its blood vessels, a contrast dye administered by intravenous injection, wherein said subject is prepared by the method of claim **1**.

* * * * *

METASTASIS MODELS USING GREEN FLUORESCENT PROTEIN (GFP) AS A MARKER

This application is a Continuation of U.S. Ser. No. 09/226,856, filed Jan. 7, 1999, now U.S. Pat. No. 6,251,384, which is a Continuation-in-part of U.S. Ser. No. 09/067,734, filed Apr. 28, 1998, now U.S. Pat. No. 6,235,968, which is a Continuation-in-part of U.S. Ser. No. 09/049,544, filed March 27, 1998, now U.S. Pat. No. 5,235,967, which is a Continuation-in-part of U.S. Ser. No. 08/848,539, filed April 28, 1997, now U.S. Pat. No. 6,232,523, the contents of which are incorporated by reference.

TECHNICAL FIELD

The invention relates to the study of tumor progression. Specifically, it concerns model systems for studying the metastasis of tumors in vertebrate systems and to models and methods for evaluating candidate drugs.

BACKGROUND ART

It has long been recognized that the ability of tumor tissues to metastasize constitutes a major portion of the life-threatening aspects of malignancy. Metastasis is the growth of secondary tumors at sites different from the primary tumor. Thus, despite surgical removal of the primary tumor, it may not be possible to arrest the progress of this condition. An understanding of the mechanism whereby metastasis occurs will be crucial to the development of protocols whereby the growth of secondary tumors can be controlled. In order to understand the mechanism of metastasis, it will be necessary to provide a model which permits identification of small numbers of tumor cells against a background of many host cells so that secondary tumor emboli and micrometastases can be observed over the course of real time.

Others have demonstrated extravasation and initial seeding steps in tumor metastasis in vitro using externally fluorescently labeled tumor cells. Khokha, R. et al., *Cancer Metastasis Rev* (1995) 14:279–301; Koop, S. et al., *Cancer Res* (1995) 55:2520–2523. Further, Margolis, L. B. et al., *In Vitro Cell Dev Biol* (1995) 31:221–226 was able to visualize the migration of externally fluorescently labeled lung tumor cells in host mouse lung in histoculture. In all cases, however, long-term observation was not possible due to the limitation of exogenous fluorescent labels. Retroviral transfer of a green fluorescent protein (GFP) gene has been shown to result in stable transfectants of human cancer cells in vitro (Levy, J. P. et al., *Nature Biotechnol* (1996) 14:610–614), as well as of hematopoietic cells (Grignani, F. et al. *Cancer Res* (1998) 58:14–19 and by Cheng, L. et al. *Gene Therapy* (1997) 4:1013–1022).

Attempts have been made to provide such a model using the β-galactosidase gene as a marker (Lin, W. C. et al., *Cancer Res* (1990) 50:2808–2817; Lin, W. C. et al., *Invasion and Metastasis* (1992) 12:197–209). However, this marker has not proved satisfactory, as fresh or processed tissue cannot be used. The present invention provides a marker which permits visualization of tumor invasion and micrometastasis formation in viable fresh tissue. In addition, by providing suitable contrast media, the method of the invention can be adapted to visualize angiogenesis in established and growing tumors. The methods of the invention can be applied not only to models of tumor growth and metastasis, but, through the use of retroviral vectors, can be employed to obtain clinical data in human subjects bearing tumors.

The present invention utilizes green fluorescent protein (GFP) as a marker. Heterologous expression of this protein, principally to monitor expression of fused DNA, was disclosed in U.S. Pat. No. 5,491,084. This document describes the expression of GFP in *E. coli* and *C. elegans* and postulates that cells in general can be modified to express GFP. Such expression, according to this document, permits not only a method to monitor expression of fused DNA, but also a means of monitoring protein localization within the cell.

The aspect of the invention which provides a metastatic model has been reported and described in a series of publications. Chishima, T. et al. *Cancer Research* (1997) 57:2042–2047 describe the construction of a dicistronic vector containing the gene for humanized green fluorescent protein (GFP) and dihydrofolate reductase (DHFR). This vector was transfected into CHO-K1 cells to obtain clone-38. Clone-38 showed stable GFP expression which was maintained in the presence of methotrexate (MTX). Clone-38 cells were injected into mice to obtain tumor fragments which were then implanted by surgical orthotopic implantation (SOI) on the ovarian serosa in nude mice. Metastasis could be followed in this model.

Chishima, T. et al. *Proc Natl Acad Sci USA* (1997) 94:11573–11576 describe the preparation of clone-26 by transfection of Anip 973 human lung adenocarcinoma cells with the codon optimized hGFP-S65T clone obtained from Clontech. Clone-26 was injected intravenously into nude mice and the resulting tumors were followed in histoculture.

Chishima, T. et al. *Clin Exp Metastasis* (1997) 15:547–552 and Chishima, T. et al. *Anticancer Res* (1997) 17:2377–2384 describe similar work with clone-26 wherein the cells were inoculated subcutaneously into nude mice resulting in a visualizable tumor which was then implanted into the visceral pleura of nude mice by SOI. Metastases were observed in this model as well.

Chishima, T. et al. *In Vitro Cell Dev Biol* (1997) 33:745–747 describe histoculture of clone-26 and visualization of growth using the fluorescence emitted by GFP.

Yang, M., et al., *Cancer Res* (1998) 58:4217–4221 describe transduction of the human lung cancer cell line H460 with a retroviral expression vector containing enhanced GFP to obtain a stable high-GFP-expressing clone. Cells from this cell line were injected into nude mice and the resulting subcutaneously growing labeled tumors were transplanted by SOI into the left lung of nude mice. Fluorescence could then be observed from the metastases in the collateral lung, pleural membrane and throughout the skeletal system.

Yang, M., et al., *Cancer Res* (In Press) report similar studies using a model for prostate tumor and showing fluorescence throughout the skeletal system in nude mice.

The contents of the foregoing publications are incorporated herein by reference.

In addition to the foregoing, Cheng, L., et al., *Gene Therapy* (1997) 4:1013–1022, describe the modification of hematopoietic stem cells using the GFP gene under control of a retroviral promoter. Although the authors state that human stem cells are transfected with this system only with difficulty, by using an enhanced form of the GFP, satisfactory brightness could be achieved.

In addition, Grignani, F., et al., *Cancer Res* (1998) 58:14–19, report the use of a hybrid EBV/retroviral vector expressing GFP to effect high-efficiency gene transfer into human hematopoietic progenitor cells.

Vectors containing various modified forms of GFP to provide various colors are marketed by Clontech. The Clon-

tech vectors intended for mammalian cell expression place the GFP under control of the cytomegalovirus (CMV) promoter.

DISCLOSURE OF THE INVENTION

The invention provides models which permit the intimate study of formation of metastases from primary tumors in a realistic and real-time setting. By using green fluorescent protein (GFP) as a stable and readily visualized marker, the progression of such metastasis can be modeled and the mechanism elucidated.

Thus, in one aspect, the invention is directed to a method to follow the progression of metastasis of a primary tumor, which method comprises removing fresh organ tissues from a vertebrate subject which has been modified to contain tumor cells that express GFP and observing the excised tissues for the presence of fluorescence.

In one embodiment, however, it is unnecessary to remove organ tissues; rather, the fluorescence can be visualized in the whole animal by real-time fluorescence optical tumor imaging (FOTI).

In another aspect, the invention is directed to a vertebrate subject which has been modified to contain tumor cells expressing GFP.

In these aspects, the vertebrate subject may constitute a model system, such as an immunocompromised mouse wherein tumor cells or a tumor, modified to express green fluorescent protein has been introduced into the subject. The model system may be used to evaluate candidate drugs for their capacity to inhibit metastasis. Alternatively, the subject may be a human or other vertebrate which natively contains the tumor, but wherein the tumor has been subjected to viral infection or to transfection with a retroviral vector so as to produce said GFP. The efficacy of drugs administered to such patients can be evaluated by following the course of metastasis in the subject.

In still other aspects, the invention is directed to tumor cells modified to produce GFP under control of heterologous control elements, to cells that are immortalized to provide stable cell lines as well as comprising visible amounts of GFP, to tissues containing metastatic tumors that produce GFP, and to histocultures of tissues which contain such metastasized tumors.

The invention also includes a method to observe and follow angiogenesis in solid tumors which method comprises (usually) exposing and observing said tumors. The tumors will have been modified to express GFP, and the subject will have been administered a contrast dye to permit this observation.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1*a* and 1*b* show the construction of expression vectors useful in the invention.

MODES OF CARRYING OUT THE INVENTION

The invention provides model systems for the study of the mechanism of metastasis of tumors generally, as well as to study angiogenesis in solid tumors. Advantage is taken of the visible marker green fluorescence protein (GFP) to label the tumor cells so that their migration and colonization in tissues distal to the tumor can be followed as the migration and colonization progresses. Further, by administering to the subject a contrast dye, such as rhodamine, the growth of blood vessels in solid tumors which have been labeled with GFP can also be observed.

Since sufficient intensity can be achieved to observe the migration of fluorescent cells in the intact animal, in addition to determining the migration of the cells by excising organs, the progression of metastasis can be observed in the intact subject. Either or both methods may be employed to observe metastasis in evaluating, in model systems, the efficacy of potential antimetastatic drugs. The success or failure of treatments provided to patients with potentially metastatic cancers can also be followed using the materials and methods of the invention.

The label used in the various aspects of the invention is green fluorescent protein (GFP). The native gene encoding this protein has been cloned from the bioluminescent jellyfish *Aequorea victoria* (Morin, J. et al., *J Cell Physiol* (1972) 77:313–318). The availability of the gene has made it possible to use GFP as a marker for gene expression. GFP itself is a 283 amino acid protein with a molecular weight of 27 kD. It requires no additional proteins from its native source nor does it require substrates or cofactors available only in its native source in order to fluoresce. (Prasher, D.C. et al., *Gene* (1992) 111:229–233; Yang, F. et al., *Nature Biotechnol* (1996) 14:1252–1256; Cody, C. W. et al., *Biochemistry* (1993) 32:1212–1218.) Mutants of the GFP gene have been found useful to enhance expression and to modify excitation and fluorescence. GFP-S65T (wherein serine at 65 is replaced with threonine) is particularly useful in the invention method and has a single excitation peak at 490 nm. (Heim, R. et al., *Nature* (1995) 373:663–664); U.S. Pat. No. 5,625,048. Other mutants have also been disclosed by Delagrade, S. et al., *Biotechnology* (1995) 13:151–154; Cormack, B. et al., *Gene* (1996) 173:33–38 and Cramer, A. et al. *Nature Biotechnol* (1996) 14:315–319. Additional mutants are also disclosed in U.S. Pat. No. 5,625,048. By suitable modification, the spectrum of light emitted by the GFP can be altered. Thus, although the term "GFP" is used in the present application, the proteins included within this definition are not necessarily green in appearance. Various forms of GFP exhibit colors other than green and these, too, are included within the definition of "GFP" and are useful in the methods and materials of the invention. In addition, it is noted that green fluorescent proteins falling within the definition of "GFP" herein have been isolated from other organisms, such as the sea pansy, *Renilla reriformis*. Any suitable and convenient form of the GFP gene can be used to modify the tumor cells useful in the models of the invention, and for retroviral transformation of endogenous tumors. The particular humanized hGFP-S65T clone is used in the examples set forth below for illustration.

Techniques for labeling cells in general using GFP are disclosed in U.S. Pat. No. 5,491,084 (supra).

In one application, the method of the invention provides a model system for studying the effects of various therapeutic candidate protocols and substances on metastatic growth of tumors.

In general, the model involves modifying a vertebrate, preferably a mammal, so as to contain tumor tissue, wherein the tumor cells have, themselves, been modified to contain an expression system for GFP. The tumor cells may arise from cell lines of the invention wherein tumor cells have been modified to contain expression systems for GFP and SV40 T-antigen. Tumors can be formed in such vertebrate systems by administering the transformed cells containing the GFP expression system and permitting these transformed cells to form tumors. Typically such administration is subcutaneous and the tumors are formed as solid masses. The tumors thus formed can be implanted in any suitable host tissue and allowed to progress, metastasize and develop.

Suitable procedures for growing the initial tumor, thus, involve transcutaneous injection of the tumor cells producing GFP, such as CHO cells, HeLa cells, carcinoma and sarcoma cell lines, well established cell lines such as the human lung adenocarcinoma line Anip 973, or lung cancer cell line H460 as well as GFP-containing human breast cancer lines MDA-MB468 and MDA-MB435; human prostate cancer lines PC3 and DU-145, human glioblastoma line 324, mouse melanoma B16 and others that may become available in the art, including the immortalized cells of the invention. The administered cells will have been modified to contain an expression system for GFP. After administration, solid tumors generally develop, typically at the site of subcutaneous injection. These tumors, which are themselves fluorescent, can then be removed and used for implantation in the model vertebrate.

Techniques for implantation of the solid tumors, now labeled with GFP, into vertebrates include direct implantation by surgical orthotopic implantation (SOI) at the desired site, typically the site from which the tumor cells were derived. Suitable sites include lung, liver, pancreas, stomach, breast, ovary, prostate, bone marrow, brain, and other tissues susceptible to malignancy. Once the solid tumors have been implanted, the vertebrate becomes a model system for studying metastasis. The tumor is thus allowed to progress and develop and the vertebrate is monitored for appearance of the GFP labeled cells at sites distal from the original implantation site. The monitoring can occur either on the whole vertebrate by opening the animal and observing the organs directly with a fluorescent microscope, or the tissues may be excised and examined microscopically. In some cases the tumors are sufficiently bright that opening the animal is unnecessary—they can be seen directly through the skin. In any case, as GFP is visible to the naked eye, no development systems to stain the tissue samples are required. Tissue samples are simply properly processed as fresh samples in slices of suitable size, typically 1 mm thick, and placed under a microscope for examination. Even colonies of less than 10 cells are thus visible. A variety of microscopic visualization techniques is known in the art and any appropriate method can be used.

It is particularly convenient to visualize the migration of tumor cells in the intact animal through fluorescent optical tumor imaging (FOTI). This permits real-time observation and monitoring of progression of metastasis on a continuous basis, in particular, in model systems, in evaluation of potential anti-metastatic drugs. Thus, the relative lack of metastasis observed directly in test animals administered a candidate drug in comparison to controls which have not been administered the drugs indicates the efficacy of the candidate and its potential as a treatment. In subjects being treated for cancer, the availability of FOTI permits those devising treatment protocols to be informed on a continuous basis of the advisability of modifying or not modifying the protocol.

In addition, the development of the tumor can be studied in vitro in histological culture. Suitable systems for such study include solid supported cultures such as those maintained on collagen gels and the like.

Suitable vertebrate subjects for use as models are preferably mammalian subjects, most preferably convenient laboratory animals such as rabbits, rats, mice, and the like. For closer analogy to human subjects, primates could also be used. Particularly useful are subjects that are particularly susceptible to tumor development, such as subjects with impaired immune systems, typically nude mice or SCID mice. Any appropriate vertebrate subject can be used, the choice being dictated mainly by convenience and similarity to the system of ultimate interest.

Any suitable expression system operable in the tumor cells to be implanted may be used. A number of vectors are commercially available that will effect expression in tumor cells of various types. The nature of the vector may vary with the nature of the tumor and the vertebrate in which it finds its origin. However, when GFP is used to visualize metastasis in a model system, it is preferred to utilize vectors which do not use retroviral or other viral promoters which may complicate the nature of the model.

In order to provide cell lines that are helpful in establishing tumors for these model systems, it is also advantageous to employ expression vectors which provide the cells with the SV40 T-antigen. The presence of this antigen ensures immortality of the culture. Thus, particularly useful in the invention are vectors which comprise expression systems that result in the production both of GFP and SV40 T-antigen.

In order to transfect and modify the transformed cells which are effective in generating tumors, any suitable transfection method may be used, such as liposomes, calcium phosphate precipitation, electroporation and use of a gene gun. Lipofection is preferred.

In contrast, when the method of the invention is used to visualize metastasis in tumors that natively occur in a subject such as a human cancer patient, vectors that employ retroviral or other viral promoters are preferred. The use of such vectors permits the insertion of an expression system for GFP into the already existent tumor. In addition, the expression system may contain nucleotide sequence encoding other useful proteins such as therapeutic proteins which permit simultaneous diagnosis of metastasis and treatment. Among such suitable proteins are included methioninase (see, for example, PCT/US93/11311 and PCT/US96/09935). Such proteins may be produced either as fusions with the GFP, or independently either using a dicistronic expression system or independent expression systems, one for the therapeutic protein and the other for the GFP.

Retroviral based expression systems for GFP have already been described by Grignani, F. et al. *Cancer Res* (1998) 58:14–19 and by Cheng, L. et al. *Gene Therapy* (1997) 4:1013–1022. In these reports, the retroviral expression system itself was used to transfect hematopoietic progenitor cells or packaging cells were employed to provide virus-containing supernatants which can be used directly for infection of the mammalian cells. Thus, in the method of the invention, the tumor contained in the vertebrate subject is typically infected with virus which has been modified and packaged to contain the expression system for GFP. In situ infection with virus results in the ability of the tumor to produce GFP and, in effect, label itself.

Various retroviral systems useful in producing proteins in mammalian cells are known in the art. Examples include commercially available vector and packaging systems such as those sold by Clontech, San Diego, Calif., including their Retro-X vectors pLNCX and pLXSN which permit expression of GFP under a variety of promoters by insertion into the multiple cloning site. These vectors contain ψ* (the extended viral packaging signal) and antibiotic resistance genes for selection. A number of these systems have been developed for use in gene therapy, including vectors which provide a multiple cloning site sandwiched between 5' and 3' LTR derived from retroviral sources, and thus would be useful in labeling the tumors of human patients.

Thus, retroviral based vectors such as those set forth in FIGS. 1*a*–1*b* can be transfected into packaging cells and

transferred directly to targeted cancer cells or supernatants from the packaging cells can be used to infect tumor cells with the retrovirus. Preferred combinations of retrovirus and packaging cells include the GFP-retrovirus vector pLEIN in PT-67 packaging cells. Co-culture of the packaging cells with colon cancer cells results in transfer of the GFP-retrovirus to the cancer cells.

Using histoculture techniques, and supernatants from PT-67 packaging cells generating GFP-pLEIN virus, the successful modification of a human cancer tissue to display the fluorescence associated with GFP has been demonstrated. For use in vivo, the virus is administered, preferably locally to the tumor, which can be observed within hours after injection either of packaging cells or of the viral containing supernatants. The malignant cells can be identified by their green color, sometimes sufficiently bright so that the tumors can be seen through the skin.

In addition to direct observation of tumor metastasis and growth either in a model system or in a vertebrate, typically mammalian and more typically a human subject which is already afflicted by a tumor, the methods of the invention can be adapted to observe angiogenesis in solid tumors. The tumor is itself labeled with GFP as described above. The subject is then administered a contrast dye, typically by injection, preferably intravenous injection, which allows blood vessels in the tumor to be observed. Suitable dyes include rodamine and other contrast dyes. Any dye which forms a contrasting color with the green color of the GFP can be used. Preferably, the dye is coupled to an inert polymer such as polyethylene glycol to increase the length of time the dye will remain in the blood vessel. A sufficient amount of dye is provided to permit ready visualization; the amount of dye required will depend on the choice of dye, the location of the tumor, the nature of the background GFP, and the method used for observation. Within a few minutes, vessels growing into the solid tumors in such areas as the mesentery, colon wall, and omentum can be observed. Observations can be continued over substantial periods; for example, angiogenesis after several hours is still observed by using this method.

The following examples are intended to illustrate but not to limit the invention.

EXAMPLE 1

Preparation of Tumor Cells that Produce GFP

The humanized hGFP-S65T clone described by Zolotukhin, S. et al., *J Virol* (1996) 70:4646–4654 was used as the green fluorescent protein coding sequence. This codon-optimized gene was purchased from Clontech Laboratories, Inc. (Palo Alto, Calif.) and ligated into the dicistronic expression vector (pED-mtx[1]) obtained from Genetics Institute, Cambridge, Mass. and described in Kaufman, R. J. et al., *Nucleic Acids Res* (1991) 19:4485–4490. hGFP-S65T was digested with HindIII and blunted; the entire hGFP coding region was excised with XbaI and then unidirectionally subcloned into pED-mtx[1] which had been digested with PstI, blunted and then further digested with XbaI.

CHO-K1 cells were cultured in DMEM containing 10% fetal calf serum, 2 mM L-glutamine and 100 μM nonessential amino acids. Near confluent cells were incubated with a precipitated mixture of LipofectAMINE™ reagent (GIBCO) and saturating amounts of plasmids for six hours and then replenished with fresh medium. The cells were harvested by trypsin/EDTA 48 hours later and subcultured at 1:15 into selective medium containing 1.5 μM methotrexate (MTX). Cells with stably integrated plasmids were selected in MTX-containing medium and isolated with cloning cylinders (Bel-Art Products, Pequannock, N.J.) by EDTA. After amplification and transfer, Clone-38 was selected because of its high-intensity GFP fluorescence and stability.

In a similar manner, Anip 973 cells, a human lung cancer cell line obtained from Harbin Medical University, China, were cultured as described above for CHO-K1 cells except using RPMI1640 (GIBCO) in place of DMEM. Transfection, selection and amplification and transfer were conducted as described above. Clone-26 was chosen because of its high-intensity GFP fluorescence and stability.

EXAMPLE 2

Mouse Model Using Modified CHO Cells

Clone-38, which was stable at 1.5 μM MTX and which proliferated at the same rate as the parental CHO-K1 cells as ascertained by comparing doubling times, was used in this model.

Three six-week old Balb/C nu/nu female mice were injected subcutaneously with a single dose of 10^7 Clone-38 cells that had been harvested by trypsinization and washed three times with cold serum-containing medium and then kept on ice. The cells were injected in a total volume of 0.4 ml within 40 minutes of harvesting and the nude mice sacrificed three weeks after injection. All of the mice had a subcutaneous tumor ranging in diameter from 13.0 mm to 18.5 mm (mean=15.2 mm±2.9 mm). The tumor tissue was strongly fluorescent. It was shown by extracting GFP from cultured Clone-38 cells in comparison to Clone-38 cells prepared from the tumor that the levels of production of GFP were the same in both.

To construct the model, tumor fragments (1 mm^3) derived from the nude mouse subcutaneous Clone-38 tumor grown as described above, were implanted by surgical or surgical orthotopic implantation (SOI) on the ovarian serosa in six nude mice as described by Fu, X. et al., *Anticancer Res* (1993) 13:283–286, incorporated herein by reference. Briefly, the mice were anesthetized by isofluran inhalation and an incision was made through the left lower abdominal pararectal line and peritoneum to expose the left ovary and part of the serosal membrane, which was scraped with a forceps. Four 1 mm^3 tumor pieces were fixed on the scraped site with an 8–0 nylon suture and the ovary then returned to the peritoneal cavity. The abdominal wall and skin were closed with 6–0 silk sutures.

Four weeks later, the mice were sacrificed and lung and various other organs were removed. The fresh samples were sliced at approximately 1 mm thickness and observed directly under fluorescent and confocal microscopy. Samples were also processed for histological examination for fluorescence and conventional staining. Frozen sections were prepared wherein the slides were rinsed with phosphate buffer saline and fixed for 10 minutes at 4° C.; 10% formaldehyde plus 0.2% glutaraldehyde and PBS were added and the slides were then washed with PBS. The fixed tissue was stained with hematoxylin and eosin using standard techniques.

Light and fluorescence microscopy were carried out using a Nikon microscope equipped with a Xenon lamp power supply and a GFP filter set (Chromotechnology Corp., Brattleboro, Vt.). Confocal microscopy was with an MRC-600 Confocal Imaging System (Bio-Rad) mounted on a Nikon microscope with an argon laser.

The mice, at sacrifice, had tumors in the ovaries ranging in diameter from 18.7 mm–25.3 mm (mean 21.9±3.1 mm). The fresh organ tissues examined under fluorescence microscopy with no treatment of the tissues showed seeding of the tumor throughout the peritoneal cavity, including the colon (6/6 mice), cecum (5/6), small intestine (4/6), spleen (1/6), and peritoneal wall (6/6). Numerous micrometastases were detected in the lungs of all mice and multiple micrometastases were also detected on the liver (1/6), kidney (1/6), contralateral ovary (3/6), adrenal gland (2/6), para-aortic lymph node (5/6) and pleural membrane (5/6). Single-cell micrometastases could not be detected by the standard histological techniques described above and even the multiple cell colonies were difficult to detect using them. As the colonies developed, the density of tumor cells decreased markedly in the center.

In an additional experiment, 5×10^6 Clone-38 cells were injected into a nude mouse through the tail vein and the mouse sacrificed after two minutes. Fresh visceral organs were analyzed by fluorescence microscopy and showed the presence of fluorescent cells in peritoneal wall vessels which formed emboli in the capillaries of the lung, liver, kidney, spleen, ovary, adrenal gland, thyroid gland and brain.

Thus, using these techniques, progression of micrometastasis can be observed as seeded cells develop into colonies within the relevant target organs. Further, screening for micrometastases can be done easily and quickly in all systemic organs.

EXAMPLE 3

Murine Model Using Human Lung Cancer Cells

The procedures are generally those set forth in Example 2 except that Clone-26 cells as prepared in Example 1 were used instead of Clone-38 CHO cells.

A. As in Example 2, tumors were grown in six-week-old Balb/C nu/nu male mice injected subcutaneously with a single 0.4 ml dose of 10^7 Clone-26 cells within 40 minutes of harvesting by trypsinization and washing three times with cold serum-containing medium. The cells were kept on ice prior to injection. The animals were sacrificed when the tumors had reached approximately 1.2 cm diameters. The 1.2 cm tumors formed after about 5 weeks.

B. The tumor pieces, 1 mm^3, were implanted by SOI into the left visceral pleura of 8 mice as described by Astoul, P. et al., *Anticancer Research* (1994) 14:85–92; Astoul, P. *J Cell Biochem* (1994) 56:9–15, both incorporated herein by reference. Briefly, the mice were anesthetized by isofluoran inhalation an a small 1 cm transverse incision made on the left lateral chest, via the fourth intercostal space, resulting in total lung collapse. Five tumor pieces were sewn together with a 7–0 nylon surgical suture and fixed by making one knot. The lung was taken up by forceps and the tumor sewn into the lower part of the lung with one suture, after which the lung was returned to the chest cavity and the muscles and skin closed with a single layer of 6–0 silk sutures. The lung was reinflated by withdrawing air from the chest cavity with a 23-gauge needle.

C. Four of the mice were sacrificed at 4 weeks and another 4 at 8 weeks. Pleural tumors for the 4-week group ranged from 244.40 mm^3–522.88 mm^3; those from the 8 week group from 1279.08 mm^3–2714.40 mm^3. This represented mean volumes of 371 mm^3 and 1799 mm^3. Specimens of tissue were sliced at 1 mm thickness and observed directly under fluorescent microscopy using a Nikon microscope equipped with a Xenon lamp power supply and a Leica stereo fluorescence microscope equipped with a mercury lamp power supply and GFP filter sets. All of the animals showed chest wall invasion and local and regional spread of the tumor, but in the 8-week mice, all tumors involved the mediastinum and contralateral pleural cavity as well as metastases on the visceral and parietal pleura. Pulmonary hilum lymph nodes were involved in 3 of 4 mice of the 4-week group and all of the mice in the 8-week group. Cervical node involvement was detected in one of the mice of the 8-week group, but no other metastases were observed. The animals were also observed directly before the tissues were excised. The margin of the invading tumor in normal lung tissue could be detected by GFP fluorescence and a small vessel could be seen developing at the margin of the tumor.

D. In an additional experiment, 8 nude mice were injected in the tail vein with a single dose of 1×10^7 Clone-26 cells that had been harvested by trypsinization and washed 3 times with cold serum-containing medium. The injection contained a total volume of 0.8 ml within 40 min. of harvesting. Again, 4 mice were sacrificed at 4 weeks and another 4 at 8 weeks and tissue specimens were obtained and studied by microscopy as described above. Numerous micrometastatic colonies were detected in whole lung tissue in both groups ranging from 5.2 μm to 32.5 μm in the 4-week group and 5.5 μm–178.3 μm in the 8-week group. The colonies from the 8-week group did not appear further developed as compared with those from the 4-week group. Numerous small colonies ranging in number to less than 10 cells were detected at the lung surface in both groups and brain metastases were detected in 1 mouse of the 4-week group and 2 from the 8-week group. One mouse in the 8-week group had systemic metastases in the brain, the submandibular gland, the whole lung, the pancreas, the bilateral adrenal glands, the peritoneum and the pulmonary hilum lymph nodes.

E. In an additional experiment, similar to that set forth in the previous paragraph, the mice injected in a tail vein with 10^7 Clone-26 cells were sacrificed at 4, 8 and 12 weeks and the tissues examined as described. Most of the colonies and mice sacrificed at 8 weeks were not obviously further developed compared with those sacrificed at 4 weeks, but numerous small quantities ranging in number down to less than 10 cells and ranging in size from 5.5 μm–110 μm were detected at the lung surface. At 12 weeks, there were many small metastatic colonies which appeared dormant, although other colonies grew extensively by this time, reaching a size up to 1100 μM, suggesting a heterogeneity of dormant and active tumor colonies in the lung.

EXAMPLE 4

Growth of Clone-26 Tumor Cells in Histoculture

Six-week old SCID/SCID mice were injected intravenously with a single dose of 7.5×10^7 Clone-26 cells which had been harvested by trypsinization and washed 3 times with cold serum-containing medium and kept on ice as described above. The cells were injected in a total volume of 0.5 ml within 40 minutes of harvesting. After 3 weeks, numerous micrometastatic colonies were detected in whole lung tissue up to approximately 550 μm. After 5 weeks, the mice were sacrificed and the Clone-26 seeded mouse lungs were removed and histocultured on spun gels using the histoculture methods developed by Leighton, J. *Cancer Res* (1957) 17:929–941; Leighton, J. et al., *Cancer Res* (1960) 20:575–597; Hoffman, R. M. *Cancer Cells* (1991) 3:86–92. Tumor colonies spread rapidly in the lung tissue over time

and after 1 week the tumor cells started to invade and colonize supporting collagen sponge-gel. After 2 weeks, tumor cells formed satellite colonies in the sponge-gel distant from the primary colonies in the lung tissue, thus growing faster in histoculture than in SCID mice. Tumor colonies could grow in histoculture for more than 1 month.

EXAMPLE 5

Construction of a Retroviral Expression Vector for GFP and Preparation of Labeled Tumor Cell Lines

FIGS. 1*a* and 1*b* show the construction of expression vectors for GFP under control of the SV40 promoter. The constructs employ commercially available pEGFP series vectors available from Clontech. Both bacterial and mammalian expression vectors are available which permit production of additional proteins, as well as GFP, either as fusions or in dicistronic systems. FIG. 1*a* shows the construction of an expression vector, pGFP/Met, for a fusion of GFP with methioninase; FIG. 1*b* shows the construction of a vector pGFP/SV40 for production of a fusion protein of GFP with the SV40 T-antigen.

Commercial vectors containing the GFP coding sequence of the desired spectral characteristics using the pLEIN system described in Example 6 were transfected into cell lines originating from tumors, such as human breast cancer, human prostate cancer, human glioblastoma and mouse melanoma. In this manner, human breast cancer cell lines MF-7, MDA-MB468 and MDA-MB435, human prostate cancer cell lines PC3 and DU145, human glioblastoma cell line 324, human lung cancer cells Anip-73 and H460, human colon cancer cells lines Colo-205, HCT-15 and WiDr, human gastric cancer cell line NVGC-4, human kidney cancer cell line SN12C, human tongue cancer cell line SCC-25, human melanomas LOX and SK-mel-5, labeled Chinese hamster ovary cells from cell line CHO-K1 and mouse melanoma cell line B16 labeled with green fluorescent protein were established.

The SV40 T-antigen protein is useful to immortalize cultured cells so as to establish permanent cell lines. Accordingly, the vector pGFP/SV40 is transfected into a series of tumor cell cultures to provide fluorescent immortalized cell lines.

EXAMPLE 6

In Vivo Labeling of Established Tumors

Unlabeled tumors derived from the human lung cancer cell line Anip973 were established in mice using the procedure set forth in Example 3, paragraphs A and B, but substituting unlabeled Anip973 cells for clone 26. The mice were then injected with 1×10^7 packaging cells containing the retroviral vector GFP-retrovirus pLEIN contained in PT67 cells. This virus packaging system is available from Clontech, San Diego, Calif. pLEIN contains an insert of the coding sequence for EGFP, a red-shifted variant of wild-type GFP that has been optimized for brighter fluorescence and higher expression in mammalian cells. It has an excitation maximum of 488 nm and an emission maximum at 507 nm. This mutant contains a double amino acid substitution at position 64 from Phe to Leu and at position 65 from Ser to Thr. It is described by Comack, B. et al. *Gene* (1996) 173:31–38. There are more than 190 silent base changes to maximize human codon usage preferences as described by Haas, J. et al. *Curr Biol* (1996) 6:315–324. Thus, pLEIN contains the above-described GFP coding sequence inserted into the multiple cloning site of pLXIN to obtain a dicistronic expression system which permits coordinated translation of the GFP and neomycin resistance. Three days after injection of the cells into the peritoneal cavity of the mice, the tumor cells could be seen in the seminal vesicles under bright-field microscopy and under fluorescent microscopy.

EXAMPLE 7

Observation of Angiogenesis

A suspension containing 1×10^7 clone-38 cells, described in Example 1, were injected into the peritoneal cavity of a mouse. Five days later, the mouse was injected in the tail with rhodamine and the mouse was then put under anesthesia and the abdominal cavity opened sufficiently to visualize the tumor. Recovery from this surgery is straightforward. In some cases, abdominal opening is unnecessary as the intraperitoneal tumors can be visualized through intact skin. Tumors were visible in the abdominal cavity and angiogenesis was apparent as identified by the rhodamine fluorescence. Similar results were found in tumors growing in the omentum in the wall of the small intestine, and in the mesentery.

In an analogous experiment, a suspension containing 1×10^7 cells of clone-26, described in Example 1, were injected into the peritoneal cavity of a mouse. After one day, tumors appeared in the mesentery and in the colon wall. These were observed by anesthetizing the mouse and a minimal opening of the abdomen. Observations on day 3 of a similarly treated mouse showed tumors in the wall of the small intestine and in the omentum as well as in the colon wall and mesentery. On day 5, a similarly treated mouse was injected in the tail with 100 μl of 2×10^3 M rhodamine and a few vessels could be seen in the tumor growing in the mesentery. After day 60, numerous vessels were seen in the tumor growing in the colon wall.

EXAMPLE 8

Construction of Metastatic Models

Using the labeled human cancer cell lines described in Example 5, murine models are established for various types of cancer. The cell lines are implanted into 6-week-old nu/nu female mice with a single dose of 107 GFP expressing human tumor cells which had been harvested by trypsinization and washed three time with cold, serum-containing medium and then kept on ice. The cells are injected in subcutaneous space in the flank of the animal at a total volume of 0.4 ml within 40 min of harvesting. The nude mice are sacrificed to harvest the tumor fragments 3 weeks after tumor cell injection. These tumor fragments are then used for surgical implantation into the corresponding tissue (surgical orthotopic implantation (SOI)) in nude mice as recipients.

The recipient mice are first anesthetized and then implanted using established SOI techniques with fragments of the subcutaneously grown colon cancer, lung cancer, breast cancer, prostate cancer or melanoma. In all cases, except for melanoma, the size of the fragment is 1 mm^3; for melanoma, 0.025 mm^3 fragments are prepared from the human melanoma LOX-GFP subcutaneous tumor and 5–6 fragments are implanted. The progress of metastasis is then observed using FOTI with a Leica Stereomicroscope MZ12 with a mercury lamp source. GFP is excited with a D425/60 bandpass filter and a 470DCXR dichroic mirror; fluorescence is emitted through a GG475 longpass filter (Chroma

Technology, Brattle-boro, Vt.) and collected by a thermoelectrically cooled ST-133 Micromass High-Speed Controlled Camera—TEA/CCD-1317K1 (Princeton Instruments, Trenton, N.J.) with a 17×1035 pixels chip. The images are processed and analyzed with ImagePro+3.1 Software (Media Cybernetics, Silver Spring, Md.). High resolution images are captured by computer, or continuously through video output onto video tape.

In the colon cancer model, a small midline incision is made in the abdomen and the colorectal part of the intestine is exteriorized. The serosa is removed and 8–15 pieces of tumor fragments are implanted. An 8–0 surgical suture is used to penetrate the small tumor pieces and suture them to the wall of the intestine. The intestine is returned to the abdominal cavity and abdominal wall is closed. The animals are then observed for metastases.

For lung cancer models, a small 1 cm transverse incision is made on the left lateral chest via the fourth intercostal space; total lung collapse results. Five tumor pieces sewn together with 8–0 nylon surgical suture are fixed by making one knot; the lung is taken out by forceps and the tumor sewn into the lower part of the lung with one suture. After returning the lung to the chest cavity, the chest muscles and skin are closed. The lung is reinflated by withdrawing air from the chest cavity with a 23-gauge needle. The animals can then be observed for metastasis either by FOTI or by excising various tissues.

For breast cancer, an incision of 1.5 cm is made along the medial side of the nipple and after blunt dissection, the fat pad is exposed. A small incision is made and a small pocket formed to accommodate 2 fragments of the tumor tissue; an 8–0 suture is made to close the pocket. The skin layer is then closed. The animals are then observed by FOTI or by tissue excision.

For prostate cancer, an opening is made above the pubic symphysis to expose the prostate gland. The fascia surrounding the dorsal portion of the prostate and the dorsal lateral lobes of the gland are separated by a small incision. Five randomized fragments are sutured into the incision using a 8–0 nylon suture. The two parts of the separated lobes are sutured together and the surrounding fascia used to wrap this portion of the gland to consolidate the incision. The abdomen is then closed and the animals maintained for observation.

For melanoma, 5–6 fragments are transplanted subdermally into the flank with a 13×¼ cancer implant needle (Popper & Sons, New Hyde Park, N.Y.).

Images can be obtained as described above showing metastases to various locations in the animal.

The animals treated as described above, can then be used to evaluate potential protocols for treatment of cancer and metastasis inhibition. The metastatic progress of the fluorescent tumors in animals administered the protocols is compared to similar animals lacking treatment. The efficacy of the protocols can then be directly observed.

What is claimed is:

1. A method to evaluate a candidate protocol or drug for the inhibition of metastasis of a primary tumor which method comprises:

administering said protocol or drug to a subject which is a mouse, rat or rabbit which contains a primary tumor that stably expresses green fluorescent protein (GFP) in cells of said tumor when said tumor metastasizes and monitoring the progression of metastasis by observing the presence, absence or intensity of the fluorescence at various locations in the treated subject;

wherein said subject contains said tumor that expresses GFP and wherein said subject is a genetically immunocompromised mouse, rat or rabbit, or a mouse, rat or rabbit which is syngeneic to said tumor;

monitoring the progression of metastasis in a control, which contains a similar tumor that expresses green fluorescent protein;

wherein said control subject contains said tumor that expresses GFP wherein said control subject is an immunocompromised mouse, rat or rabbit, or a mouse, rat or rabbit which is syngeneic to said tumor; and

comparing the progression of metastasis in said treated subject with the progression of metastasis in said control subject wherein the control subject and treated subject are intact;

whereby a diminution of the progression of metastasis in said treated subject as compared to said control subject identifies the protocol or drug as effective in inhibiting metastasis.

2. The method of claim **1** wherein the progression of metastasis is monitored by fluorescent optical tumor imaging in the intact subject.

3. The method of claim **1** wherein said subject contains said tumor by virtue of surgical orthotopic implantation of said tumor.

4. The method of claim **1** wherein said subject contains said tumor by virtue of injecting cells of a stably transformed tumor cell line which has been transfected with an expression vector containing a first nucleotide sequence encoding green fluorescent protein (GFP) and a second nucleotide sequence encoding a selection marker, both said first and second nucleotide sequences being under control of a viral promoter and wherein said cell line stably effects high level expression of said GFP in the absence of selection agent and maintains a high level expression of GFP when said cell line proliferates through multiple passages of said cell line.

5. A method to monitor metastasis of a primary tumor in a subject which is a mouse, rat or rabbit which contains said primary tumor, and wherein said tumor stably expresses green fluorescent protein (GFP) in cells of said tumor when said tumor metastasizes,

wherein said subject contains said tumor that expresses GFP and wherein said subject is a genetically immunocompromised mouse, rat or rabbit, or a mouse, rat or rabbit which is syngeneic to said tumor;

which method comprises monitoring the progression of metastasis by observing the presence, absence or intensity of the fluorescence as a function of time at various locations in said subject wherein the subject is intact.

6. The method of claim **5** wherein the progression of metastasis is monitored by fluorescent optical tumor imaging in the intact subject.

7. The method of claim **5** wherein said subject contains said tumor by virtue of surgical orthotopic implantation of said tumor.

8. The method of claim **5** wherein said subject contains said tumor by virtue of injecting cells of a stably transformed tumor cell line which has been transfected with an expression vector in containing a first nucleotide sequence encoding green fluorescent protein (GFP) and a second nucleotide sequence encoding a selection marker, both said first and second nucleotide sequences being under control of a viral promoter and wherein said cell line stablely effects high level expression of said GFP in the absence of selection agent and maintains a high level expression of GFP when said cell line proliferates through multiple passages of said cell line.

9. A method to monitor metastasis of a primary tumor in a mammalian subject which contains said primary tumor, and wherein said tumor stably expresses green fluorescent protein (GFP) in cells of said tumor when said tumor metastasizes,

wherein said primary tumor is endogenous to said mammalian subject and expresses said GFP as a result of locally administering a retroviral vector to said subject in the vicinity of said tumor, said retroviral vector containing an expression system for said GFP;

which method comprises monitoring the progression of metastasis by observing the presence, absence or intensity of the fluorescence as a function of time at various locations in said subject.

10. The method of claim 9 wherein the subject is human.

11. The method of claim 9 wherein the progression of metastasis is monitored by excising fresh organ tissues from various locations in said subject.

12. The method of claim 11 wherein said excised tissues are observed by microscopic examination of fresh tissue slices.

* * * * *

US006759038B2

(12) **United States Patent**
Tan et al.

(10) **Patent No.: US 6,759,038 B2**
(45) **Date of Patent: *Jul. 6, 2004**

(54) **METASTASIS MODELS USING GREEN FLUORESCENT PROTEIN (GFP) AS A MARKER**

(75) Inventors: **Yuying Tan**, San Diego, CA (US); **Takashi Chishima**, Yokohama (JP)

(73) Assignee: **AntiCancer, Inc.**, San Diego, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **09/870,268**

(22) Filed: **May 29, 2001**

(65) **Prior Publication Data**

US 2002/0026649 A1 Feb. 28, 2002

Related U.S. Application Data

(63) Continuation of application No. 09/226,856, filed on Jan. 7, 1999, now Pat. No. 6,251,384, which is a continuation-in-part of application No. 09/067,734, filed on Apr. 28, 1998, now Pat. No. 6,235,968, which is a continuation-in-part of application No. 09/049,544, filed on Mar. 27, 1998, now Pat. No. 5,235,967, which is a continuation-in-part of application No. 08/848,539, filed on Apr. 28, 1997, now Pat. No. 6,232,523.

(51) **Int. Cl.**[7] **A61K 48/00**; C12N 5/00; C12N 15/63

(52) **U.S. Cl.** **424/93.21**; 800/8; 800/9; 800/10; 435/320.1; 435/325; 435/455; 514/44

(58) **Field of Search** 435/325, 320.1, 435/455; 800/8, 9, 10; 514/44; 424/93.2, 93.21

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,650,135 A	7/1997	Contag et al.	424/9.1
5,874,304 A	2/1999	Zolotukhin et al.	435/366

FOREIGN PATENT DOCUMENTS

WO	WO 97/18841	5/1997
WO	WO 97/45550	12/1997
WO	WO 98/49336	11/1998

OTHER PUBLICATIONS

Merriam-Webster Online, http://www.m-w.com/cgi-bin/dictionary, definition of "intact". 2003.*
Fu, X. et al., *Anticancer Res.* (1993) 13:283–286.
Heim, R. et al., *Nature* (1995) 373:663–664.
Hoffman, R.M., *Cancer Cells* (1991) 3:86–92.
Hoffman, *Investigational New Drugs* (1999) 17(4):343–359.
Hyer, M. L. et al., *Cancer Gene Therapy* (1997) 4(6):s29–s30.
Kaufman, R.J. et al., *Nucleic Acids Res.* (1991) 19:4485–4490.
Kaufman et al., *Annu. Rev. Immunol.* (1995) 13:339–367.
Khokha, R. et al., *Cancer Metastasis Rev.* (1995) 14:279–301.
Koop, S. et al., *Cancer Res.* (1995) 55:2520–2523.
Leighton, J., *Cancer Res.* (1957) 17:929–941.
Leighton, J., *Cancer Res.* (1960) 20:575–597.
Levy, J.P. et al., *Nature Biotechnol.* (1996) 14:610–614.
Li, Y. et al., Biotechnologies, (1997) 23:1026–1029.
Lin, W.C. et al., *Cancer Res.* (1990) 50:2808–2817.
Lin, W.C. et al., *Invasion and Metastasis* (1992) 12:197–209.
Margolis, L.B. et al., *In Vitro Cell Dev. Biol.* (1995) 31:221–226.
Margolis et al., *Annu. Rev. Immunol.* (1995) 13:339–367.
Miller et al., *FASEB* (1995) 9:190–199.
Morin, J. et al., *J. Cell. Physiol.* (1972) 77:313–318.
Plautz, J. D. et al., *Gene* (1996) 173:83–87.
Prasher, D.C. et al., *Gene* (1992) 111:229–233.
Romer et al., *APMIS* (1995) 103:582–587.
Vieweg et al., *Cancer Invest.* (1995) 13(2):193–201.
Yang et al., *Cancer Research* (1999) 59(4):781–786.
Yang et al., *Proc. SPIE–Int. Soc. Opt. Eng.* (1999) 117–124.
Yokoe, H. et al., *Nature Biotechnol.* (1996) 14:1252–1256.
Zolotukhin, S. et al., *J. Virol.* (1996) 70:4646–4654.
Aboody-Guterman et al., *Society for Neuroscience Abstracts* (1996) 22:949–1998.
Astoul, P. et al., *Anticancer Res.* (1994) 14:85–92.
Astoul, P. et al., *J. Cell Biochem.* (1994) 56:9–15.
Chalfie, M. et al., *Science* (1994) 263:802–805.
Chishima et al., *Proc. Am. Assoc. Canc. Res* (1997) 38:489 (#3276–3/97).
Chishima, T. et al., *Clinical and Experimental Metastais* (1997) 15(5):547–552.
Chishima, T. et al., *AnticancerRes.* (1997) 17:2377–2384.
Chishima, T. et al., *Cancer Res.* (1997) 57(10):2042–2047.
Chishima, T. et al., *Proc. Nat'l Academy of Sciences of USA* (1997) 94:11573–11576.
Cody, C.W. et al., *Biochemistry* (1993) 32:1212–1218.
Cormack, B. et al., *Gene* (1996) 173:33–38.
Crameri, A. et al., *Nature Biotechnol.* (1996) 14:315–319.
Delagrave, S. et al., *Biotechnology* (1995) 13:151–154.

* cited by examiner

Primary Examiner—Anne M. Wehbe'
(74) *Attorney, Agent, or Firm*—Morrison & Foerster LLP

(57) **ABSTRACT**

A method to follow the progression of metastasis of a primary tumor, which method comprises removing fresh organ tissues from a vertebrate subject which has been modified to contain tumor cells that express GFP and observing the excised tissues for the presence of fluorescence is disclosed. The fluorescence can also be monitored by observing the tissues in situ. Vertebrate subjects which contain GFP producing tumors are useful models to study the mechanism of metastasis, as well as to evaluate candidate protocols and drugs. In addition, subjects already harboring tumors can be treated so as to modify the endogenous tumors to contain GFP. This permits clinical applications. Finally, by injecting a contrast dye into a subject harboring a GFP-labeled tumor, angiogenesis in the tumor can be observed directly.

12 Claims, 2 Drawing Sheets

US006251384B1

(12) **United States Patent**
Tan et al.

(10) **Patent No.: US 6,251,384 B1**
(45) **Date of Patent: Jun. 26, 2001**

(54) **METASTASIS MODELS USING GREEN FLUORESCENT PROTEIN (GFP) AS A MARKER**

(75) Inventors: **Yuying Tan**, San Diego, CA (US); **Takashi Chishima**, Yokohama (JP)

(73) Assignee: **AntiCancer, Inc.**, San Diego, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/226,856**

(22) Filed: **Jan. 7, 1999**

Related U.S. Application Data

(63) Continuation-in-part of application No. 09/067,734, filed on Apr. 28, 1998, which is a continuation-in-part of application No. 09/049,544, filed on Mar. 27, 1998, which is a continuation-in-part of application No. 08/848,539, filed on Apr. 28, 1997.

(51) **Int. Cl.**[7] **A61K 35/00**; A61K 48/00; C12N 15/63; C12N 15/85

(52) **U.S. Cl.** **424/93.21**; 514/44; 435/320.1; 800/9; 800/10

(58) **Field of Search** 514/44; 424/93.21; 800/9, 10; 435/320.1

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,650,135 * 7/1997 Contag et al. 424/9.1
5,874,304 * 2/1999 Zolotukhin et al. 435/366

FOREIGN PATENT DOCUMENTS

WO 97/18841 5/1997 (WO) .
WO 97 45550 12/1997 (WO) .
WO 98/49336 11/1998 (WO) .

OTHER PUBLICATIONS

Kaufman et al. (1995) Annu. Rev. Immunol. , vol. 13, 339–367, 1995.*
Miller et al. (1995) FASEB, vol. 9, 190–199, 1995.*
Vieweg et al. (1995) Cancer Invest., vol. 13(2), 193–201, 1995.*

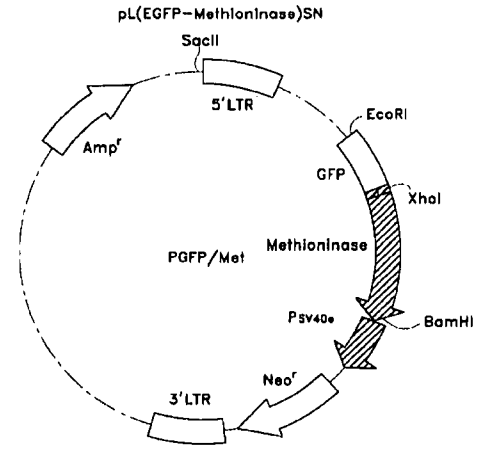
(List continued on next page.)

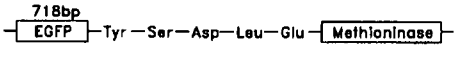
Primary Examiner—Karen M. Hauda
Assistant Examiner—Anne Marie S. Beckerleg
(74) *Attorney, Agent, or Firm*—Morrison & Foerster LLP

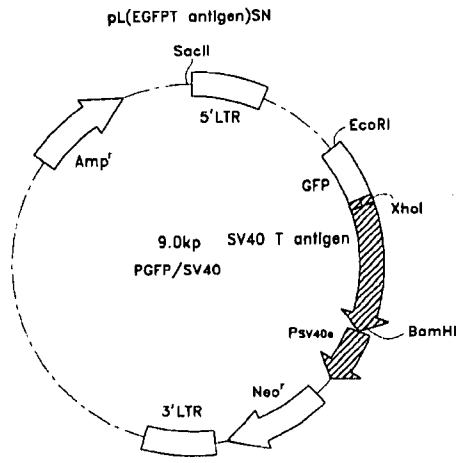
(57) **ABSTRACT**

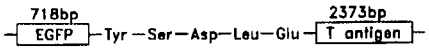
A method to follow the progression of metastasis of a primary tumor, which method comprises removing fresh organ tissues from a vertebrate subject which has been modified to contain tumor cells that express GFP and observing the excised tissues for the presence of fluorescence is disclosed. The fluorescence can also be monitored by observing the tissues in situ. Vertebrate subjects which contain GFP producing tumors are useful models to study the mechanism of metastasis, as well as to evaluate candidate protocols and drugs. In addition, subjects already harboring tumors can be treated so as to modify the endogenous tumors to contain GFP. This permits clinical applications. Finally, by injecting a contrast dye into a subject harboring a GFP-labeled tumor, angiogenesis in the tumor can be observed directly.

6 Claims, 2 Drawing Sheets





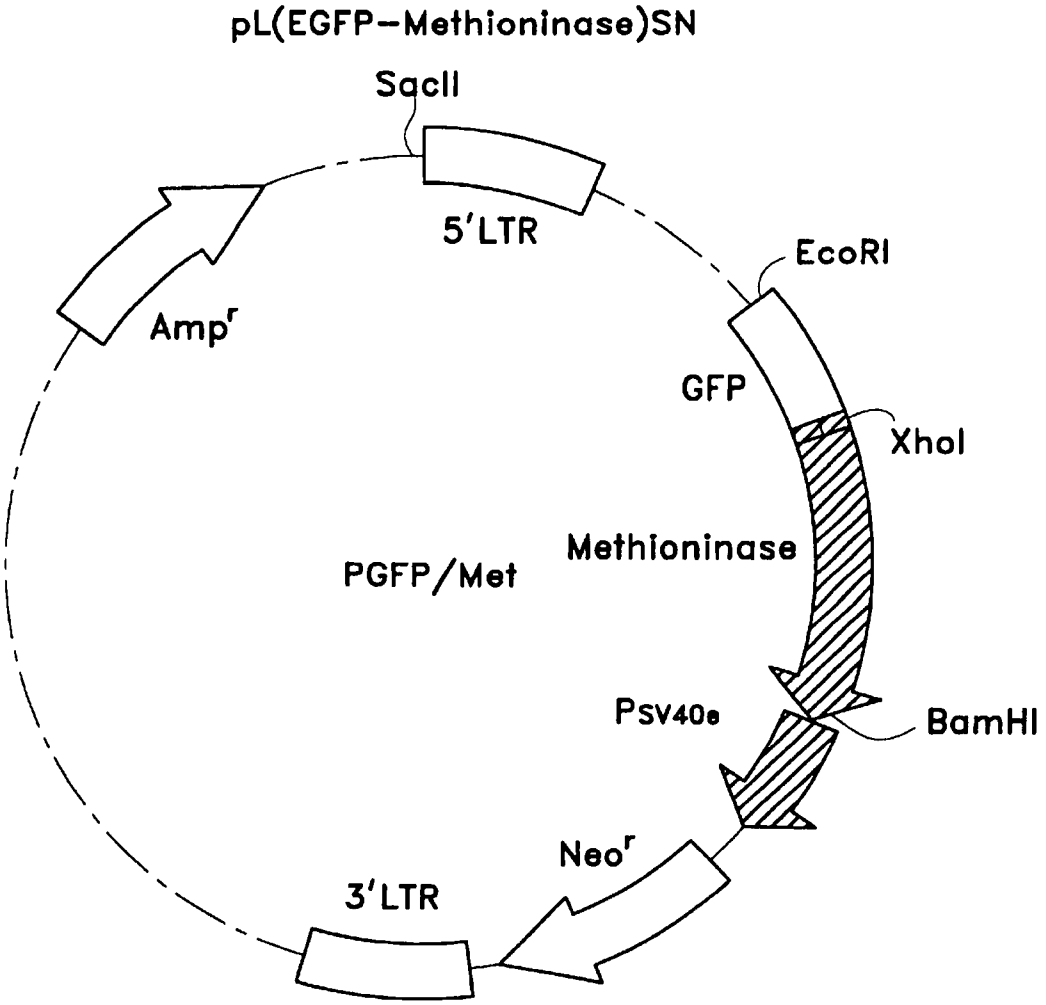




OTHER PUBLICATIONS

Romer et al. (1995) APMIS, vol. 103, 582–587, 1995.*
Astoul et al. (1994) J. Cell. Biochem., vol. 56, 9–15, 1994.*
Hoffman, "Orthotopic Metastatic Mouse Models for Anticancer Drug Discovery and Evaluation: A Bridge to the Clinic," *Investigational New Drugs* (1999) 17(4):343–359 (Abstract).
Yang et al., "Widespread Skeletal Metastatic Potential of Human Lung Cancer Revealed by Green Fluorescent Protein Expression," *Cancer Research* (1998) 58(19):4217–4221.
Yang et al., "A Fluorescent Orthotopic Bone Metastasis Model of Human Prostate Cancer," *Cancer Research* (1999) 59(4):781–786.
Yang et al., "Green Fluorescent Protein: New Light to Visualize Metastasis and Angiogenesis in Cancer," *Proc SPIE—Int Soc Opt Eng* (1999) 117–124 (Abstract).
Levy, J P et al., "Retroviral Transfer and Expression of a Humanized, Red–Shifted Green Fluorescent Protein Gene Into Human Tumor Cells", *Nature Biotechnology*, vol. 14, No. 5, May 1996, pp 610–614.
Plautz, J. D. et al., "Green fluorescent protein and its derivatives as versatile markers for gene expression in living *Drosophila melanogaster*, plant and mammalian cells", *Gene*, vol. 173, 1996, pp. 83–87.
Chalfie, M. et al., "Green fluorescent protein as a marker for gene expression", *Science*, vol. 263, Feb. 11, 1994, pp 802–805.
Lin, W. C., et al., "Bacterial lacZ gene as a highly sensitive marker to detect micrometastasis formation during tumor progression", *Cancer Research*, vol. 50, No. 9, May 1, 1990, pp. 2808–2817.
Lin, W. C., et al., "Altered establishment/clearance mechanisms during experimental micrometastasis with live and/or disabled bacterial lacZ–tagged tumor cells", *Invasion Metastasis*, vol. 12, May 1992–Aug. 1992.
Chishisma, T. et al., "Metastatic patterns of lung cancer visualized live and in process by green fluorescence protein expression", *Clinical and Experimental Metastasis*, vol. 15, No. 5, Sep. 1997, pp. 547–552.
Chishima, T. et al., "Visualization of the metastatic process by green fluorescent protein expression", *Anticancer Research*, vol. 17, No. 4a, Jul. 1997, pp. 2377–2384.
Chishima, T. et al., "Cancer Invasion and micrometastasis visualized in live tissue by green fluorescent protein expression", *Cancer Research*, vol. 57, No. 10, May 15, 1997, pp 2042–2047.
Chishima, T. et al., "Governing step of metastasis visualized in vitro", *Proceedings of the National Academy of Sciences of USA*, vol. 94, No. 21, Oct. 1997, pp. 11573–11576.
Hyer, M. L. et al., "Adenovirus–mediated gene transfer of GFP (green fluorescent protein) to mouse and human prostate cancer cells", *Cancer Gene Therapy*, vol. 4, No. 6, Nov. 1997, pp. s29–s30.
Li, Y. et al., "Use of a Green Fluorescent Protein in Studies of Apoptosis of Transfected Cells", *Biotechnologies*, vol. 23, No. 6, Dec. 1997, pp. 1026–1029.
Chishima et al. (1997) Proc. Am. Assoc. Canc. Res., vol. 38, p. 489 #3276—Dated Mar. 1997.
Aboody–Guterman et al. (1996) Society for Neuroscience Abstracts, vol. 22, 949–1998.
Romer et al. (1995) APMIS, vol. 103,582–587.
Levy et al (1996) Nature Biotech., vol. 14, 610–614.
Astoul et al. (1994) J. Cell, Biochem., vol. 56, 9–15.
Margolis et al. (1995) Annu. Rev. Immunol., vol. 13, 339–367.
Aboody–Guterman et al., *Society for Neuroscience Abstracts* (1996) 22:949–1998.
Astoul, P. et al., *Anticancer Res.* (1994) 14:85–92.
Astoul, P. et al., *J. Cell Biochem.* (1994) 56:9–15.
Chalfie, M. et al., *Science* (1994) 263:802–805.
Chishima et al., *Proc. Am. Assoc. Canc. Res* (1997) 38:489 (#3276—Mar. 1997).
Chishima, T. et al., *Clinical and Experimental Metastais* (1997) 15(5):547–552.
Chishima, T. et al., *AnticancerRes.* (1997) 17:2377–2384.
Chishima, T. et al., *Cancer Res.* (1997) 57(10):2042–2047.
Chishima, T. et al., *Proc. Nat'l Academy of Sciences of USA* (1997) 94:11573–11576.
Cody, C.W. et al., *Biochemistry* (1993) 32:1212–1218.
Cormack, B. et al., *Gene* (1996) 173:33–38.
Cramcri, A. et al., *Nature Biotechnol.* (1996) 14:315–319.
Delagrave, S. et al., *Biotechnology* (1995) 13:151–154.
Fu, X. et al., *Anticancer Res.* (1993) 13:283–286.
Heim, R. et al., *Nature* (1995) 373:663–664.
Hoffman, R.M., *Cancer Cells* (1991) 3:86–92.
Hyer, M. L. et al., *Cancer Gene Therapy* (1997) 4(6):s29–s30.
Kaufman, R.J. et al., *Nucleic Acids Res.* (1991) 19:4485–4490.
Khokha, R. et al., *Cancer Metastasis Rev.* (1995) 14:279–301.
Koop, S. et al., *Cancer Res.* (1995) 55:2520–2523.
Leighton, J., *Cancer Res.* (1957) 17:929–941.
Leighton, J., *Cancer Res.* (1960) 20:575–597.
Levy, J.P. et al., *Nature Biotechnol.* (1996) 14:610–614.
Li, Y. et al., *Biotechnologies*, (1997) 23:1026–1029.
Lin, W.C. et al., *Cancer Res.* (1990) 50:2808–2817.
Lin, W.C. et al., *Invasion and Metastasis* (1992) 12:197–209.
Margolis, L.B. et al., *In Vitro Cell Dev. Biol.* (1995) 31:221–226.
Margolis et al., *Annu. Rev. Immunol.* (1995) 13:339–367.
Morin, J. et al., *J. Cell. Physiol.* (1972) 77:313–318.
Plautz, J. D. et al., *Gene* (1996) 173:83–87.
Prasher, D.C. et al., *Gene* (1992) 111:229–233.
Romer et al., *APMIS* (1995) 103:582–587.
Yokoe, H. et al., *Nature Biotechnol.* (1996) 14:1252–1256.
Zolotukhin, S. et al., *J. Virol.* (1996) 70:4646–4654.

* cited by examiner



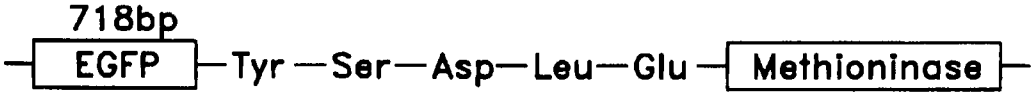


Fig. 1a

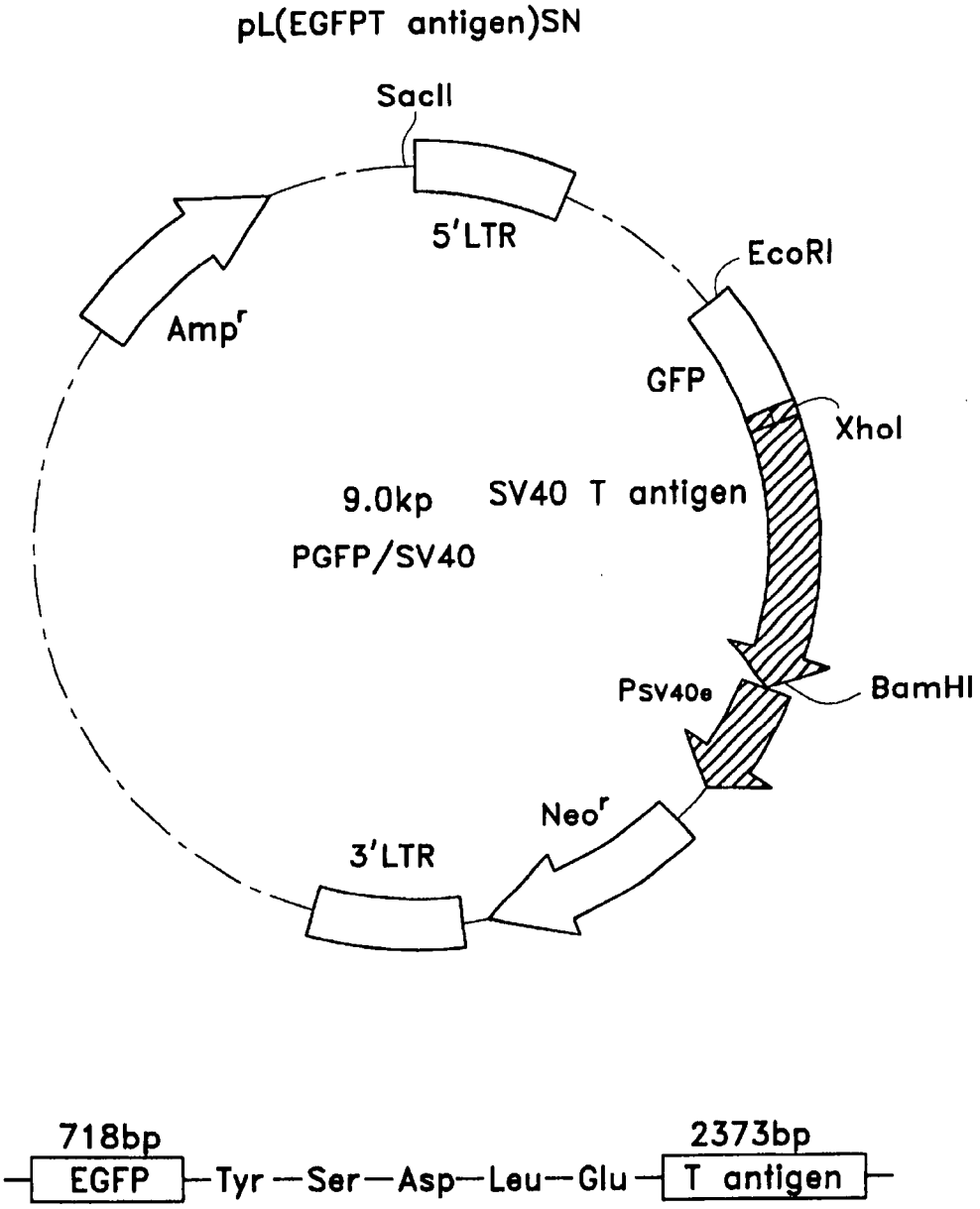


Fig. 1b

METASTASIS MODELS USING GREEN FLUORESCENT PROTEIN (GFP) AS A MARKER

This application is a continuation-in-part of U.S. Ser. No. 09/067,734 filed Apr. 28, 1998 which is a continuation-in-part of U.S. Ser. No. 09/049,544 filed Mar. 27, 1998 which is a continuation-in-part of U.S. Ser. No. 08/848,539 filed Apr. 28, 1997, the contents of which are incorporated herein by reference.

TECHNICAL FIELD

The invention relates to the study of tumor progression. Specifically, it concerns model systems for studying the metastasis of tumors in vertebrate systems and to models and methods for evaluating candidate drugs.

BACKGROUND OF THE INVENTION

It has long been recognized that the ability of tumor tissues to metastasize constitutes a major portion of the life-threatening aspects of malignancy. Metastasis is the growth of secondary tumors at sites different from the primary tumor. Thus, despite surgical removal of the primary tumor, it may not be possible to arrest the progress of this condition. An understanding of the mechanism whereby metastasis occurs will be crucial to the development of protocols whereby the growth of secondary tumors can be controlled. In order to understand the mechanism of metastasis, it will be necessary to provide a model which permits identification of small numbers of tumor cells against a background of many host cells so that secondary tumor emboli and micrometastases can be observed over the course of real time.

Others have demonstrated extravasation and initial seeding steps in tumor metastasis in vitro using externally fluorescently labeled tumor cells. Khokha, R. et al., *Cancer Metastasis Rev* (1995) 14:279–301; Koop, S. et al., *Cancer Res* (1995) 55:2520–2523. Further, Margolis, L. B. et al., *In Vitro Cell Dev Biol* (1995) 31:221–226 was able to visualize the migration of externally fluorescently labeled lung tumor cells in host mouse lung in histoculture. In all cases, however, long-term observation was not possible due to the limitation of exogenous fluorescent labels. Retroviral transfer of a green fluorescent protein (GFP) gene has been shown to result in stable transfectants of human cancer cells in vitro (Levy, J. P. et al., *Nature Biotechnol* (1996) 14:610–614), as well as of hematopoietic cells (Grignani, F. et al. *Cancer Res* (1998) 58:14–19 and by Cheng, L. et al. *Gene Therapy* (1997) 4:1013–1022).

Attempts have been made to provide such a model using the β-galactosidase gene as a marker (Lin, W. C. et al., *Cancer Res* (1990) 50:2808–2817; Lin, W. C. et al., *Invasion and Metastasis* (1992) 12:197–209). However, this marker has not proved satisfactory, as fresh or processed tissue cannot be used. The present invention provides a marker which permits visualization of tumor invasion and micrometastasis formation in viable fresh tissue. In addition, by providing suitable contrast media, the method of the invention can be adapted to visualize angiogenesis in established and growing tumors. The methods of the invention can be applied not only to models of tumor growth and metastasis, but, through the use of retroviral vectors, can be employed to obtain clinical data in human subjects bearing tumors.

The present invention utilizes green fluorescent protein (GFP) as a marker. Heterologous expression of this protein, principally to monitor expression of fused DNA, was disclosed in U.S. Pat. No. 5,491,084. This document describes the expression of GFP in *E. coli* and *C. elegans* and postulates that cells in general can be modified to express GFP. Such expression, according to this document, permits not only a method to monitor expression of fused DNA, but also a means of monitoring protein localization within the cell.

The aspect of the invention which provides a metastatic model has been reported and described in a series of publications. Chishima, T. et al. *Cancer Research* (1997) 57:2042–2047 describe the construction of a dicistronic vector containing the gene for humanized green fluorescent protein (GFP) and dihydrofolate reductase (DHFR). This vector was transfected into CHO-K1 cells to obtain clone-38. Clone-38 showed stable GFP expression which was maintained in the presence of methotrexate (MTX). Clone-38 cells were injected into mice to obtain tumor fragments which were then implanted by surgical orthotopic implantation (SOI) on the ovarian serosa in nude mice. Metastasis could be followed in this model.

Chishima, T. et al. *Proc Natl Acad Sci USA* (1997) 94:11573–11576 describe the preparation of clone-26 by transfection of Anip 973 human lung adenocarcinoma cells with the codon optimized hGFP-S65T clone obtained from Clontech. Clone-26 was injected intravenously into nude mice and the resulting tumors were followed in histoculture.

Chishima, T. et al. *Clin Exp Metastasis* (1997) 15:547–552 and Chishima, T. et al. *Anticancer Res* (1997) 17:2377–2384 describe similar work with clone-26 wherein the cells were inoculated subcutaneously into nude mice resulting in a visualizable tumor which was then implanted into the visceral pleura of nude mice by SOI. Metastases were observed in this model as well.

Chishima, T. et al. *In Vitro Cell Dev Biol* (1997) 33:745–747 describe histoculture of clone-26 and visualization of growth using the fluorescence emitted by GFP.

Yang, M., et al., *Cancer Res* (1998) 58:4217–4221 describe transduction of the human lung cancer cell line H460 with a retroviral expression vector containing enhanced GFP to obtain a stable high-GFP-expressing clone. Cells from this cell line were injected into nude mice and the resulting subcutaneously growing labeled tumors were transplanted by SOI into the left lung of nude mice. Fluorescence could then be observed from the metastases in the collateral lung, pleural membrane and throughout the skeletal system.

Yang, M., et al., *Cancer Res* (In Press) report similar studies using a model for prostate tumor and showing fluorescence throughout the skeletal system in nude mice.

The contents of the foregoing publications are incorporated herein by reference.

In addition to the foregoing, Cheng, L., et al., *Gene Therapy* (1997) 4:1013–1022, describe the modification of hematopoietic stem cells using the GFP gene under control of a retroviral promoter. Although the authors state that human stem cells are transfected with this system only with difficulty, by using an enhanced form of the GFP, satisfactory brightness could be achieved.

In addition, Grignani, F., et al., *Cancer Res* (1998) 58:14–19, report the use of a hybrid EBV/retroviral vector expressing GFP to effect high-efficiency gene transfer into human hematopoietic progenitor cells.

Vectors containing various modified forms of GFP to provide various colors are marketed by Clontech. The Clontech vectors intended for mammalian cell expression place the GFP under control of the cytomegalovirus (CMV) promoter.

DISCLOSURE OF THE INVENTION

The invention provides models which permit the intimate study of formation of metastases from primary tumors in a realistic and real-time setting. By using green fluorescent protein (GFP) as a stable and readily visualized marker, the progression of such metastasis can be modeled and the mechanism elucidated.

Thus, in one aspect, the invention is directed to a method to follow the progression of metastasis of a primary tumor, which method comprises removing fresh organ tissues from a vertebrate subject which has been modified to contain tumor cells that express GFP and observing the excised tissues for the presence of fluorescence.

In one embodiment, however, it is unnecessary to remove organ tissues; rather, the fluorescence can be visualized in the whole animal by real-time fluorescence optical tumor imaging (FOTI).

In another aspect, the invention is directed to a vertebrate subject which has been modified to contain tumor cells expressing GFP.

In these aspects, the vertebrate subject may constitute a model system, such as an immunocompromised mouse wherein tumor cells or a tumor, modified to express green fluorescent protein has been introduced into the subject. The model system may be used to evaluate candidate drugs for their capacity to inhibit metastasis. Alternatively, the subject may be a human or other vertebrate which natively contains the tumor, but wherein the tumor has been subjected to viral infection or to transfection with a retroviral vector so as to produce said GFP. The efficacy of drugs administered to such patients can be evaluated by following the course of metastasis in the subject.

In still other aspects, the invention is directed to tumor cells modified to produce GFP under control of heterologous control elements, to cells that are immortalized to provide stable cell lines as well as comprising visible amounts of GFP, to tissues containing metastatic tumors that produce GFP, and to histocultures of tissues which contain such metastasized tumors.

The invention also includes a method to observe and follow angiogenesis in solid tumors which method comprises (usually) exposing and observing said tumors. The tumors will have been modified to express GFP, and the subject will have been administered a contrast dye to permit this observation.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1*a* and 1*b* show the construction of expression vectors useful in the invention.

MODES OF CARRYING OUT THE INVENTION

The invention provides model systems for the study of the mechanism of metastasis of tumors generally, as well as to study angiogenesis in solid tumors. Advantage is taken of the visible marker green fluorescence protein (GFP) to label the tumor cells so that their migration and colonization in tissues distal to the tumor can be followed as the migration and colonization progresses. Further, by administering to the subject a contrast dye, such as rhodamine, the growth of blood vessels in solid tumors which have been labeled with GFP can also be observed.

Since sufficient intensity can be achieved to observe the migration of fluorescent cells in the intact animal, in addition to determining the migration of the cells by excising organs, the progression of metastasis can be observed in the intact subject. Either or both methods may be employed to observe metastasis in evaluating, in model systems, the efficacy of potential antimetastatic drugs. The success or failure of treatments provided to patients with potentially metastatic cancers can also be followed using the materials and methods of the invention.

The label used in the various aspects of the invention is green fluorescent protein (GFP). The native gene encoding this protein has been cloned from the bioluminescent jellyfish *Aequorea victoria* (Morin, J. et al., *J Cell Physiol* (1972) 77:313–318). The availability of the gene has made it possible to use GFP as a marker for gene expression. GFP itself is a 283 amino acid protein with a molecular weight of 27 kD. It requires no additional proteins from its native source nor does it require substrates or cofactors available only in its native source in order to fluoresce. (Prasher, D. C. et al., *Gene* (1992) 111:229–233; Yang, F. et al., *Nature Biotechnol* (1996) 14:1252–1256; Cody, C. W. et al., *Biochemistry* (1993) 32:1212–1218.) Mutants of the GFP gene have been found useful to enhance expression and to modify excitation and fluorescence. GFP-S65T (wherein serine at 65 is replaced with threonine) is particularly useful in the invention method and has a single excitation peak at 490 nm. (Heim, R. et al., *Nature* (1995) 373:663–664); U.S. Pat. No. 5,625,048. Other mutants have also been disclosed by Delagrade, S. et al., *Biotechnology* (1995) 13:151–154; Cormack, B. et al., *Gene* (1996) 173:33–38 and Cramer, A. et al. *Nature Biotechnol* (1996) 14:315–319. Additional mutants are also disclosed in U.S. Pat. No. 5,625,048. By suitable modification, the spectrum of light emitted by the GFP can be altered. Thus, although the term "GFP" is used in the present application, the proteins included within this definition are not necessarily green in appearance. Various forms of GFP exhibit colors other than green and these, too, are included within the definition of "GFP" and are useful in the methods and materials of the invention. In addition, it is noted that green fluorescent proteins falling within the definition of "GFP" herein have been isolated from other organisms, such as the sea pansy, *Renilla reriformis*. Any suitable and convenient form of the GFP gene can be used to modify the tumor cells useful in the models of the invention, and for retroviral transformation of endogenous tumors. The particular humanized hGFP-S65T clone is used in the examples set forth below for illustration.

Techniques for labeling cells in general using GFP are disclosed in U.S. Pat. No. 5,491,084 (supra).

In one application, the method of the invention provides a model system for studying the effects of various therapeutic candidate protocols and substances on metastatic growth of tumors.

In general, the model involves modifying a vertebrate, preferably a mammal, so as to contain tumor tissue, wherein the tumor cells have, themselves, been modified to contain an expression system for GFP. The tumor cells may arise from cell lines of the invention wherein tumor cells have been modified to contain expression systems for GFP and SV40 T-antigen. Tumors can be formed in such vertebrate systems by administering the transformed cells containing the GFP expression system and permitting these transformed cells to form tumors. Typically such administration is subcutaneous and the tumors are formed as solid masses. The tumors thus formed can be implanted in any suitable host tissue and allowed to progress, metastasize and develop.

Suitable procedures for growing the initial tumor, thus, involve transcutaneous injection of the tumor cells produc-

ing GFP, such as CHO cells, HeLa cells, carcinoma and sarcoma cell lines, well established cell lines such as the human lung adenocarcinoma line Anip 973, or lung cancer cell line H460 as well as GFP-containing human breast cancer lines MDA-MB468 and MDA-MB435; human prostate cancer lines PC3 and DU-145, human glioblastoma line 324, mouse melanoma B16 and others that may become available in the art, including the immortalized cells of the invention. The administered cells will have been modified to contain an expression system for GFP. After administration, solid tumors generally develop, typically at the site of subcutaneous injection. These tumors, which are themselves fluorescent, can then be removed and used for implantation in the model vertebrate.

Techniques for implantation of the solid tumors, now labeled with GFP, into vertebrates include direct implantation by surgical orthotopic implantation (SOI) at the desired site, typically the site from which the tumor cells were derived. Suitable sites include lung, liver, pancreas, stomach, breast, ovary, prostate, bone marrow, brain, and other tissues susceptible to malignancy. Once the solid tumors have been implanted, the vertebrate becomes a model system for studying metastasis. The tumor is thus allowed to progress and develop and the vertebrate is monitored for appearance of the GFP labeled cells at sites distal from the original implantation site. The monitoring can occur either on the whole vertebrate by opening the animal and observing the organs directly with a fluorescent microscope, or the tissues may be excised and examined microscopically. In some cases the tumors are sufficiently bright that opening the animal is unnecessary—they can be seen directly through the skin. In any case, as GFP is visible to the naked eye, no development systems to stain the tissue samples are required. Tissue samples are simply properly processed as fresh samples in slices of suitable size, typically 1 mm thick, and placed under a microscope for examination. Even colonies of less than 10 cells are thus visible. A variety of microscopic visualization techniques is known in the art and any appropriate method can be used.

It is particularly convenient to visualize the migration of tumor cells in the intact animal through fluorescent optical tumor imaging (FOTI). This permits real-time observation and monitoring of progression of metastasis on a continuous basis, in particular, in model systems, in evaluation of potential anti-metastatic drugs. Thus, the relative lack of metastasis observed directly in test animals administered a candidate drug in comparison to controls which have not been administered the drugs indicates the efficacy of the candidate and its potential as a treatment. In subjects being treated for cancer, the availability of FOTI permits those devising treatment protocols to be informed on a continuous basis of the advisability of modifying or not modifying the protocol.

In addition, the development of the tumor can be studied in vitro in histological culture. Suitable systems for such study include solid supported cultures such as those maintained on collagen gels and the like.

Suitable vertebrate subjects for use as models are preferably mammalian subjects, most preferably convenient laboratory animals such as rabbits, rats, mice, and the like. For closer analogy to human subjects, primates could also be used. Particularly useful are subjects that are particularly susceptible to tumor development, such as subjects with impaired immune systems, typically nude mice or SCID mice. Any appropriate vertebrate subject can be used, the choice being dictated mainly by convenience and similarity to the system of ultimate interest.

Any suitable expression system operable in the tumor cells to be implanted may be used. A number of vectors are commercially available that will effect expression in tumor cells of various types. The nature of the vector may vary with the nature of the tumor and the vertebrate in which it finds its origin. However, when GFP is used to visualize metastasis in a model system, it is preferred to utilize vectors which do not use retroviral or other viral promoters which may complicate the nature of the model.

In order to provide cell lines that are helpful in establishing tumors for these model systems, it is also advantageous to employ expression vectors which provide the cells with the SV40 T-antigen. The presence of this antigen ensures immortality of the culture. Thus, particularly useful in the invention are vectors which comprise expression systems that result in the production both of GFP and SV40 T-antigen.

In order to transfect and modify the transformed cells which are effective in generating tumors, any suitable transfection method may be used, such as liposomes, calcium phosphate precipitation, electroporation and use of a gene gun. Lipofection is preferred.

In contrast, when the method of the invention is used to visualize metastasis in tumors that natively occur in a subject such as a human cancer patient, vectors that employ retroviral or other viral promoters are preferred. The use of such vectors permits the insertion of an expression system for GFP into the already existent tumor. In addition, the expression system may contain nucleotide sequence encoding other useful proteins such as therapeutic proteins which permit simultaneous diagnosis of metastasis and treatment. Among such suitable proteins are included methioninase (see, for example, PCT/US93/11311 and PCT/US96/09935). Such proteins may be produced either as fusions with the GFP, or independently either using a dicistronic expression system or independent expression systems, one for the therapeutic protein and the other for the GFP.

Retroviral based expression systems for GFP have already been described by Grignani, F. et al. *Cancer Res* (1998) 58:14–19 and by Cheng, L. et al. *Gene Therapy* (1997) 4:1013–1022. In these reports, the retroviral expression system itself was used to transfect hematopoietic progenitor cells or packaging cells were employed to provide virus-containing supernatants which can be used directly for infection of the mammalian cells. Thus, in the method of the invention, the tumor contained in the vertebrate subject is typically infected with virus which has been modified and packaged to contain the expression system for GFP. In situ infection with virus results in the ability of the tumor to produce GFP and, in effect, label itself.

Various retroviral systems useful in producing proteins in mammalian cells are known in the art. Examples include commercially available vector and packaging systems such as those sold by Clontech, San Diego, Calif., including their Retro-X vectors pLNCX and pLXSN which permit expression of GFP under a variety of promoters by insertion into the multiple cloning site. These vectors contain ψ^* (the extended viral packaging signal) and antibiotic resistance genes for selection. A number of these systems have been developed for use in gene therapy, including vectors which provide a multiple cloning site sandwiched between **540** and 3' LTR derived from retroviral sources, and thus would be useful in labeling the tumors of human patients.

Thus, retroviral based vectors such as those set forth in FIGS. 1*a*–1*b* can be transfected into packaging cells and transferred directly to targeted cancer cells or supernatants

from the packaging cells can be used to infect tumor cells with the retrovirus. Preferred combinations of retrovirus and packaging cells include the GFP-retrovirus vector pLEIN in PT-67 packaging cells. Co-culture of the packaging cells with colon cancer cells results in transfer of the GFP-retrovirus to the cancer cells.

Using histoculture techniques, and supernatants from PT-67 packaging cells generating GFP-pLEIN virus, the successful modification of a human cancer tissue to display the fluorescence associated with GFP has been demonstrated. For use in vivo, the virus is administered, preferably locally to the tumor, which can be observed within hours after injection either of packaging cells or of the viral containing supernatants. The malignant cells can be identified by their green color, sometimes sufficiently bright so that the tumors can be seen through the skin.

In addition to direct observation of tumor metastasis and growth either in a model system or in a vertebrate, typically mammalian and more typically a human subject which is already afflicted by a tumor, the methods of the invention can be adapted to observe angiogenesis in solid tumors. The tumor is itself labeled with GFP as described above. The subject is then administered a contrast dye, typically by injection, preferably intravenous injection, which allows blood vessels in the tumor to be observed. Suitable dyes include rodamine and other contrast dyes. Any dye which forms a contrasting color with the green color of the GFP can be used. Preferably, the dye is coupled to an inert polymer such as polyethylene glycol to increase the length of time the dye will remain in the blood vessel. A sufficient amount of dye is provided to permit ready visualization; the amount of dye required will depend on the choice of dye, the location of the tumor, the nature of the background GFP, and the method used for observation. Within a few minutes, vessels growing into the solid tumors in such areas as the mesentery, colon wall, and omentum can be observed. Observations can be continued over substantial periods; for example, angiogenesis after several hours is still observed by using this method.

The following examples are intended to illustrate but not to limit the invention.

EXAMPLE 1

Preparation of Tumor Cells that Produce GFP

The humanized hGFP-S65T clone described by Zolotukhin, S. et al., *J Virol* (1996) 70:4646–4654 was used as the green fluorescent protein coding sequence. This codon-optimized gene was purchased from Clontech Laboratories, Inc. (Palo Alto, Calif.) and ligated into the dicistronic expression vector (pED-mtx^{40}) obtained from Genetics Institute, Cambridge, Mass. and described in Kaufinan, R. J. et al., *Nucleic Acids Res* (1991) 19:4485–4490. hGFP-S65T was digested with HindIII and blunted; the entire hGFP coding region was excised with XbaI and then unidirectionally subcloned into pED-mtx^{1} which had been digested with PstI, blunted and then further digested with XbaI.

CHO-K1 cells were cultured in DMEM containing 10% fetal calf serum, 2 mM L-glutamine and 100 μM nonessential amino acids. Near confluent cells were incubated with a precipitated mixture of LipofectAMIN™ reagent (GIBCO) and saturating amounts of plasmids for six hours and then replenished with fresh medium. The cells were harvested by trypsin/EDTA 48 hours later and subcultured at 1:15 into selective medium containing 1.5 μM methotrexate (MTX). Cells with stably integrated plasmids were selected in MTX-containing medium and isolated with cloning cylinders (Bel-Art Products, Pequannock, N.J.) by EDTA. After amplification and transfer, Clone-38 was selected because of its high-intensity GFP fluorescence and stability.

In a similar manner, Anip 973 cells, a human lung cancer cell line obtained from Harbin Medical University, China, were cultured as described above for CHO-K1 cells except using RPMI1640 (GIBCO) in place of DMEM. Transfection, selection and amplification and transfer were conducted as described above. Clone-26 was chosen because of its high-intensity GFP fluorescence and stability.

EXAMPLE 2

Mouse Model Using Modified CHO Cells

Clone-38, which was stable at 1.5 μM MTX and which proliferated at the same rate as the parental CHO-K1 cells as ascertained by comparing doubling times, was used in this model.

Three six-week old Balb/C nu/nu female mice were injected subcutaneously with a single dose of 10^7 Clone-38 cells that had been harvested by trypsinization and washed three times with cold serum-containing medium and then kept on ice. The cells were injected in a total volume of 0.4 ml within 40 minutes of harvesting and the nude mice sacrificed three weeks after injection. All of the mice had a subcutaneous tumor ranging in diameter from 13.0 mm to 18.5 mm (mean=15.2 mm±2.9 mm). The tumor tissue was strongly fluorescent. It was shown by extracting GFP from cultured Clone-38 cells in comparison to Clone-38 cells prepared from the tumor that the levels of production of GFP were the same in both.

To construct the model, tumor fragments (1 mm^3) derived from the nude mouse subcutaneous Clone-38 tumor grown as described above, were implanted by surgical or surgical orthotopic implantation (SOI) on the ovarian serosa in six nude mice as described by Fu, X. et al., *Anticancer Res* (1993) 13:283–286, incorporated herein by reference. Briefly, the mice were anesthetized by isofluran inhalation and an incision was made through the left lower abdominal pararectal line and peritoneum to expose the left ovary and part of the serosal membrane, which was scraped with a forceps. Four 1 mm^3 tumor pieces were fixed on the scraped site with an 8-0 nylon suture and the ovary then returned to the peritoneal cavity. The abdominal wall and skin were closed with 6-0 silk sutures.

Four weeks later, the mice were sacrificed and lung and various other organs were removed. The fresh samples were sliced at approximately 1 mm thickness and observed directly under fluorescent and confocal microscopy. Samples were also processed for histological examination for fluorescence and conventional staining. Frozen sections were prepared wherein the slides were rinsed with phosphate buffer saline and fixed for 10 minutes at 4° C.; 10% formaldehyde plus 0.2% glutaraldehyde and PBS were added and the slides were then washed with PBS. The fixed tissue was stained with hematoxylin and eosin using standard techniques.

Light and fluorescence microscopy were carried out using a Nikon microscope equipped with a Xenon lamp power supply and a GFP filter set (Chromotechnology Corp., Brattleboro, Vt.). Confocal microscopy was with an MRC-600 Confocal Imaging System (Bio-Rad) mounted on a Nikon microscope with an argon laser.

The mice, at sacrifice, had tumors in the ovaries ranging in diameter from 18.7 mm–25.3 mm (mean 21.9±3.1 mm).

The fresh organ tissues examined under fluorescence microscopy with no treatment of the tissues showed seeding of the tumor throughout the peritoneal cavity, including the colon (6/6 mice), cecum (5/6), small intestine (4/6), spleen (1/6), and peritoneal wall (6/6). Numerous micrometastases were detected in the lungs of all mice and multiple micrometastases were also detected on the liver (1/6), kidney (1/6), contralateral ovary (3/6), adrenal gland (2/6), para-aortic lymph node (5/6) and pleural membrane (5/6). Single-cell micrometastases could not be detected by the standard histological techniques described above and even the multiple cell colonies were difficult to detect using them. As the colonies developed, the density of tumor cells decreased markedly in the center.

In an additional experiment, 5×10^6 Clone-38 cells were injected into a nude mouse through the tail vein and the mouse sacrificed after two minutes. Fresh visceral organs were analyzed by fluorescence microscopy and showed the presence of fluorescent cells in peritoneal wall vessels which formed emboli in the capillaries of the lung, liver, kidney, spleen, ovary, adrenal gland, thyroid gland and brain.

Thus, using these techniques, progression of micrometastasis can be observed as seeded cells develop into colonies within the relevant target organs. Further, screening for micrometastases can be done easily and quickly in all systemic organs.

EXAMPLE 3

Murine Model Using Human Lung Cancer Cells

The procedures are generally those set forth in Example 2 except that Clone-26 cells as prepared in Example 1 were used instead of Clone-38 CHO cells.

A. As in Example 2, tumors were grown in six-week-old Balb/C nu/nu male mice injected subcutaneously with a single 0.4 ml dose of 10^7 Clone-26 cells within 40 minutes of harvesting by trypsinization and washing three times with cold serum-containing medium. The cells were kept on ice prior to injection. The animals were sacrificed when the tumors had reached approximately 1.2 cm diameters. The 1.2 cm tumors formed after about 5 weeks.

B. The tumor pieces, 1 mm^3, were implanted by SOI into the left visceral pleura of 8 mice as described by Astoul, P. et al., *Anticancer Research* (1994) 14:85–92; Astoul, P. *J Cell Biochem* (1994) 56:9–15, both incorporated herein by reference. Briefly, the mice were anesthetized by isofluoran inhalation an a small 1 cm transverse incision made on the left lateral chest, via the fourth intercostal space, resulting in total lung collapse. Five tumor pieces were sewn together with a 7-0 nylon surgical suture and fixed by making one knot. The lung was taken up by forceps and the tumor sewn into the lower part of the lung with one suture, after which the lung was returned to the chest cavity and the muscles and skin closed with a single layer of 6-0 silk sutures. The lung was reinflated by withdrawing air from the chest cavity with a 23-gauge needle.

C. Four of the mice were sacrificed at 4 weeks and another 4 at 8 weeks. Pleural tumors for the 4-week group ranged from 244.40 mm^3–522.88 mm^3; those from the 8 week group from 1279.08 mm^3–2714.40 mm^3. This represented mean volumes of 371 mm^3 and 1799 mm^3. Specimens of tissue were sliced at 1 mm thickness and observed directly under fluorescent microscopy using a Nikon microscope equipped with a Xenon lamp power supply and a Leica stereo fluorescence microscope equipped with a mercury lamp power supply and GFP filter sets. All of the animals showed chest wall invasion and local and regional spread of the tumor, but in the 8-week mice, all tumors involved the mediastinum and contralateral pleural cavity as well as metastases on the visceral and parietal pleura. Pulmonary hilum lymph nodes were involved in 3 of 4 mice of the 4-week group and all of the mice in the 8-week group. Cervical node involvement was detected in one of the mice of the 8-week group, but no other metastases were observed. The animals were also observed directly before the tissues were excised. The margin of the invading tumor in normal lung tissue could be detected by GFP fluorescence and a small vessel could be seen developing at the margin of the tumor.

D. In an additional experiment, 8 nude mice were injected in the tail vein with a single dose of 1×10^7 Clone-26 cells that had been harvested by trypsinization and washed 3 times with cold serum-containing medium. The injection contained a total volume of 0.8 ml within 40 min. of harvesting. Again, 4 mice were sacrificed at 4 weeks and another 4 at 8 weeks and tissue specimens were obtained and studied by microscopy as described above. Numerous micrometastatic colonies were detected in whole lung tissue in both groups ranging from 5.2 μm to 32.5 μm in the 4-week group and 5.5 μm–178.3 μm in the 8-week group. The colonies from the 8-week group did not appear further developed as compared with those from the 4-week group. Numerous small colonies ranging in number to less than 10 cells were detected at the lung surface in both groups and brain metastases were detected in 1 mouse of the 4-week group and 2 from the 8-week group. One mouse in the 8-week group had systemic metastases in the brain, the submandibular gland, the whole lung, the pancreas, the bilateral adrenal glands, the peritoneum and the pulmonary hilum lymph nodes.

E. In an additional experiment, similar to that set forth in the previous paragraph, the mice injected in a tail vein with 10^7 Clone-26 cells were sacrificed at 4,8 and 12 weeks and the tissues examined as described. Most of the colonies and mice sacrificed at 8 weeks were not obviously further developed compared with those sacrificed at 4 weeks, but numerous small quantities ranging in number down to less than 10 cells and ranging in size from 5.5 μm–110 μm were detected at the lung surface. At 12 weeks, there were many small metastatic colonies which appeared dormant, although other colonies grew extensively by this time, reaching a size up to 1100 μm, suggesting a heterogeneity of dormant and active tumor colonies in the lung.

EXAMPLE 4

Growth of Clone-26 Tumor Cells in Histoculture

Six-week old SCID/SCID mice were injected intravenously with a single dose of 7.5×10^7 Clone-26 cells which had been harvested by trypsinization and washed 3 times with cold serum-containing medium and kept on ice as described above. The cells were injected in a total volume of 0.5 ml within 40 minutes of harvesting. After 3 weeks, numerous micrometastatic colonies were detected in whole lung tissue up to approximately 550 μm. After 5 weeks, the mice were sacrificed and the Clone-26 seeded mouse lungs were removed and histocultured on spun gels using the histoculture methods developed by Leighton, *J. Cancer Res* (1957) 17:929–941; Leighton, J. et al., *Cancer Res* (1960) 20:575–597; Hoffmnan, R. M. *Cancer Cells* (1991) 3:86–92. Tumor colonies spread rapidly in the lung tissue over time and after 1 week the tumor cells started to invade and colonize supporting collagen sponge-gel. After 2 weeks, tumor cells formed satellite colonies in the sponge-gel

distant from the primary colonies in the lung tissue, thus growing faster in histoculture than in SCID mice. Tumor colonies could grow in histoculture for more than 1 month.

EXAMPLE 5

Construction of a Retroviral Expression Vector for GFP and Preparation of Labeled Tumor Cell Lines

FIGS. 1*a* and 1*b* show the construction of expression vectors for GFP under control of the SV40 promoter. The constructs employ commercially available pEGFP series vectors available from Clontech. Both bacterial and mammalian expression vectors are available which permit production of additional proteins, as well as GFP, either as fusions or in dicistronic systems. FIG. 1*a* shows the construction of an expression vector, pGFP/Met, for a fusion of GFP with methioninase; FIG. 1*b* shows the construction of a vector pGFP/SV40 for production of a fusion protein of GFP with the SV40 T-antigen.

Commercial vectors containing the GFP coding sequence of the desired spectral characteristics using the pLEIN system described in Example 6 were transfected into cell lines originating from tumors, such as human breast cancer, human prostate cancer, human glioblastoma and mouse melanoma. In this manner, human breast cancer cell lines MF-7, MDA-MB468 and MDA-MB435, human prostate cancer cell lines PC3 and DU145, human glioblastoma cell line 324, human lung cancer cells Anip-73 and H460, human colon cancer cells lines Colo-205, HCT-15 and WiDr, human gastric cancer cell line NVGC-4, human kidney cancer cell line SN12C, human tongue cancer cell line SCC-25, human melanomas LOX and SK-mel-5, labeled Chinese hamster ovary cells from cell line CHO-K1 and mouse melanoma cell line B16 labeled with green fluorescent protein were established.

The SV40 T-antigen protein is useful to immortalize cultured cells so as to establish permanent cell lines. Accordingly, the vector pGFP/SV40 is transfected into a series of tumor cell cultures to provide fluorescent immortalized cell lines.

EXAMPLE 6

In Vivo Labeling of Established Tumors

Unlabeled tumors derived from the human lung cancer cell line Anip973 were established in mice using the procedure set forth in Example 3, paragraphs A and B, but substituting unlabeled Anip973 cells for clone 26. The mice were then injected with 1×10^7 packaging cells containing the retroviral vector GFP-retrovirus pLEIN contained in PT67 cells. This virus packaging system is available from Clontech, San Diego, Calif. pLEIN contains an insert of the coding sequence for EGFP, a red-shifted variant of wild-type GFP that has been optimized for brighter fluorescence and higher expression in mammalian cells. It has an excitation maximum of 488 nm and an emission maximum at 507 nm. This mutant contains a double amino acid substitution at position 64 from Phe to Leu and at position 65 from Ser to Thr. It is described by Comack, B. et al. *Gene* (1996) 173:31–38. There are more than 190 silent base changes to maximize human codon usage preferences as described by Haas, J. et al. *Curr Biol* (1996) 6:315–324. Thus, pLEIN contains the above-described GFP coding sequence inserted into the multiple cloning site of pLXIN to obtain a dicistronic expression system which permits coordinated translation of the GFP and neomycin resistance. Three days after injection of the cells into the peritoneal cavity of the mice, the tumor cells could be seen in the seminal vesicles under bright-field microscopy and under fluorescent microscopy.

EXAMPLE 7

Observation of Angiogenesis

A suspension containing 1×10^7 clone-38 cells, described in Example 1, were injected into the peritoneal cavity of a mouse. Five days later, the mouse was injected in the tail with rhodamine and the mouse was then put under anesthesia and the abdominal cavity opened sufficiently to visualize the tumor. Recovery from this surgery is straightforward. In some cases, abdominal opening is unnecessary as the intraperitoneal tumors can be visualized through intact skin. Tumors were visible in the abdominal cavity and angiogenesis was apparent as identified by the rhodamine fluorescence. Similar results were found in tumors growing in the omentum in the wall of the small intestine, and in the mesentery.

In an analogous experiment, a suspension containing 1×10^7 cells of clone-26, described in Example 1, were injected into the peritoneal cavity of a mouse. After one day, tumors appeared in the mesentery and in the colon wall. These were observed by anesthetizing the mouse and a minimal opening of the abdomen. Observations on day 3 of a similarly treated mouse showed tumors in the wall of the small intestine and in the omentum as well as in the colon wall and mesentery. On day 5, a similarly treated mouse was injected in the tail with 100 μl of 2×10^{-3} M rhodamine and a few vessels could be seen in the tumor growing in the mesentery. After day 60, numerous vessels were seen in the tumor growing in the colon wall.

EXAMPLE 8

Construction of Metastatic Models

Using the labeled human cancer cell lines described in Example 5, murine models are established for various types of cancer. The cell lines are implanted into 6-week-old nu/nu female mice with a single dose of 10^7 GFP expressing human tumor cells which had been harvested by trypsinization and washed three time with cold, serum-containing medium and then kept on ice. The cells are injected in subcutaneous space in the flank of the animal at a total volume of 0.4 ml within 40 min of harvesting. The nude mice are sacrificed to harvest the tumor fragments 3 weeks after tumor cell injection. These tumor fragments are then used for surgical implantation into the corresponding tissue (surgical orthotopic implantation (SOI)) in nude mice as recipients.

The recipient mice are first anesthetized and then implanted using established SOI techniques with fragments of the subcutaneously grown colon cancer, lung cancer, breast cancer, prostate cancer or melanoma. In all cases, except for melanoma, the size of the fragment is 1 mm^3; for melanoma, 0.025 mm^3 fragments are prepared from the human melanoma LOX-GFP subcutaneous tumor and 5–6 fragments are implanted. The progress of metastasis is then observed using FOTI with a Leica Stereomicroscope MZ12 with a mercury lamp source. GFP is excited with a D425/60 bandpass filter and a 470DCXR dichroic mirror; fluorescence is emitted through a GG475 longpass filter (Chroma Technology, Brattle -boro, Vt.) and collected by a thermoelectrically cooled ST-133 Micromass High-Speed Controlled Camera—TEA/CCD-1317K1 (Princeton

Instruments, Trenton, N.J.) with a 1317×1035 pixels chip. The images are processed and analyzed with ImagePro+ 3.1 Software (Media Cybernetics, Silver Spring, Md.). High resolution images are captured by computer, or continuously through video output onto video tape.

In the colon cancer model, a small midline incision is made in the abdomen and the colorectal part of the intestine is exteriorized. The serosa is removed and 8–15 pieces of tumor fragments are implanted. An 8-0 surgical suture is used to penetrate the small tumor pieces and suture them to the wall of the intestine. The intestine is returned to the abdominal cavity and abdominal wall is closed. The animals are then observed for metastases.

For lung cancer models, a small 1 cm transverse incision is made on the left lateral chest via the fourth intercostal space; total lung collapse results. Five tumor pieces sewn together with 8-0 nylon surgical suture are fixed by making one knot; the lung is taken out by forceps and the tumor sewn into the lower part of the lung with one suture. After returning the lung to the chest cavity, the chest muscles and skin are closed. The lung is reinflated by withdrawing air from the chest cavity with a 23-gauge needle. The animals can then be observed for metastasis either by FOTI or by excising various tissues.

For breast cancer, an incision of 1.5 cm is made along the medial side of the nipple and after blunt dissection, the fat pad is exposed. A small incision is made and a small pocket formed to accommodate 2 fragments of the tumor tissue; an 8-0 suture is made to close the pocket. The skin layer is then closed. The animals are then observed by FOTI or by tissue excision.

For prostate cancer, an opening is made above the pubic symphysis to expose the prostate gland. The fascia surrounding the dorsal portion of the prostate and the dorsal lateral lobes of the gland are separated by a small incision. Five randomized fragments are sutured into the incision using a 8-0 nylon suture. The two parts of the separated lobes are sutured together and the surrounding fascia used to wrap this portion of the gland to consolidate the incision. The abdomen is then closed and the animals maintained for observation.

For melanoma, 5–6 fragments are transplanted subdermally into the flank with a 13×¼ cancer implant needle (Popper & Sons, New Hyde Park, N.Y.).

Images can be obtained as described above showing metastases to various locations in the animal.

The animals treated as described above, can then be used to evaluate potential protocols for treatment of cancer and metastasis inhibition. The metastatic progress of the fluorescent tumors in animals administered the protocols is compared to similar animals lacking treatment. The efficacy of the protocols can then be directly observed.

What is claimed is:

1. A method to evaluate a candidate protocol or drug for the inhibition of metastasis of a primary tumor which method comprises:

administering said protocol or drug to a mammalian subject which contains a primary tumor that stably expresses green fluorescent protein (GFP) in cells of said tumor when said tumor metastasizes and monitoring the progression of metastasis by observing the presence, absence or intensity of the fluorescence at various locations in the treated subject by fluorescent optical tumor imaging in the intact subject;

wherein said subject has been modified to contain said tumor that expresses GFP by surgical orthotopic implantation of said tumor wherein said subject is a nude or SCID mouse or is a mouse which is syngeneic to said tumor;

monitoring the progression of metastasis in a control subject by fluorescent optical tumor imaging in the intact control subject, which contains a similar tumor that expresses green fluorescent protein;

wherein said control subject has been modified to contain said tumor that expresses GFP by surgical orthotopic implantation of said tumor wherein said control subject is a nude or SCID mouse or is a mouse which is syngeneic to said tumor; and

comparing the progression of metastasis in said treated subject with the progression of metastasis in said control subject;

whereby a diminution of the progression of metastasis in said treated subject as compared to said control subject identifies the protocol or drug as effective in inhibiting metastasis.

2. The method of claim **1** wherein the progression of metastasis is further monitored by excising fresh organ tissues from various locations in said subject.

3. The method of claim **2** wherein said excised tissues are observed by microscopic examination of fresh tissue slices.

4. A method to monitor metastasis of a primary tumor in a mammalian subject which contains said primary tumor, and wherein said tumor stably expresses green fluorescent protein (GFP) in cells of said tumor when said tumor stably metastasizes,

wherein said subject has been modified to contain said tumor that expresses GFP by surgical orthotopic implantation of said tumor and wherein said subject is a nude or SCID mouse or is a mouse which is syngeneic to said tumor;

which method comprises monitoring the progression of metastasis by observing the presence, absence or intensity of the fluorescence as a function of time at various locations in said subject by fluorescent optical tumor imaging in the intact subject.

5. A method to monitor metastasis of a primary tumor in a mammalian subject which contains said primary tumor, and wherein said tumor stably expresses green fluorescent protein (GFP) in cells of said tumor when said tumor metastasizes,

wherein said primary tumor is endogenous to said mammalian subject and expresses said GFP as a result of locally administering a retroviral vector to said subject in the vicinity of said tumor, said retroviral vector containing an expression system for said GFP;

which method comprises monitoring the progression of metastasis by observing the presence, absence or intensity of the fluorescence as a function of time at various locations in said subject by fluorescent optical tumor imaging in the intact subject.

6. The method of claim **5** wherein the subject is human.

* * * * *

(12) **United States Patent**
Yang et al.

(10) **Patent No.: US 6,649,159 B2**
(45) **Date of Patent: Nov. 18, 2003**

(54) **WHOLE-BODY OPTICAL IMAGING OF GENE EXPRESSION AND USES THEREOF**

(75) Inventors: **Meng Yang**, San Diego, CA (US); **Eugene Baranov**, San Diego, CA (US)

(73) Assignee: **AntiCancer, Inc.**, San Diego, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/812,710**

(22) Filed: **Mar. 19, 2001**

(65) **Prior Publication Data**

US 2002/0013954 A1 Jan. 31, 2002

Related U.S. Application Data

(60) Provisional application No. 60/190,196, filed on Mar. 17, 2000.

(51) **Int. Cl.**[7] **A61K 48/00**; A61K 49/00; A01N 63/00; C12N 15/63; C12N 15/85

(52) **U.S. Cl.** **424/93.21**; 424/93.1; 424/93.2; 424/9.1; 435/320.1; 435/325

(58) **Field of Search** 800/8; 435/320.1, 435/325; 536/24.5; 424/93.1

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,650,135	A	7/1997	Contag et al.	424/9.1
5,876,711	A	3/1999	Fattaey	424/932
6,020,192	A	2/2000	Muzyczka et al.	435/320.1

OTHER PUBLICATIONS

Yang et al., Whole-body optical imaging of green fluorescent protein-expressing tumors and metastates, 2000, PNAS, vol. 97, pp. 1206–1211.*

Edinger et al., Noninvasive assessment of tumor cell proliferation in animal models, 1999, NEOPLASIA, vol. 1, pp. 303–310.*

Zhang et al., An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells, 1996, Biochemical and Biophysical Communications, vol. 227, pp. 707–711.*

Yang, M. et al. "Whole-Body Optical Imaging of Green Fluorescent Protein-Expressing Tumors and Metastases" PNAS 97(3): 1206–1211 (2000).

Yang, M. et al. "Visualizing Gene Expression by Whole-Body Fluorescence Imaging" PNAS 97(22):12278–12282 (2000).

Benard et al., J. Nucl. Med. (1999) 40(8):1257–1263.

Brenner et al., Eur. J. Nucl. Med. (1999) 26(12):1567–1571.

Engelson et al., Am. J. Clin. Nutr. (1999) 69(9):1162–1169.

Eustace et al., Magn. Reson. Imaging Clin. (N. Am.) (1999) 7(2):209–236.

Jerusalem et al., Blood (1999) 9492):429–433.

Saunders et al., Ann. Thorac. Surg. (1999) 67(3):790–797.

Valk et al., Arch. Surg. (1999) 134(5):503–511.

Yang et al., Proc. Natl. Acad. Sci. USA (2000) 97(3):1206–1211.

* cited by examiner

Primary Examiner—Anne-Marie Falk
Assistant Examiner—Celine Qian
(74) *Attorney, Agent, or Firm*—Morrison & Foerster LLP

(57) **ABSTRACT**

The invention relates to the whole-body external optical imaging of gene expression. Specifically, methods for whole-body external optical imaging of gene expression and methods for evaluating a candidate protocol or drug for treating diseases or disorders using a fluorophore operatively linked to the promoter of a gene and external optical imaging are provided herein. Methods to screen for substances or genes that regulate target promoters are also provided.

13 Claims, 2 Drawing Sheets

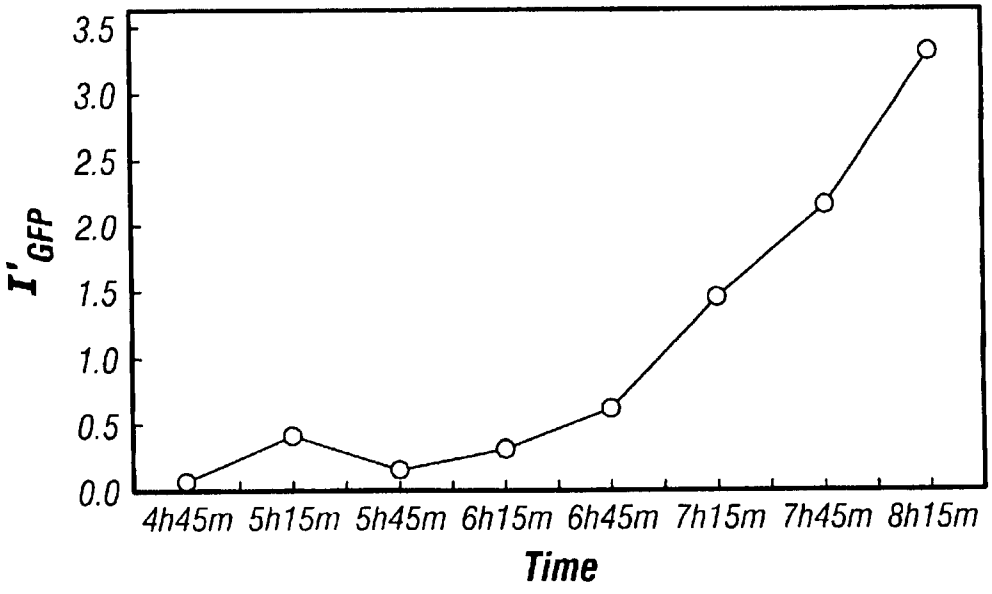


FIG. 1A

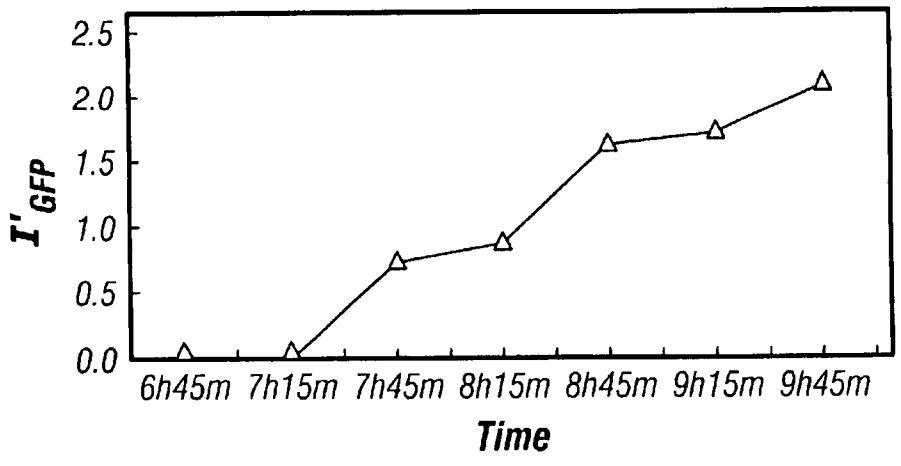


FIG. 1B

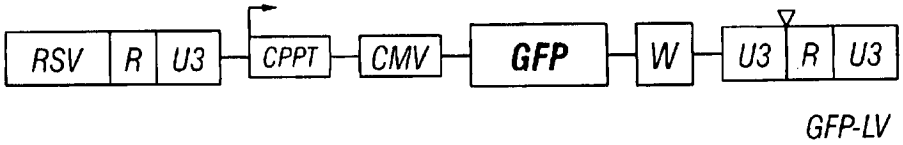

RSV
R
U3
CPPT
CMV
GFP
W
U3
R
U3
GFP-LV

FIG. 2A

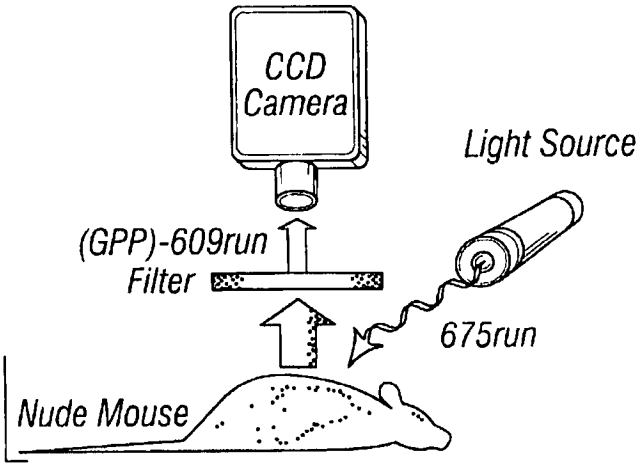
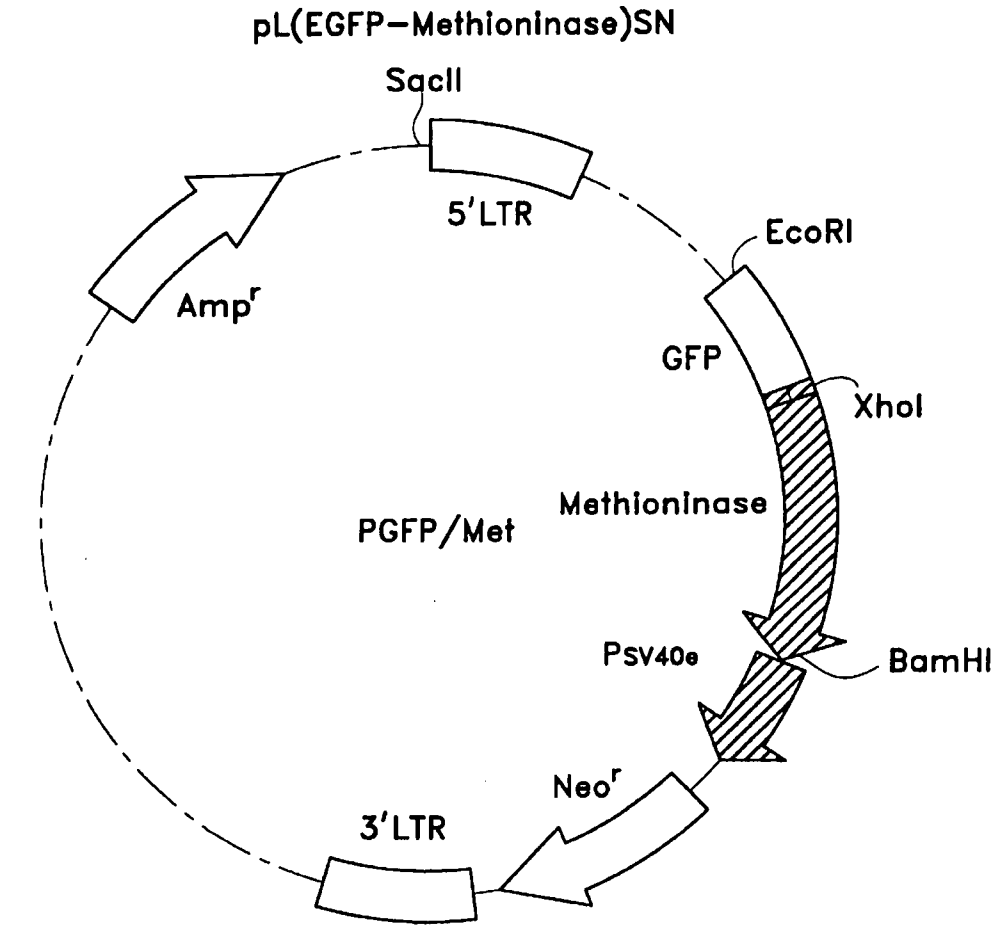

CCD Camera
Light Source
(GPP)-609run Filter
675run
Nude Mouse

FIG. 2B



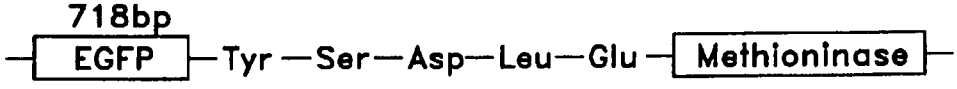


Fig. 1a

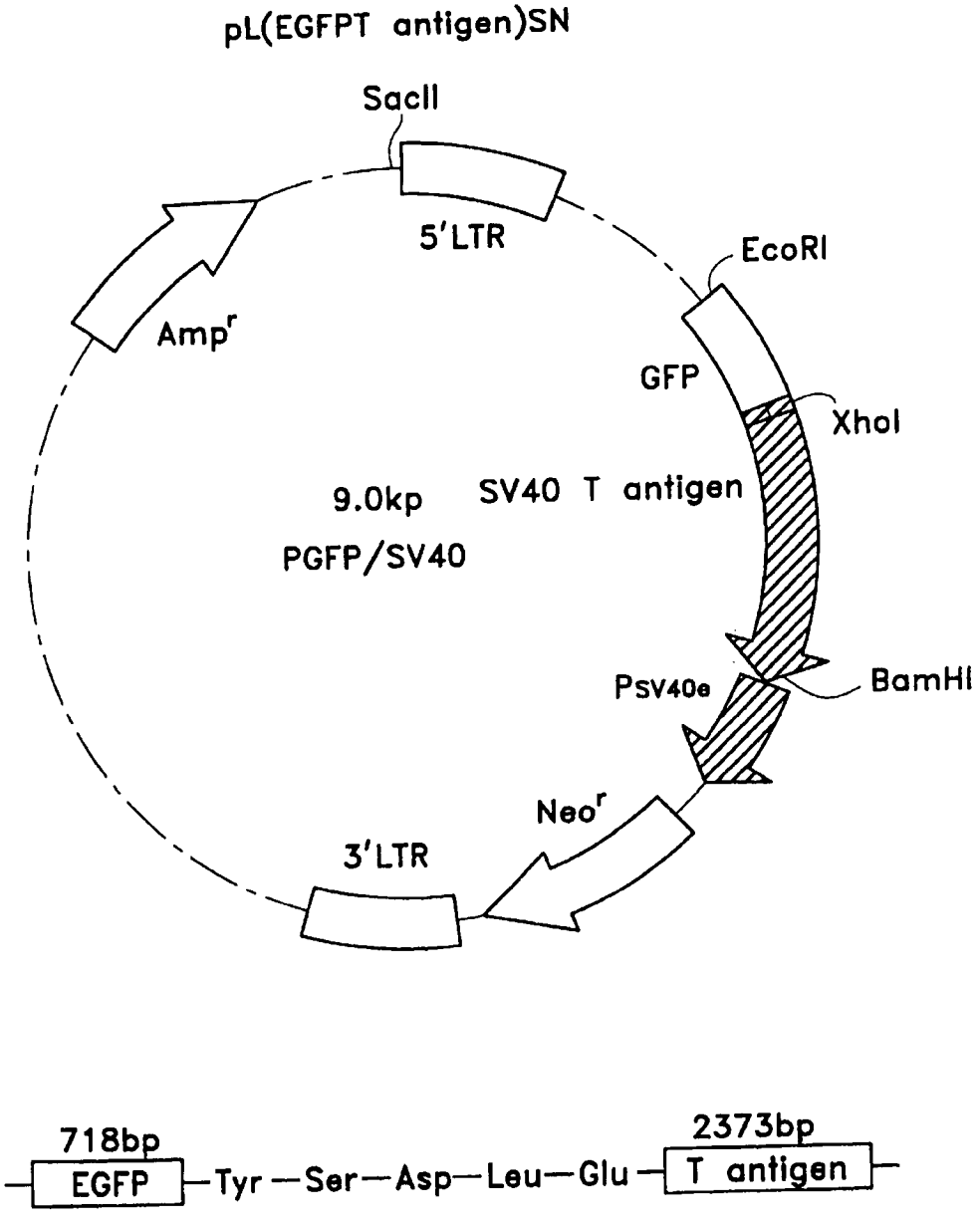


Fig. 1b

WHOLE-BODY OPTICAL IMAGING OF GENE EXPRESSION AND USES THEREOF

This application claims priority under 35 U.S.C. 119 from provisional application U.S. Ser. No. 60/190,196 filed Mar. 17, 2000, the contents of which are incorporated herein by reference.

TECHNICAL FIELD

The invention relates to the whole-body external optical imaging of gene expression. Specifically, methods for whole-body external optical imaging of gene expression and methods for evaluating a candidate protocol or drug for treating diseases or disorders using a fluorophore operatively linked to the promoter of a gene and external optical imaging are provided herein. Methods to screen for substances or genes that regulate target promoters are also provided.

BACKGROUND ART

Whole-body imaging technology has been used to monitor "tracer molecules" in the intact body. For example, Brenner et al. studied the diagnostic value of iodine-123-2-hydroxy-3-iodo-6-methoxy-N-[(1-ethyl-2-pyrrolidinyl) methyl] benzamide (IBZM) whole-body imaging in comparison to thallium-201 scintigraphy in patients with metastatic malignant melanoma (Brenner et al., *Eur. J. Nucl. Med.,* 26(12):1567–71 (1999)). Benard et al. conducted clinical evaluation of processing techniques for attenuation correction with ^{137}Cs in whole-body PET imaging (Benard et al., *J. Nucl. Med.,* 40(8):1257–63 (1999)). Jerusalem et al. showed that whole-body positron emission tomography using ^{18}F-fluorodeoxyglucose for posttreatment evaluation in Hodgkin's disease and non-Hodgkin's lymphoma has higher diagnostic and prognostic value than classical computed tomography scan imaging (Jerusalem et al., *Blood,* 94(2):429–33 (1999)). Eustace et al. discussed practical issues, clinical applications, and future directions of whole-body MR imaging (Eustace et al., *Magn. Reson. Imaging Clin. (N. Am),* 7(2):209–36 (1999)). Engelson et al. studied fat distribution in HIV-infected patients reporting truncal enlargement quantified by whole-body magnetic resonance imaging (Engelson et al., *Am. J. Clin. Nutr.,* 69(6 :1162–9 (1999)). Valk et al. used whole-body positron emission tomography (PET) imaging with [^{18}F]fluorodeoxyglucose in management of recurrent colorectal cancer (Valk et al., Arch. Surg., 134(5):503–11 (1999)). Saunders et al. evaluated fluorine-18-fluorodeoxyglucose whole body positron emission tomography imaging in the staging of lung cancer (Saunders et al., *Ann. Thorac. Surg.,* 67(3):790–7 (1999)).

U.S. Pat. No. 5,650,135 discloses a noninvasive method for detecting the localization of an entity under study from within a mammalian subject, which method comprises: (a) administering to the subject a conjugate of the entity and a light-generating moiety or a transformed cell expressing the light-generating moiety; (b) after a period of time in which the conjugate or transformed cell can achieve localization in the subject, immobilizing the subject within the detection field of a photodetector device; (c) maintaining the subject in an immobilized condition, (d) during said maintaining, measuring photon emission from the light-generating moiety, localized in the subject, with the photodetector device until an image of photon emission can be constructed; and (e) detecting said image through an opaque tissue of said mammal. U.S. Pat. No. 5,650,135 also discloses a noninvasive method for detecting the level of an entity under study in a mammalian subject over time, which

method comprises: (a) administering to the subject a conjugate of the entity and a light-generating moiety or a transformed cell expressing the light-generating moiety; (b) placing the subject within the detection field of a photodetector device; (c) maintaining the subject in the detection field of the device; (d) during said maintaining, measuring photon emission from the light-generating moiety, in the subject, with the photodetector device; and (e) repeating steps (b) through (d) at selected intervals, wherein said repeating is effective to detect changes in the level of the entity in the subject over time.

Recently, Yang et al. conducted whole-body optical imaging of green fluorescent protein-expressing tumors and metastases (Yang et al., *Proc. Natl. Acad. Sci. (USA),* 97(3): 1206–11 (2000)). Yang et al. have imaged, in real time, fluorescent tumors growing and metastasizing in live mice. The whole-body optical imaging system is external and noninvasive. It affords unprecedented continuous visual monitoring of malignant growth and spread within intact animals. Yang et al. have established new human and rodent tumors that stably express very high levels of the Aequorea victoria green fluorescent protein (GFP) and transplanted these to appropriate animals. B16F0-GFP mouse melanoma cells were injected into the tail vein or portal vein of 6-week-old C57BL/6 and nude mice. Whole-body optical images showed metastatic lesions in the brain, liver, and bone of B 16F0-GFP that were used for real time, quantitative measurement of tumor growth in each of these organs. The AC3488-GFP human colon cancer was surgically implanted orthotopically into nude mice. Whole-body optical images showed, in real time, growth of the primary colon tumor and its metastatic lesions in the liver and skeleton. Imaging was with either a trans-illuminated epifluorescence microscope or a fluorescence light box and thermoelectrically cooled color charge-coupled device camera. The depth to which metastasis and micrometastasis could be imaged depended on their size. A 60-micrometer diameter tumor was detectable at a depth of 0.5 mm whereas a 1, 800-micrometer tumor could be visualized at 2.2-mm depth. The simple, noninvasive, and highly selective imaging of growing tumors, made possible by strong GFP fluorescence, enables the detailed imaging of tumor growth and metastasis formation. This should facilitate studies of modulators of cancer growth including inhibition by potential chemotherapeutic agents.

Methods for monitoring gene expression are known in the art (see generally, Ausubel et al. (Ed.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc.). However, whole-body external optical imaging of gene expression, which offers simple, noninvasive, highly selective, and real-time recording and analysis of gene expression in an intact multi-cellular organisms, e.g., animals, is not available currently. The present invention addresses this and other related needs in the art.

DISCLOSURE OF THE INVENTION

The invention provides for whole-body external optical imaging of gene expression and methods for evaluating a candidate protocol or drug for treating diseases or disorders. The method uses a fluorophore operatively linked to the promoter of a gene and external optical imaging. Methods to screen for substances or genes that regulate target promoters are also provided.

In a specific embodiment, a method to monitor the expression of a gene is provided, which method comprises: a) delivering to a multi-cellular organism a nucleic acid encod-

ing a fluorophore operatively linked to the promoter of a gene whose expression is to be analyzed or delivering a cell containing said nucleic acid; and b) observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said organism by whole-body external fluorescent optical imaging, whereby the expression of said gene is monitored.

In a preferred embodiment, a nucleic acid encoding a fluorophore operatively linked to the promoter of the gene is delivered directly to the organism. Also preferably, the nucleic acid encoding a fluorophore operatively linked to the promoter of the gene is in a viral vector such as a viral vector derived from adenovirus or a lentivirus.

In another preferred embodiment, a cell containing a nucleic acid encoding a fluorophore operatively linked to the promoter of the gene is delivered to the organism. More preferably, the cell is delivered to the organism via a surgical procedure such as direct implantation by surgical orthotopic implantation (SOI) at a desired site.

In still another preferred embodiment, the fluorophore operatively linked to the promoter of a gene is a humanized fluorophore. Also preferably, the fluorophore is a green fluorescent protein (GFP), a blue fluorescent protein (BFP) or a red fluorescent protein (RFP). More preferably, the GFP is the humanized hGFP-S65T.

In yet another preferred embodiment, the multi-cellular organism to be analyzed is a plant or an animal, including a transgenic animal. More preferably, the animal is a mammal. A human can also be analyzed by the present method.

In yet another preferred embodiment, the gene to be analyzed is expressed in a tissue or organ specific manner. More preferably, the gene is expressed in connective, epithelium, muscle or nerve tissue. Also more preferably, the gene is expressed in an internal animal organ such as brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, gland, internal blood vessels, etc. Yet more preferably, the gene to be analyzed is a tumor or cancer associated gene such as an oncogene or a tumor suppressor gene.

In yet another preferred embodiment, the expression of more than one gene is monitored simultaneously.

In another specific embodiment, a method to evaluate a candidate protocol or drug for treating a disease or disorder is provided, which method comprises: a) administering said protocol or drug to a non-human mammalian subject which expresses a fluorophore under the direction of a promoter of a gene associated with a disease or disorder, and determining the expression of said promoter via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said mammalian subject by whole-body external fluorescent optical imaging; b) determining the expression of said promoter, via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said mammalian subject by whole-body external fluorescent optical imaging, in a control non-human mammalian subject which expresses said fluorophore under the direction of said promoter of said gene; and c) comparing the expression of said promoter determined in steps a) and b), wherein the expression determined in step a) is different from that in step b) identifies said protocol or drug as effective in treating the disease or disorder.

If overexpression of the gene is associated with the disease or disorder, the expression determined in step a) is lower than that in step b) when said protocol or drug is effective in treating the disease or disorder.

If underexpression of the gene is associated with the disease or disorder, the expression determined in step a) is higher than that in step b) when said protocol or drug is effective in treating the infection.

Preferably, the disease or disorder is a cancer, an immune system disease or disorder, a metabolism disease or disorder, a muscle and bone disease or disorder, a nervous system disease or disorder, a signal disease or disorder, or a transporter disease or disorder.

Preferably, the non-human mammalian subject which expresses a fluorophore under the direction of a promoter of the gene is produced by delivering a nucleic acid encoding the fluorophore operatively linked to the promoter of the gene, or a cell containing the nucleic acid, to the non-human mammalian subject. Alternatively, the non-human mammalian subject used in the screen is a transgenic animal.

The non-human mammalian subject used in the screening is preferably a well established laboratory animal such as a mice, a rabbit or a non-human primate.

The fluorophore used in the screening is preferably a green fluorescent protein (GFP), a blue fluorescent protein (BFP) or a red fluorescent protein (RFP).

More than one candidate protocol or candidate drug is preferably screened for simultaneously.

If the non-human mammalian subject expresses a fluorophore under the direction of a promoter of an infectious organism, the expression determined in step a) is lower than that in step b) when said protocol or drug is effective in treating infection caused by the infectious organism.

The non-human mammalian subject used in the screening is preferably an infectious disease animal model.

The infectious organism screened against is preferably a fungus such as a yeast, a bacterium such as an eubacteria or an archaebacteria, or a virus such as a Class I virus, a Class II virus, a Class III virus, a Class IV virus, a Class V virus or a Class VI virus.

If the infection is caused by a bacterium, the candidate drug to be screened is preferably an antibiotic.

In still another specific embodiment, a method to screen for a modulator of the expression of a gene in a non-human multi-cellular organism is provided, which method comprises: a) administering a test substance to a non-human multi-cellular organism which expresses a fluorophore under the direction of a promoter of a gene, and determining the expression of said promoter via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said multi-cellular organism by whole-body external fluorescent optical imaging; b) determining the expression of said promoter, via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations by whole-body external fluorescent optical imaging, in a control multi-cellular organism which expresses said fluorophore under the direction of said promoter of said gene; and c) comparing the expression of said promoter determined in steps a) and b), wherein the expression determined in step a) is different from that in step b) identifies said test substance as a modulator of said gene expression. Preferably, the promoter is an endogenous promoter of the multi-cellular organism.

In yet another specific embodiment, a method to screen for a non-human multi-cellular organism that expresses a gene at an altered level is provided, which method com-

prises: a) administering a mutation-inducing agent or treatment to a non-human multi-cellular organism which expresses a fluorophore under the direction of a promoter of a gene, and determining the expression of said promoter via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said multi-cellular organism by whole-body external fluorescent optical imaging; b) determining the expression of said promoter, via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations by whole-body external fluorescent optical imaging in an untreated control multi-cellular organism which expresses said fluorophore under the direction of said promoter of said gene; and c) comparing the expression of said promoter determined in steps a) and b), wherein the expression determined in step a) is different from that in step b) identifies a multi-cellular organism that expresses said gene at the altered level. Preferably, the mutation-inducing agent or treatment causes a mutation in germ-line cells of the multi-cellular organism so that the desired mutation is stably-transferable to offspring of the multi-cellular organism.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A and 1B show the time course of expression of adenoviral-administered GFP in brain and liver respectively. Fluorescence first becomes visible in the brain within six (6) hours after local delivery and liver fluorescence became detectable at about seven (7) hours after injection into the tail vein.

FIGS. 2A and 2B art pertinent to administration of lentiviral vectors. FIG. 2A is a diagram of lentiviral vector GFP-LV. FIG. 2B is a diagram of a control observation method; whole body measurement involved use of a light box.

MODES FOR CARRYING OUT THE INVENTION

A. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. All patents, applications, published applications and other publications and sequences from GenBank and other data bases referred to herein are incorporated by reference in their entirety.

As used herein, "delivering a nucleic acid to a multi-cellular organism" refers to a process in which the nucleic acid is either administered directly into the body of the multi-cellular organism, or the nucleic acid is administered into a cell first, and then the cell containing the nucleic acid is administered into the body of the multi-cellular organism. After delivery into the organism, the nucleic acid may exist independently from the genome of the host organism or may be integrated into the genome of the host organism. If the nucleic acid is integrated into a germline cell of the host organism, such nucleic acid may be transmitted into the host organism's offspring.

As used herein, "whole-body external fluorescent optical imaging" refers to an imaging process in which the presence, absence or intensity of the fluorescence generated by the fluorophore at various locations in the host organism is monitored, recorded and/or analyzed externally without any procedure, e.g., surgical procedure, to expose and/or to excise the desired observing site from the host organism. To achieve the whole-body external fluorescent optical imaging, it is necessary that the intensity of the fluorescence generated by the fluorophore is sufficiently high so that, even when the fluorescence site is an internal one within the host organism body, the fluorescence signal can be analyzed externally without exposing or excising the site from the host body, or while the animal is not controlled.

As no invasive procedures are required and the intensity of the signal is sufficiently great for direct observation, the animal may remain completely mobile and need not be restrained. The ability to provide a completely non-invasive observation protocol is highly significant. If the animal is traumatized either by, e.g., incision or by physical restraint, e.g., straps or pins, the alteration in metabolism may affect the expression of the genes in organs or tissues.

Since whole-body external fluorescent optical imaging are quick and easily amenable to automation, it can be used for monitoring large number of gene expression simultaneously. In addition, it can be employed in high-throughput screening methods for identifying protocols, substances including candidate drugs, and cis-acting regulators that regulate the expression of a target gene. Using the whole-body external fluorescent optical imaging provided in this application, multiple candidate protocols, substances, drugs, and cis-acting regulators can be screened for, either against a single target gene or against multiple target genes, in either a single animal or in multiple animals, simultaneously.

As used herein, "fluorophore" refers to a protein that is auto-fluorescent such that no other substrates or co-factors are needed for it to fluoresce. Non-limiting examples of such fluorophores include green fluorescent proteins (GFPs), blue fluorescent proteins (BFPs) and red fluorescent protein (RFPs), and functional fragments, derivatives and analogues thereof.

As used herein, "a promoter region or promoter element" refers to a segment of DNA or RNA that controls transcription of the DNA or RNA to which it is operatively linked. The promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of RNA polymerase. These sequences may be cis acting or may be responsive to trans acting factors. Promoters, depending upon the nature of the regulation, may be constitutive or regulated.

As used herein, "operatively linked or operationally associated" refers to the functional relationship of DNA with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA. In order to optimize expression and/or in vitro transcription, it may be necessary to remove, add or alter 5' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation (i.e., start) codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, e.g., Kozak, *J. Biol. Chem.*, 266:19867–19870 (1991)) can be inserted immediately 5' of the start codon and may enhance expression. The desirability of (or need for) such modification may be empirically determined.

As used herein, "humanized fluorophore" refers to a fluorophore whose codon is modified according to the codon usage pattern in human genome to enhance its expression while substantially maintaining its fluorescent characteristics.

As used herein, "multi-cellular organism" refers to an organism with certain cell numbers, mass, and internal structure so that internal sites of such multi-cellular organism are not externally detectable by non-fluorescent optical imaging without exposing the internal sites. Sufficiently high intensity of internal fluorescence is needed for external fluorescent optical imaging of the internal site.

As used herein, "plant" refers to any of various photosynthetic, eucaryotic multi-cellular organisms of the kingdom Plantae, characteristically producing embryos, containing chloroplasts, having cellulose cell walls and lacking locomotion.

As used herein, "animal" refers to a multi-cellular organism of the kingdom of Animalia, characterized by a capacity for locomotion, nonphotosynthetic metabolism, pronounced response to stimuli, restricted growth and fixed bodily structure. Non-limiting examples of animals include birds such as chickens, vertebrates such fish and mammals such as mice, rats, rabbits, cats, dogs, pigs, cows, ox, sheep, goats, horses, monkeys and other non-human primates.

As used herein, "expressed in a tissue or organ specific manner" refers to a gene expression pattern in which a gene is expressed, either transiently or constitutively, only in certain tissues or organs, but not in other tissues or organs.

As used herein, "tissue" refers to a collection of similar cells and the intracellular substances surrounding them. There are four basic tissues in the body: 1) epithelium; 2) connective tissues, including blood, bone, and cartilage; 3) muscle tissue; and 4) nerve tissue.

As used herein, "organ" refers to any part of the body exercising a specific function, as of respiration, secretion or digestion.

As used herein, "disease or disorder" refers to a pathological condition in an organism resulting from, e.g., infection or genetic defect, and characterized by identifiable symptoms.

As used herein, neoplasm (neoplasia) refers to abnormal new growth, and thus means the same as tumor, which may be benign or malignant. Unlike hyperplasia, neoplastic proliferation persists even in the absence of the original stimulus.

As used herein, cancer refers to a general term for diseases caused by any type of malignant tumor.

As used herein, "oncogene" refers to a mutated and/or overexpressed version of a normal gene of animal cells (the proto-oncogene) that in a dominant fashion can release the cell from normal restraints on growth, and thus alone, or in concert with other changes, convert a cell into a tumor cell. Exemplary oncogenes include, but are not limited to, abl, erbA, erbB, ets, fes (fps), fgr, fms, fos, hst, int1, int2, jun, hit, B-lym, mas, met, mil (raf), mos, myb, myc, N-myc, neu (ErbB2), ral (mil), Ha-ras, Ki-ras, N-ras, rel, ros, sis, src, ski, trk and yes.

As used herein, "tumor suppressor gene" (or anti-oncogene, cancer susceptibility gene) refers to a gene that encodes a product which normally negatively regulates the cell cycle, and which must be mutated or otherwise inactivated before a cell can proceed to rapid division. Exemplary tumor suppressor genes include, but are not limited to, p16, p21, p53, RB (retinoblastoma), WT-1 (Wiln's tumor), DCC (deleted in colonic carcinoma), NF-1 (neurofibrosarcoma) and APC (adenomatous polypospis coli).

As used herein, "an immune system disease or disorder" refers to a pathological condition caused by a defect in the immune system. The immune system is a complex and highly developed system, yet its mission is simple: to seek and kill invaders. If a person is born with a severely defective immune system, death from infection by a virus, bacterium, fungus or parasite will occur. In severe combined immunodeficiency, lack of an enzyme means that toxic waste builds up inside immune system cells, killing them and thus devastating the immune system. A lack of immune system cells is also the basis for DiGeorge syndrome: improper development of the thymus gland means that T cell production is diminished. Most other immune disorders result from either an excessive immune response or an 'autoimmune attack'. For example, asthma, familial Mediterranean fever and Crohn disease (inflammatory bowel disease) all result from an over-reaction of the immune system, while autoimmune polyglandular syndrome and some facets of diabetes are due to the immune system attacking 'self' cells and molecules. A key part of the immune system's role is to differentiate between invaders and the body's own cells—when it fails to make this distinction, a reaction against 'self' cells and molecules causes autoimmune disease.

As used herein, "a metabolism disease or disorder" refers to a pathological condition caused by errors in metabolic processes. Metabolism is the means by which the body derives energy and synthesizes the other molecules it needs from the fats, carbohydrates and proteins we eat as food, by enzymatic reactions helped by minerals and vitamins. There is a significant level of tolerance of errors in the system: often, a mutation in one enzyme does not mean that the individual will suffer from a disease. A number of different enzymes may compete to modify the same molecule, and there may be more than one way to achieve the same end result for a variety of metabolic intermediates. Disease will only occur if a critical enzyme is disabled, or if a control mechanism for a metabolic pathway is affected.

As used herein, "a muscle and bone disease or disorder" refers to a pathological condition caused by defects in genes important for the formation and function of muscles, and connective tissues. Connective tissue is used herein as a broad term that includes bones, cartilage and tendons. For example, defects in fibrillin—a connective tissue protein that is important in making the tissue strong yet flexible—cause Marfan syndrome, while diastrophic dysplasia is caused by a defect in a sulfate transporter found in cartilage. Two diseases that originate through a defect in the muscle cells themselves are Duchenne muscular dystrophy (DMD) and myotonic dystrophy (DM). DM is another 'dynamic mutation' disease, similar to Huntington disease, that involves the expansion of a nucleotide repeat, this time in a muscle protein kinase gene. DMD involves a defect in the cytoskeletal protein, dystrophin, which is important for maintaining cell structure.

As used herein, "a nervous system disease or disorder" refers to a pathological condition caused by defects in the nervous system including the central nervous system, i.e., brain, and the peripheral nervous system. The brain and nervous system form an intricate network of electrical signals that are responsible for coordinating muscles, the senses, speech, memories, thought and emotion. Several diseases that directly affect the nervous system have a genetic component: some are due to a mutation in a single gene, others are proving to have a more complex mode of

inheritance. As our understanding of the pathogenesis of neurodegenerative disorders deepens, common themes begin to emerge: Alzheimer brain plaques and the inclusion bodies found in Parkinson disease contain at least one common component, while Huntington disease, fragile X syndrome and spinocerebellar atrophy are all 'dynamic mutation' diseases in which there is an expansion of a DNA repeat sequence. Apoptosis is emerging as one of the molecular mechanisms invoked in several neurodegenerative diseases, as are other, specific, intracellular signaling events. The biosynthesis of myelin and the regulation of cholesterol traffic also figure in Charcot-Marie-Tooth and Neimann-Pick disease, respectively.

As used herein, "a signal disease or disorder" refers to a pathological condition caused by defects in the signal transduction process. Signal transduction within and between cells mean that they can communicate important information and act upon it. Hormones released from their site of synthesis carry a message to their target site, as in the case of leptin, which is released from adipose tissue (fat cells) and transported via the blood to the brain. Here, the leptin signals that enough has been eaten. Leptin binds to a receptor on the surface of hypothalamus cells, triggering subsequent intracellular signaling networks. Intracellular signaling defects account for several diseases, including cancers, ataxia telangiectasia and Cockayne syndrome. Faulty DNA repair mechanisms are also invoked in pathogenesis, since control of cell division, DNA synthesis and DNA repair all are inextricably linked. The end-result of many cell signals is to alter the expression of genes (transcription) by acting on DNA-binding proteins. Some diseases are the result of a lack of or a mutation in these proteins, which stop them from binding DNA in the normal way. Since signaling networks impinge on so many aspects of normal function, it is not surprising that so many diseases have at least some basis in a signaling defect.

As used herein, "a transporter disease or disorder" refers to a pathological condition caused by defects in a transporter, channel or pump. Transporters, channels or pumps that reside in cell membranes are key to maintaining the right balance of ions in cells, and are vital for transmitting signals from nerves to tissues. The consequences of defects in ion channels and transporters are diverse, depending on where they are located and what their cargo is. For example, in the heart, defects in potassium channels do not allow proper transmission of electrical impulses, resulting in the arrhythmia seen in long QT syndrome. In the lungs, failure of a sodium and chloride transporter found in epithelial cells leads to the congestion of cystic fibrosis, while one of the most common inherited forms of deafness, Pendred syndrome, looks to be associated with a defect in a sulphate transporter.

As used herein, "infection" refers to invasion of the body of a multi-cellular organism with organisms that have the potential to cause disease.

As used herein, "infectious organism" refers to an organism that is capable to cause infection of a multi-cellular organism. Most infectious organisms are microorganisms such as viruses, bacteria and fungi.

As used herein, "bacteria" refers to small prokaryotic organisms (linear dimensions of around 1 μm) with non-compartmentalized circular DNA and ribosomes of about 70S. Bacteria protein synthesis differs from that of eukaryotes. Many anti-bacterial antibiotics interfere with bacteria proteins synthesis but do not affect the infected host.

As used herein, "eubacteria" refers to a major subdivision of the bacteria except the archaebacteria. Most Gram-positive bacteria, cyanobacteria, mycoplasmas, enterobacteria, pseudomonas and chloroplasts are eubacteria. The cytoplasmic membrane of eubacteria contains ester-linked lipids; there is peptidoglycan in the cell wall (if present); and no introns have been discovered in eubacteria.

As used herein, "archaebacteria" refers to a major subdivision of the bacteria except the eubacteria. There are 3 main orders of archaebacteria: extreme halophiles, methanogens and sulphur-dependent extreme thermophiles. Archaebacteria differs from eubacteria in ribosomal structure, the possession (in some case) of introns, and other features including membrane composition.

As used herein, "virus" refers to obligate intracellular parasites of living but non-cellular nature, consisting of DNA or RNA and a protein coat. Viruses range in diameter from about 20 to about 300 nm. Class I viruses (Baltimore classification) have a double-stranded DNA as their genome; Class II viruses have a single-stranded DNA as their genome; Class III viruses have a double-stranded RNA as their genome; Class IV viruses have a positive single-stranded RNA as their genome, the genome itself acting as mRNA; Class V viruses have a negative single-stranded RNA as their genome used as a template for mRNA synthesis; and Class VI viruses have a positive single-stranded RNA genome but with a DNA intermediate not only in replication but also in mRNA synthesis. The majority of viruses are recognized by the diseases they cause in plants, animals and prokaryotes. Viruses of prokaryotes are known as bacteriophages.

As used herein, "fungi" refers to a division of eucaryotic organisms that grow in irregular masses, without roots, stems, or leaves, and are devoid of chlorophyll or other pigments capable of photosynthesis. Each organism (thallus) is unicellular to filamentous, and possess branched somatic structures (hyphae) surrounded by cell walls containing glucan or chitin or both, and containing true nuclei.

As used herein, "antibiotic" refers to a substance either derived from a mold or bacterium or organically synthesized, that inhibits the growth of certain microorganisms without substantially harming the host of the microorganisms to be killed or inhibited.

As used herein, "test substance" refers to a chemically defined compound (e.g., organic molecules, inorganic molecules, organic/inorganic molecules, proteins, peptides, nucleic acids, oligonucleotides, lipids, polysaccharides, saccharides, or hybrids among these molecules such as glycoproteins, etc.) or mixtures of compounds (e.g., a library of test compounds, natural extracts or culture supernatants, etc.) whose effect on the promoter to be analyzed is determined by the disclosed and/or claimed methods herein.

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections that follow.

B. Methods of Whole-Body External Optical Imaging of Gene Expression

In a specific embodiment, a method to monitor the expression of a gene is provided herein, which method comprises: a) delivering to a multi-cellular organism a nucleic acid encoding a fluorophore operatively linked to the promoter of a gene whose expression is to be analyzed or a cell containing said nucleic acid; and b) observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said organism by whole-body external fluorescent optical imaging, whereby the expression of said gene is monitored.

The present methods can be used to monitor gene expression for any suitable purposes including prognostic, diag-

nostic and screening purposes. For example, if abnormal gene expression is associated with a disease or disorder in a multi-cellular organism such as a plant or an animal, the present method can be used in prognosis or diagnosis by monitoring the abnormal gene expression. The present monitoring methods are advantageous over the currently available gene expression monitoring methods in several aspects. First, the present monitoring methods can avoid any invasive procedures and this is particularly advantageous for human clinical uses. Second, the present monitoring methods offer in vivo, real-time and continuous monitor and analysis of gene expression in plants or animals, which cannot be accomplished using the currently available monitoring methods. Third, the present monitoring methods are quick and easily amenable to automation, which are important for monitoring large number of gene expression simultaneously. Since many diseases or disorders involve abnormal gene expression of more than gene, the present monitoring methods are particularly suitable for the prognosis and diagnosis of these diseases or disorders. Besides prognosis or diagnosis, if expression of certain genes is a good indicator of tissue or organ health or functionality, the present monitoring methods can also be used in monitoring the health or functionality of these tissues or organs without any invasive procedures.

1. Methods for Delivering the Nucleic Acids into the Multi-Cellular Organism

The nucleic acids encoding a fluorophore operatively linked to the promoter of a gene whose expression is to be analyzed can be a DNA or a RNA. Such nucleic acids can be delivered into the body of the multi-cellular organism by any methods known in the art.

For example, if the host multi-cellular organism is an animal, the DNA or RNA sequence can be delivered to the interstitial space of tissues of the animal body, including those of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular, fluid, mucopolysaccharide matrix among the reticular fibers or organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation of the lymph fluid of the lymphatic channels.

The DNA or RNA sequence can be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression can be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts.

In a specific embodiment, the DNA or RNA sequence is delivered directly to a tissue of the host animal. Preferably, the DNA or RNA sequence is delivered directly to muscle, skin or mucous membrane. Delivery to the interstitial space of muscle tissue is preferred because muscle cells are particularly competent in their ability to take up and express polynucleotides.

The DNA or RNA sequence can be delivered directly to a tissue of the host animal by injection, by gene gun technology or by lipid mediated delivery technology. The injection can be conducted via a needle or other injection devices. The gene gun technology is disclosed in U.S. Pat. No. 5,302,509 and the lipid mediated delivery technology is disclosed in U.S. Pat. No. 5,703,055.

In still another specific embodiment, the DNA or RNA sequence is delivered to a cell of host animal and said cell containing the DNA or RNA sequence is delivered to a suitable tissue of the host animal. Preferably, the DNA or RNA sequence is delivered to tail or portal vein of the host animal.

The DNA or RNA sequence can be delivered to the cells of the host animal by a number of methods (see generally Koprowski & Weiner, DNA vaccination/genetic vaccination, 1998. Springer-verlag Berlin Heidelberg) including $Ca_3(PO_4)_2$-DNA transfection (Sambrook et al., *Molecular Cloning*, 2nd Edition, Plainview, N.Y. Cold Spring Harbor Press, 1989), DEAE dextran-DNA transfection (Sambrook et al., *Molecular Cloning*, 2nd Edition, Plainview, N.Y. Cold Spring Harbor Press, 1989), electroporation (e.g., protocols from Bio-Rad), transfection using "LIPOFECTIN"™ reagent (e.g., protocols from BRL-Life Science), gene gun technology (U.S. Pat. No. 5,302,509), or viral gene delivery system (Kaplitt et al., *Viral Vectors, Academic Press, Inc.*, 1995).

Gold-particle based gene gun delivery is disclosed in U.S. Pat. No. 5,302,509. In a specific embodiment, Bio-Rad helios gene gun system is used in the DNA delivery. (BIO-RAD Inc. New England). The helios gene gun is a convenient, hand-held device that provides rapid and direct gene transfer in vivo. The device employs an adjustable, helium pulse to sweep DNA coated gold microcarriers from the inner wall of a small plastic cartridge directly into the target cells. The tubing prepstation and tubing cutter provide a simple way to prepare 50 cartridge "bullets" at a time.

In a preferred embodiment, a nucleic acid encoding a fluorophore operatively linked to the promoter of the gene is delivered directly to the organism. More preferably, the nucleic acid encoding a fluorophore operatively linked to the promoter of the gene is delivered to the organism, or to a cell to be delivered to the organism, in a viral vector such as a viral vector derived from adenovirus or a lentivirus.

Any viral vectors known in the art can be used. For example, vectors derived from a parvovirus (U.S. Pat. Nos. 5,252,479 and 5,624,820), a paramyxovirus such as simian virus 5 (SV5) (U.S. Pat. No. 5,962,274), a retrovirus such as HIV (U.S. Pat. Nos. 5,753,499 and 5,888,767), and a baculovirus such as a nuclear polyhedrosis virus (U.S. Pat. No. 5,674,747) can be used. Preferably, a vector derived from adenovirus can be used (U.S. Pat. Nos. 5,670,488, 5,817,492, 5,820,868, 5,856,152, 5,981,225).

U.S. Pat. No. 5,670,488 discloses an adenoviral vector comprising an adenovirus genome from which one or more of the E4 open reading frames has been deleted, but retaining sufficient E4 sequences to promote virus replication in vitro, and additionally comprising a DNA sequence of interest operably linked to expression control sequences and inserted into said adenoviral genome.

U.S. Pat. No. 5,817,492 discloses a recombinant adenoviral vector comprising: two DNA sequences which serve as a substrate for a recombinase enzyme, an origin of replication which is operable in an animal cell, a promoter, a foreign gene and a poly(A) sequence, wherein said origin of replication, promoter, foreign gene and poly(A) sequence are located between the two DNA sequences, and wherein said vector contains an E1A gene region deletion.

U.S. Pat. No. 5,820,868 discloses a live recombinant bovine adenovirus vector (BAV) wherein a part or all of the E3 multiple gene coding region is replaced by a heterolo-

gous nucleotide sequence encoding a foreign gene or fragment thereof. It also discloses a live recombinant bovine adenovirus vector (BAV) wherein part or all of the E3 multiple gene coding region is replaced by a heterologous nucleotide sequence encoding a foreign gene or fragment thereof and wherein said heterologous nucleotide sequence is optionally under the control of a promoter not normally associated with either said foreign gene or the bovine adenovirus genome.

U.S. Pat. No. 5,856,152 discloses a hybrid viral vector comprising: (a) adenovirus sequences comprising the adenovirus 5'and 3' cis-elements necessary for replication and virion encapsidation; and (b) adeno-associated virus sequences comprising the 5' and 3' ITRs of an adeno-associated virus, said adeno-associated virus sequences flanked by the adenoviral sequences of (a); and (c) a selected gene operatively linked to regulatory sequences which direct its expression in a target cell, said gene and regulatory sequences flanked by the adeno-associated virus sequences of (b).

U.S. Pat. No. 5,981,225 discloses a gene transfer vector consisting essentially of, in 5' to 3' orientation, the following elements: (i) a first adenovirus inverted terminal repeat, (ii) an adenoviral VAI gene and/or VAII gene, (iii) a gene foreign to adenovirus, wherein said gene is operably linked to a promoter functional in adenovirus target cells, and (iv) a second adenovirus inverted terminal repeat, wherein the order of elements (ii) and (iii) may be reversed; and wherein one or both of element (i) and element (iv) additionally comprise an adenovirus packaging signal, and wherein said vector is incapable of producing, in vitro, recombinant adenovirus virus particles which have encapsidated therein said vector unless said vector is co-transfected or co-infected into adenovirus host cells with adenovirus genomic DNA or adenovirus particles containing adenovirus genomic DNA, respectively.

In another preferred embodiment, cells containing a nucleic acid encoding a fluorophore operatively linked to the promoter of the gene are delivered to the organism. More preferably, the cells are delivered to the organism via a surgical procedure such as direct implantation by surgical orthotopic implantation (SOI) at a desired site (see e.g., Chang et al., *Anticancer Res.,* 19(5B):4199 (1999); and An et al., *Prostate,* 34(3 :169–74 (1998)).

It will be understood, that by introducing a nucleic acid molecule wherein a promoter is coupled to a nucleotide sequence encoding a fluorescent reporter gene, the introduced nucleic acid molecule can be used as a surrogate for the endogenous promoter. Thus, if the endogenous gene is over-expressed or under-expressed in the context of a particular condition, the behavior of the introduced construct will mimic that of the endogenous promoter. It is not necessary that the reporter-encoding nucleotide sequence be operably linked only to a promoter; the nucleotide sequence encoding reporter may be introduced into the nucleotide sequence encoding the protein normally under control of the promoter or coupled to another protein. Any method of operably linking the nucleotide sequence encoding reporter to the control sequences for the gene whose expression is to be monitored falls within the scope of the invention.

It will be seen that there are a number of ways to introduce this construct. First, the nucleic acid comprising the reporter encoding nucleotide sequence operably linked to the control sequences/promoter of interest can be introduced to the multicellular organism by direct injection, but preferably using a viral vector, such as a adenoviral vector or a antiviral vector. Since the introduced construct is not endogenous, the expression of this construct essentially functions as a surrogate for the endogenous gene. That is, the same influences which influence the endogenous gene will also influence the introduced construct. Thus, the conclusions reached by observing the expression of the construct, including the effects of various treatments on such expression, can be extrapolated to, and are equally valid for, the counterpart endogenous gene.

Second, the reporter encoding nucleotide sequence could be introduced into the cells of a particular tissue by targeting to the promoter to be studied and inserted using position-specific techniques, such as homologous recombination. When this method is used, the expression of the endogenous promoter can be observed directly as well as can the effect of various treatments thereon.

Third, a construct such as those described for the first method can be provided to embryonic tissue to obtain transgenic organisms where the reporter construct is itself endogenous, see, for example, Fukumura, D., et al., *Cell* (1998) 94:715–725, incorporated herein by reference, which describes transgenic mice which use GFP as a reporter for VEGF promoter activity.

Techniques for all three methods are well known in the art.

2. Fluorophores

Any fluorophores known in the art can be used in the present methods. In a preferred embodiment, the fluorophore operatively linked to the promoter of a gene is a humanized fluorophore. Also preferably, the fluorophore is a green fluorescent protein (GFP), a blue fluorescent protein (BFP) and a red fluorescent protein (RFP). More preferably, the GFP is the humanized hGFP-S65T.

The native gene encoding GFP has been cloned from the bioluminescent jellyfish Aequorea victoria (Morin et al., *J. Cell Physiol.,* 77:313–318 (1972)). The availability of the gene has made it possible to use GFP as a marker for gene expression. GFP itself is a 283 amino acid protein with a molecular weight of 27 kD. It requires no additional proteins from its native source nor does it require substrates or cofactors available only in its native source in order to fluoresce (Prasher et al., *Gene,* 111:229–233 (1992); Yang et al., *Nature Biotechnol.,* 14:1252–1256 (1996); and Cody et al., *Biochemistry,* 32:1212–1218 (1993)). Mutants of the GFP gene have been found useful to enhance expression and to modify excitation and fluorescence. GFP-S65T (wherein serine at 65 is replaced with threonine) is particularly useful in the invention method and has a single excitation peak at 490 nm. (Heim et al., *Nature,* 373:663–664 (1995)); and U.S. Pat. No. 5,625,048). Other mutants have also been disclosed by Delagrade et al., *Biotechnology,* 13:151–154 (1995); Cormack et al., *Gene,* 173:33–38 (1996); and Cramer et al. *Nature Biotechnol.,* 14:315–319 (1996). Additional mutants are also disclosed in U.S. Pat. No. 5,625,048. By suitable modification, the spectrum of light emitted by the GFP can be altered. Thus, although the term "GFP" is used in the present application, the proteins included within this definition are not necessarily green in appearance. Various forms of GFP exhibit colors other than green and these, too, are included within the definition of "GFP" and are useful in the methods and materials of the invention. In addition, it is noted that green fluorescent proteins falling within the definition of "GFP" herein have been isolated from other organisms, such as the sea pansy, *Renilla reriformis.* Any suitable and convenient form of the GFP gene can be used in the invention. Techniques for labeling cells in general using GFP are disclosed in U.S. Pat. No. 5,491,084 (supra).

Other GFP, BFP and RFP can be used in the present methods. For instances, the green fluorescent proteins encoded by nucleic acids with the following GenBank accession Nos. can be used: U47949 (AGP1); U43284; AF007834 (GFPuv); U89686 (*Saccharomyces cerevisiae* synthetic green fluorescent protein (cox3::GFPm-3) gene); U89685 (*Saccharomyces cerevisiae* synthetic green fluorescent protein (cox3::GFPm) gene); U87974 (Synthetic construct modified green fluorescent protein GFP5-ER (mgfp5-ER)); U87973 (Synthetic construct modified green fluorescent protein GFP5 (mgfp5)); U87625 (Synthetic construct modified green fluorescent protein GFP-ER (mfgp4-ER)); U87624 (Synthetic construct green fluorescent protein (mgfp4) mRNA)); U73901 (Aequorea victoria mutant 3); U50963 (Synthetic); U70495 (soluble-modified green fluorescent protein (smGFP)); U57609 (enhanced green fluorescent protein gene); U57608 (enhanced green fluorescent protein gene); U57607 (enhanced green fluorescent protein gene); U57606 (enhanced green fluorescent protein gene); U55763 (enhanced green fluorescent protein (egfp); U55762 (enhanced green fluorescent protein (egfp); U55761 (enhanced green fluorescent protein (egfp); U54830 (Synthetic *E. coli* Tn3-derived transposon green fluorescent protein (GF); U36202; U36201; U19282; U19279; U19277; U19276; U19281; U19280; U19278; L29345 (Aequorea victoria); M62654 (Aequorea victoria); M62653 (Aequorea victoria); AAB47853 ((U87625) synthetic construct modified green fluorescent protein (GFP-ER)); AAB47852 ((U87624) synthetic construct green fluorescent protein).

Similarly, the blue fluorescent proteins encoded by nucleic acids with the following GenBank accession Nos. can be used: U70497 (soluble-modified blue fluorescent protein (smBFP); 1BFP (blue variant of green fluorescent protein); AAB16959 (soluble-modified blue fluorescent protein).

Also similarly, the red fluorescent proteins encoded by nucleic acids with the following GenBank accession Nos. can be used: U70496 (soluble-modified red-shifted green fluorescent protein (smRSGFP); AAB16958 (U70496) soluble-modified red-shifted green fluorescent protein).

A fluorophore that changes color with time is reported by Teiskikh, A., et al., *Science* (2000) 290:1585–1588, incorporated herein by reference. This permits tracing time dependent expression.

3. Multi-Cellular Organisms

The present methods can be used in monitoring gene expression in any suitable multi-cellular organisms. In a preferred embodiment, the multi-cellular organism to be analyzed is a plant or an animal, including a transgenic animal. More preferably, the animal is a mammal including a human. Animals that can be analyzed with the present monitoring emthods include, but are not limited to, mice, rats, rabbits, cats, dogs, pigs, cows, ox, sheep, goats, horses, monkeys and other non-human primates.

4. Tissue or Organ Specific Gene Expression

The present methods can be used in monitoring expression of genes that are expressed in a tissue or organ specific manner. The present methods can be used in monitoring health and/or functionality of tissues and/or organs if expression pattern of certain genes are associated with health and/or functionality of these tissues and organs. Preferably, the gene to be monitored is expressed in connective, epithelium, muscle or nerve tissue. Also preferably, the gene to be monitored is expressed in an accessory organ of the eye, annulospiral organ, auditory organ, Chievitz organ, circumventricular organ, Corti organ, critical organ, enamel organ, end organ, external female gential organ, external male genital organ, floating organ, flower-spray organ of Ruffini, genital organ, Golgi tendon organ, gustatory organ, organ of hearing, internal female genital organ, internal male genital organ, intromittent organ, Jacobson organ, neurohemal organ, neurotendinous organ, olfactory organ, otolithic organ, ptotic organ, organ of Rosenmüller, sense organ, organ of smell, spiral organ, subcommissural organ, subfornical organ, supernumerary organ, tactile organ, target organ, organ of taste, organ of touch, urinary organ, vascular organ of lamina terminalis, vestibular organ, vestibulocochlear organ, vestigial organ, organ of vision, visual organ, vomeronasal organ, wandering organ, Weber organ and organ of Zuckerkandl. More preferably, the gene to be monitored is expressed in an internal animal organ such as brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, gland, internal blood vessels, etc.

In other embodiments, the fluorophore, e.g., GFP, BFP or RFP, can be operatively linked to the following animal transcriptional control regions that exhibit tissue specificity to monitor these tissue specific gene expressions in animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., *Cell* 38:639–646 (1984); Ornitz et al., *Cold Spring Harbor Symp. Quant. Biol.* 50:399–409 (1986); MacDonald, *Hepatology* 7:425–515 (1987)); insulin gene control region which is active in pancreatic beta cells (Hanahan et al., *Nature* 315:115–122 (1985)), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., *Cell* 38:647–658 (1984); Adams et al., *Nature* 318:533–538 (1985); Alexander et al., *Mol. Cell Biol.* 7:1436–1444 (1987)), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., *Cell* 45:485–495 (1986)), albumin gene control region which is active in liver (Pinckert et al., *Genes and Devel.* 1:268–276 (1987)), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., *Mol. Cell. Biol.* 5:1639–1648 (1985); Hammer et al., *Science* 235:53–58 1987)), alpha-1 antitrypsin gene control region which is active in liver (Kelsey et al., *Genes and Devel.* 1:161–171 (1987)), beta globin gene control region which is active in myeloid cells (Mogram et al., *Nature* 315:338–340 (1985); Kollias et al., *Cell* 46:89–94 (1986)), myelin basic protein gene control region which is active in oligodendrocyte cells of the brain (Readhead et al., *Cell* 48:703–712 (1987)), myosin light chain-2 gene control region which is active in skeletal muscle (Sani, *Nature* 314:283–286 (1985)), and gonadotrophic releasing hormone gene control region which is active in gonadotrophs of the hypothalamus (Mason et al., *Science* 234:1372–1378 (1986)).

5. Tumor or Cancer Associated Gene Expression

The present methods can be used in monitoring expression of genes that are specifically expressed in tumors or cancers. Preferably, the gene to be analyzed is a tumor or cancer associated gene such as an oncogene or a tumor suppressor gene. For instance, the expression of the oncogenes listed in the following Table 1 can be monitored by the present methods.

TABLE 1

Oncogenes and tumor viruses

Acronym	Virus	Species	Tumor origin	Comments
abl	Abelson leukemia	Mouse	Chronic myelogenous leukemia	TyrPK(src)
erbA	Erythroblastosis	Chicken		Homology to human glucocorticoid receptor
erbB	Erythroblastosis	Chicken		TryPK EGF/TGFe receptor
ets	E26 myeloblastosis	Chicken		Nuclear
fes (fps)[a]	Snyder-Thellen sarcoma Gardner-Arnstein sarcoma	Cat		TryPK(src)
fgr	Gardner-Rasheed sarcoma	Cat		TyrPK(src)
fms	McDonough sarcoma	Cat		TyrPK CSF-1 receptor
fps (fes)[a]	Fujinami sarcoma	Chicken		TyrPK(src)
fos	FBJ osteosarcoma	Mouse		Nuclear, TR
hst	NVT	Human	Stomach tumor	FGF homologue
intl	NVT	Mouse	MMTV-induced carcinoma	Nuclear, TR
int2	NVT	Mouse	MMTV-induced carcinoma	FGF homologue
jun	ASV17 sarcoma	Chicken		Nuclear, TR
hit	Hardy-Zuckerman 4 sarcoma	Cat		TyrPK GFR L
B-lym	NVT	Chicken	Bursal lymphoma	
mas	NVT	Human	Epidermoiod carcinoma	Potentiates response to angiotensin II
met	NVT	Mouse	Osteosarcoma	TyrPK GFR L
mil (raf)[b]	Mill Hill 2 acute leukemia	Chicken		Ser/ThrPK
mos	Moloney sarcoma	Mouse		Ser/ThrPK
myb	Myeloblastosis	Chicken	Leukemia	Nuclear, TR
myc	MC29 myelocytomatosis	Chicken	Lymphomas	Nuclear TR
N-myc	NVT	Human	Neuroblastomas	Nuclear
neu (ErbB2)	NVT	Rat	Neuroblastoma	TryPK GFR L
ral (mil)[b]	3611 sarcoma	Mouse		Ser/ThrPK
Ha-ras	Harvey murine sarcoma	Rat	Bladder, mammary and skin carcinomas	GTP-binding
Ki-ras	Kirsten murine sarcoma	Rat	Lung, colon carcinomas	GTP-binding
N-ras	NVT	Human	Neuroblastomas leukaemias	GTP-binding
rel	Reticuloendothe-liosis	Turkey		
ros	UR2	Chicken		TyrPK GFR L
sis	Simian sarcoma	Monkey		One chain of PDGF
src	Rous sarcoma	Chicken		TyrPK
ski	SKV770	Chicken		Nuclear
trk	NVT	Human	Colon carcinoma	TyrPK GFR L
yes	Y73, Esh sarcoma	Chicken		TyrPK(src)

Similarly, the expression of the following tumor suppressor genes can be monitored by the present methods: p16, p21, p27, p53, RB, WT-1, DCC, NF-1 and APC.

Since abnormally high level of oncogene expression and abnormally low expression of tumor suppressor gene are often good indicators of oncogenesis, the present methods can be used in prognosis or diagnosis of cancer, in monitoring the development of oncogenesis and in evaluating the efficacy of the cancer therapy.

C. Methods to Evaluate a Candidate Protocol or Drug for Treating Disease or Disorder

Since the method of the invention evaluates gene expression with regard to particular control sequences, the effect of various compounds, treatments (such as irradiation) or other perturbations of the genetic environment can be evaluated for their effect on expression using the methods of the invention. Thus, gene toxic agents, for example, can be identified.

In a specific embodiment, a method to evaluate a candidate protocol or drug for treating a disease or disorder is provide herein, which method comprises: a) administering said protocol or drug to a non-human mammalian subject which expresses a fluorophore under the direction of a promoter of a gene associated with a disease or disorder, and determining the expression of said promoter via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said mammalian subject by whole-body external fluorescent optical imaging; b) determining the expression of said promoter, via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said mammalian subject by whole-body external fluorescent optical imaging, in a control non-human mammalian subject which expresses said fluorophore under the direction of said promoter of said gene; and c) comparing the expression of said promoter determined in steps a) and b), wherein the expression determined in step a) is different from that in step b) identifies said protocol or drug as effective in treating the disease or disorder.

In a preferred embodiment, overexpression of the gene is associated with the disease or disorder and the expression determined in step a) is lower than that in step b) identifies said protocol or drug as effective in treating the disease or disorder.

In another preferred embodiment, underexpression of the gene is associated with the disease or disorder and the expression determined in step a) is higher than that in step b) identifies said protocol or drug as effective in treating the infection.

In still another preferred embodiment, the non-human mammalian subject which expresses a fluorophore under the direction of a promoter of a gene associated with a disease or disorder is produced by delivering a nucleic acid encoding the fluorophore operatively linked to the promoter, or a cell containing the nucleic acid, to the non-human mammalian subject (see Section B supra).

Any non-human mammalian subject can be used in the present screening methods. Preferably, the non-human mammalian subject used in the screening is a well established laboratory animal such as a mice, a rabbit or a non-human primate. Also preferably, the non-human mammalian subject used in the screening is an infectious disease animal model. Still preferably, the non-human mammalian subject used in the screen is a transgenic animal.

Any fluorophores known in the art, including the ones described in Section B, can used in the present screening methods. In a preferred embodiment, the fluorophore used in the screening is s a green fluorescent protein (GFP), a blue fluorescent protein (BFP) or a red fluorescent protein (RFP).

The present methods can be used to screen candidate protocols or drugs for treating any known diseases or disorders. In a preferred embodiment, the diseases or disorders to be screened against are cancers, immune system diseases or disorders, metabolism diseases or disorders, muscle and bone diseases or disorders, nervous system diseases or disorders, signal diseases or disorders and transporter diseases or disorders.

In yet another preferred embodiment, the non-human mammalian subject expresses a fluorophore under the direction of a promoter of an infectious organism and the expression determined in step a) is lower than that in step b) identifies said protocol or drug as effective in treating infection caused by the infectious organism.

The non-human mammalian subject used in the screening may be an infectious disease animal model.

The infectious organism screened against may be a fungus such as a yeast, a bacterium such as an eubacteria or an archaebacteria, or a virus such as a Class I virus, a Class II virus, a Class III virus, a Class IV virus, a Class V virus or a Class VI virus.

Any substances can be screened using the present screening methods for finding drug candidates for treating infection. In a preferred embodiment, a combinatorial library is used in the screening assays. Methods for synthesizing combinatorial libraries and characteristics of such combinatorial libraries are known in the art (See generally, *Combinatorial Libraries: Synthesis, Screening and Application Potential* (Cortese Ed.) Walter de Gruyter, Inc., 1995; Tietze and Lieb, *Curr. Opin. Chem. Biol.*, 2(3):363–71 (1998); Lam, *Anticancer Drug Des.*, 12(3 :145–67 (1997); Blaney and Martin, *Curr. Opin. Chem. Biol.*, 1(1):54–9 (1997); and Schultz and Schultz, *Biotechnol. Prog.*, 12(6 :729–43 (1996)).

If the infection is caused by bacteria, known antibiotics can be screened using the present screening methods for finding a suitable drug candidate. Preferably, the antibiotics to be screened are aminoglycosides (e.g., streptomycin, gentamicin, sisomicin, tobramycin, amicacin), ansamycins (e.g., rifamycin), antimycotics polyenes (e.g., nystatin, pimaricin, amphotericin B., pecilocin), benzofuran derivatives (e.g., griseofulvin), β-lactam antibiotics penicillins (e.g., penicillin G and its derivatives, oral penicillins, penicillinase-fixed penicillin broad-spectrum penicillins, penicillins active against Proteus and Pseudomonas), cephalosporins (e.g., cephalothin, cephaloridine, cephalexin, cefazolin, cefotaxime), chloramphenicol group (e.g., chloramphenicol, thiamphenicol, azidamphenicol), Imidazole fluconazole, itraconazole, linosamides (e.g., lincomycin, clindamycin), macrolides (e.g., azithromycin, erythromycin, oleandomycin, spiramycin, clarithromycin), peptides, peptolides, polypeptides (e.g., polymyxin B and E, bacitracin, tyrothricin, capreomycin, vancomycin), quinolones (e.g., nalidixic acid, ofloxacin, ciprofloxacin, norfioxin), tetracyclines (e.g., tetracycline, oxytetracycline, minocycline, doxycycline) and other antibiotics (e.g., phosphomycin, fusidic acid).

D. Methods to Screen for Gene Expression Modulators and Regulators

The above-described screening methods can also be used to identify gene expression modulators, i.e., trans-acting substances that modulate the expression of a target gene in a multi-cellular organism, or regulators, i.e., cis-acting genes of a multi-cellular organism that regulate the expression of the target gene. Besides for identifying disease or disorder treatment protocols or drugs, the screening methods described herein have wide applications in industrial, agricultural, environmental protection and many other fields. For example, transgenic animals such as transgenic cows are commercially used. It is desirable to find a suitable substance that increases the expression of the transgene and such substance can be added to the animal feed. Similarly, it is desirable to find and modify gene(s) within the transgenic cow that enhances the expression of the target transgene.

Once it is decided that alteration of the expression level of a target gene is desirable, a fluorophore can be operatively linked to the promoter, or other transcriptional control region, of the target gene and be expressed in a multi-cellular organism. Then, the multi-cellular organism expressing the fluorophore can be treated with a test substance to identify which substance modulates the fluorophore expression. Alternatively, the multi-cellular organism expressing the fluorophore itself can be mutagenized to identify genes within itself that alter the fluorophore expression. These screening principles have long been used to identify cis- or trans-acting regulators of gene expression in unicellular organisms such as bacteria or yeast. However, due to the lack of quick and simple screening methods, such screening are impractical for multi-cellular organisms such as plants and animals. The whole-body external optical imaging of gene expression disclosed herein makes such screening or mutant-haunt practical for multi-cellular organisms.

In a specific embodiment, a method to screen for a modulator of the expression of a gene in a multi-cellular organism is provided herein, which method comprises: a) administering a test substance to a non-human multi-cellular organism which expresses a fluorophore under the direction of a promoter of a gene, and determining the expression of said promoter via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said multi-cellular organism by whole-body external fluorescent optical imaging; b) determining the expression of said promoter, via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations by whole-body external fluorescent optical imaging, in a control multi-cellular organism which expresses said fluorophore under the direc-

tion of said promoter of said gene; and c) comparing the expression of said promoter determined in steps a) and b), wherein the expression determined in step a) is different from that in step b) identifies said test substance as a modulator of said gene expression. Preferably, the promoter is an endogenous promoter of the multi-cellular organism.

In another specific embodiment, a method to screen for a multi-cellular organism that expresses a gene at an altered level is provided herein, which method comprises: a) administering a mutation-inducing agent or treatment to a non-human multi-cellular organism which expresses a fluorophore under the direction of a promoter of a gene, and determining the expression of said promoter via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said multi-cellular organism by whole-body external fluorescent optical imaging; b) determining the expression of said promoter, via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations by whole-body external fluorescent optical imaging, in an untreated control multi-cellular organism which expresses said fluorophore under the direction of said promoter of said gene; and c) comparing the expression of said promoter determined in steps a) and b), wherein the expression determined in step a) is different from that in step b) identifies a multi-cellular organism that expresses said gene at said altered level. Preferably, the mutation-inducing agent or treatment causes a mutation in germ-line cells of the multi-cellular organism so that the desired mutation is stably-transferable to offspring of the multi-cellular organism.

In addition, the various protocols described in the art for "Big Blue" transgenic mice can be utilized in the system of the invention.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLE 1

Visualization of Gene Expression in Various Tissues using Adenovirus

Four six-week-old male of female nude/nude, nude/+, or C57BL/6 mice were used. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals under assurance number A3873-1. Mice were fed with autoclaved laboratory rodent diet (Tecklad LM-485, Western Research Products, Orange, Calif.).

The vector employed was adenorival (vAd) vector AdCMV5GFPAE1/AE3 [vAd-green fluorescent protein (GFP)] (Quantum, Montreal, Canada), which expresses enhanced GFP and the ampicillin resistance gene.

This vector was provided to various tissues to visualize expression of the CMV promoter in these tissues. Expression of reporter under control of any desired promoter can be visualized by suitable modification of this vector, as described above.

Liver: After exposure of the portal vein following an upper midline abdominal incision, total volume of 100 μl (8×10^{10} pfu/ml) vAd-GFP per mouse were injected in the portal vein using a 1 ml 39G1 latex-free syringe (Becton Dickinson, Franklin Lakes, N.J.). The puncture hole of portal vein was pressed for about 10 seconds with sterile cotton to stop any bleeding. The incision in the abdominal wall was closed with a 7-0 surgical suture in one layer. The animals were kept under Ketamine anesthesia during surgery. All procedures of the operation described above were performed with a 7× magnification microscope (Leica MZ6, Nussloch, Germany). Animals were kept in a barrier facility under HEPA filtration.

Brain: The parietal bone of the skull was exposed after an upper midline scalp incision. Twenty microliters containing 8×10^{10} plaque-forming units (pfu)/ml vAd-GFP per mouse was injected in the brain by using a 1-ml 27G1/2 latex-free syringe (Becton Dickinson). The puncture hole in the skull was plugged with bone wax. The incision in the scalp was closed with a 7-0 surgical suture in one layer. The animals were kept under isofluorane anesthesia during surgery.

Pancreas: The pancreas was exposed after an upper midline abdominal incision. One-hundred microliters containing 8×10^{10} pfu/ml vAd-GFP per mouse was injected in the pancreas by using a 1-ml $30G_{1/2}$ latex-free syringe (Becton Dickinson). The puncture hole was pressed for about 10 sec with sterile cotton for hemostasis. The incision was closed with a 7-0 surgical suture in one layer. The animals were kept under Kersel anesthesia during surgery. All procedures of the operation described above were performed with a ×7 magnification stereo microscope.

Prostate: The bladder and prostate were exposed after a lower midline abdominal incision. Thirty microliters containing 8×10^{10} pfu/ml vAd-GFP per mouse was injected in the prostate by using a 1-ml $30G_{1/2}$ latex-free syringe (Becton Dickinson). The puncture hole in the prostate was pressed for about 10 sec with sterile cotton for hemostasis. The incision in the abdominal wall was closed with a 6-0 surgical suture in one layer. The animals were kept under isofluorane anesthesia during surgery. All procedures of the operation described above were performed with a ×7 magnification stereo miscroscope.

Bone Marrow: For bone marrow injection, animals were anesthetized by inhalation of isofluorane. The skin on the hind leg was opened with a 1-cm incision to expose the tibia. A 27-gauge needle with latex-free syringe (Becton Dickinson) then was inserted in the bone marrow cavity. A total volume of 20 μl (8×10^{10} pfu/ml) vAd-GFP per mouse was injected into the bone marrow cavity. The puncture hole in the bone was plugged with bone wax, and the incision was closed with a 6-0 surgical suture.

Visualization: For visualization at high magnification, Leica fluorescence stereo microscope, model LZ12, equipped with a 50-W mercury lamp, was used. Selective excitation of GFP was produced through a D425/60 bandpass filter and 470 DCXR dichroic mirror. Emitted fluorescence was collected through a long-pass filter GG475 (Chroma Technology, Brattleboro, Vt.) on a Hamamatsu C5810—3-chip cooled color charge-coupled device camera (Hamamatsu Photonics Systems, Bridgewater, N.J.). Images were processed for contrast and brightness and analyzed with the use of IMAGE PRO PLUS 3.1 software (Media Cybernetics, Silver Springs, Md.). Images of 1,024×724 pixels were captured directly on an IBM PC or continuously through video output on a high-resolution Sony VCR model SLV-R-1000 (Sony, Tokyo).

Imaging at lower magnification that visualized the entire animal was carried out in a light box illuminated by blue light fiber optics (Lightools Research, Encinitas, Calif.) and imaged by using the thermoelectrically cooled color charge-coupled device camera, as described above.

Quantitation: The intensity of GFP fluorescence is measured to account for variations in the exciting illumination

with time and across the imaging area. These factors are corrected for by using the intrinsic red fluorescence of mouse skin as a base line to correct the increase over intrinsic green fluorescence caused by GFP. This can be done because there is relatively little red luminance in the GFP radiance. Consequently, the green fluorescence was calculated relative to red based on red and green channel composition in the skin image. A ratio (γ) of green to red channels was determined for each pixel in the image of skin without and with GFP. Values of γ for mouse skin throughout the image in the absence of GFP were fairly constant, varying between 0.7 and 1.0. The contribution of GFP fluorescence from within the animal increased the green component relative to red, which was reflected in higher γ values. The total amount of GFP fluorescence was approximated by multiplying the number of pixels in which value γ was higher than 1 times the γ value of each pixel. Such a product roughly corresponds to the integral GFP fluorescence $[\Gamma_{GFP}]$ above the maximum value of γ for skin without GFP. The number of pixels in mouse skin images with γ value >1.0 without GFP was less than 0.02% and increased with GFP expression. The value of $[\Gamma_{GFP}]$ is shown as a function of time after virus injection in FIGS. 1A and 1B for brain and liver respectively.

Images of the various organs were compared when taken at high magnification on live intact animals or similar organs viewed directly after death and dissection. The images show the distribution of gene expression in the various organs. In all cases, the images made externally are similar to those of the exposed organs.

When the live animal was viewed in a light box, it was also possible to monitor the expression of the gene, thus permitting a real time observation of the living animal and expression as it occurs in this animal. For example, a light box determination of expression of the GFP in nude mouse liver taken at 72 hours clearly shows this result. Similar results are observed in the nude mouse brain 24 hours after gene delivery. The method is quite sensitive in that the intensity of GFP fluorescence in the mouse liver at a depth of 0.8 mm under the skin was about 25% of that of the exposed organ. Gene expression is externally measurable if the average fluorescence of the GFP expressing organs is at least 20% above the average fluorescence of the surrounding skin, and at maximal level of GFP expression, the intensity in the liver exceeded more than 100 times the back dorsal and abdominal skin fluorescence.

EXAMPLE 2

Visualization of Genes Using Lentiviral Vectors

Lentiviral vectors have been shown to transduce a broad spectrum of non-dividing cells in vitro, such as neurons, retina, liver, muscle and hematopoietic stem cells (see, for example, Naldini, L. et al., *Science* (1996) 272:263–267; Kafri T. et al.; *Nat. Genet* (1997) 17:314–317; Takahashi, M. et al., *J. Virol* (1999) 73:7812–7816; Miyoshi, H. et al. *Science* (1999) 283:682–686). Although it has been reported that hepatocytes are refractory to lentiviral transductioni unless they progress into the cell cycle (Park, F. et al. *Nat. Genet* (2000) 24:49–52), it is shown below that lentiviral gene delivery to the liver for expression visualization is practical.

A lentiviral vector based on HIV1 designated GFP-LV was used. This vector contains a self-inactivating mutation in the U-3 region, a post-transcriptional element, and an internal CMV promoter. It also contains cppt, the central polypurine tract derived from HIV-pol and a woodchuck hepatitis virus post-transcriptional element (WPRE). A diagram of this vector is shown in FIG. 2A.

The vector GHP-LV at 1×10^9 IU was injected into the portal vein of nude mice; (Hsd:asymic nude-nu). Six (6) days after injection green fluorescence was testable in the liver using in-vivo fluorescence optical imaging, as shown in FIG. 2B. At day 21, all lobes of the liver of the mice injected with this vector exhibited a homogeneous green fluorescence.

GHP-LV at 1×10^9 IU was also injected intraperitoneally and this method also resulted in a high level of transduction of liver and spleen.

Western Blot demonstrated dose dependence of GFP expression in the range of $0.5–2.5\times10^9$ IU. Vector integration in the liver 3 weeks after injection was demonstrated by PCR.

Confirmation that the transduced cells were not rapidly dividing was achieved by administering 5' bromo-2' deoxyuridine (BrdU) 15 mgs/kg by daily IP injections in order to label dividing cells. While the cells in the duodenum showed high labelling, only about 3% of liver cells were BrdU positive in either control or lentiviral-treated livers.

EXAMPLE 3

Additional Applications

In addition to the procedures exemplified in Examples 1 and 2, the methods of the invention may be used to monitor expression of control sequences that are regulated by the unfolded protein response (UPR) as described, for example, by Niwa, M., et al., *Cell* (1999) 99:691–702, the contents of which are incorporated herein by reference. Another suitable target for study is the circadian rhythm controlling genes which were studied using less convenient techniques by Yamaguchi, S., et al, *Nature* (2001) 409:684, incorporated herein by reference.

Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

What is claimed is:

1. A method to monitor the ability of a promoter to promote expression in an animal of an endogenous gene that is controlled by said promoter, which method comprises:

a) delivering, to an animal, cells containing a nucleic acid encoding a fluorophore operatively linked to the promoter of said endogenous gene whose ability to promote expression is to be analyzed; and

b) observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said animal by whole-body external fluorescent optical imaging,

whereby the ability of said promoter to promote expression is monitored, and

wherein said fluorophore is a protein that is autofluorescent such that no substrates or cofactors are needed for it to fluoresce.

2. The method of claim **1**, wherein the cells are delivered to the animal via a surgical procedure.

3. The method of claim **2**, wherein the cells are delivered to the animal via direct implantation by surgical orthotopic implantation (SOI) at a desired site.

4. The method of claim **1**, wherein the animal is a human and the fluorophore is a humanized fluorophore.

5. The method of claim 1, wherein the fluorophore is selected from the group consisting of a green fluorescent protein (GFP), a blue fluorescent protein (BFP) and a red fluorescent protein (RFP).

6. The method of claim 5, wherein the animal is a human and the GFP is the humanized hGFP-S65T.

7. The method of claim 1, wherein the animal is a mammal.

8. The method of claim 7, wherein the mammal is selected from the group consisting of a mouse, a rat, a rabbit, a cat, a dog, a pig, a cow, an ox, a sheep, a goat, a horse, a monkey and a non-human primate.

9. The method of claim 1, wherein the endogenous gene is normally expressed in a tissue or organ specific manner.

10. The method of claim 9, wherein the tissue is selected from the group consisting of connective, epithelium, muscle and nerve tissues.

11. The method of claim 9, wherein the organ is selected from the group consisting of brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, gland, and internal blood vessels.

12. The method of claim 1, wherein the endogenous gene is an endogenous tumor or cancer associated gene.

13. The method of claim 12, wherein the tumor or cancer associated gene is an oncogene or a tumor suppressor gene.

* * * * *

EXHIBIT 6

US006905831B2

(12) **United States Patent**
Jiang et al.

(10) **Patent No.: US 6,905,831 B2**
(45) **Date of Patent: Jun. 14, 2005**

(54) **REAL TIME MEASUREMENT OF CELLULAR RESPONSES**

(75) Inventors: **Ping Jiang**, San Diego, CA (US); **Mingxu Xu**, La Jolla, CA (US); **Meng Yang**, San Diego, CA (US)

(73) Assignee: **Anticancer, Inc.**, San Diego, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **10/643,785**

(22) Filed: **Aug. 18, 2003**

(65) **Prior Publication Data**

US 2004/0115813 A1 Jun. 17, 2004

Related U.S. Application Data

(60) Provisional application No. 60/404,005, filed on Aug. 16, 2002, and provisional application No. 60/427,604, filed on Nov. 18, 2002.

(51) **Int. Cl.**[7] **C12Q 1/68**
(52) **U.S. Cl.** **435/6**; 435/455; 435/366
(58) **Field of Search** 435/6, 455, 366

(56) **References Cited**

PUBLICATIONS

Gerdes and Kaether, FEBS Letters 389:44–47 (1996).
Kanda et al., Curr. Biol. 8:377–385 (1998).
Kawakami et al., Immunology Lett 70:165–171 (1999).
Yang et al., Clin. Exp. Metastasis 17:417–422 (1999).
Yang et al., Proc. Natl. Acad. Sci. USA 97:1206–1211 (2000).
Yang et al., Proc. Natl. Acad. Sci. USA 97:12278–12282 (2000).
Yang et al., Proc. Natl. Acad. Sci. USA 98:2616–2621 (2001).
Yang et al., Proc. Natl. Acad. Sci. USA 99:3824–3829 (2002).
Zhao et al., Proc. Natl. Acad. Sci. 98:9814–9818 (2001).
International Search Report, mailed on Feb. 11, 2004, for PCT/US03/22308, 4 pages.
Terjung et al., Clin. Exp. Immunol. (2001) 126:37–46.
Zhai et al., J. Biol. Chem. (2001) 276(44):41318–41324.
Zhang et al., J. Cell Sci. (2001) 114(24):4485–4498.

Primary Examiner—James Ketter
(74) *Attorney, Agent, or Firm*—Morrison & Foerster LLP

(57) **ABSTRACT**

Living cells can be stably modified to emit different colors from the cytoplasm and nucleus, thus permitting analysis of the status of said cells and the effect of agents on said cells either by visual or instrumentally-aided observation. These observations may be made, if desired, in real time. In addition, rates of proliferation and drug sensitivities can be determined in vitro in real time by the use of cells modified to express a single fluorescent protein and observing fluorescence intensity as a function of time.

27 Claims, 13 Drawing Sheets

Figure 1

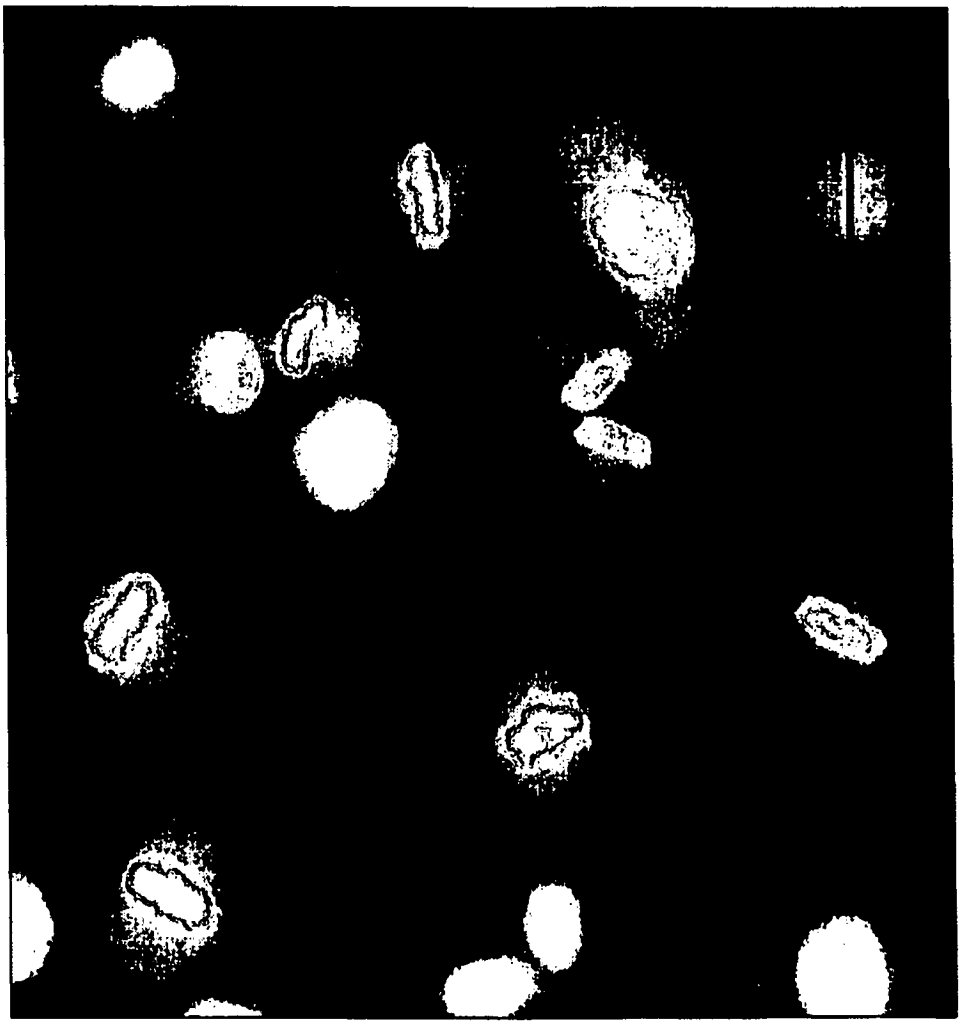


Figure 2

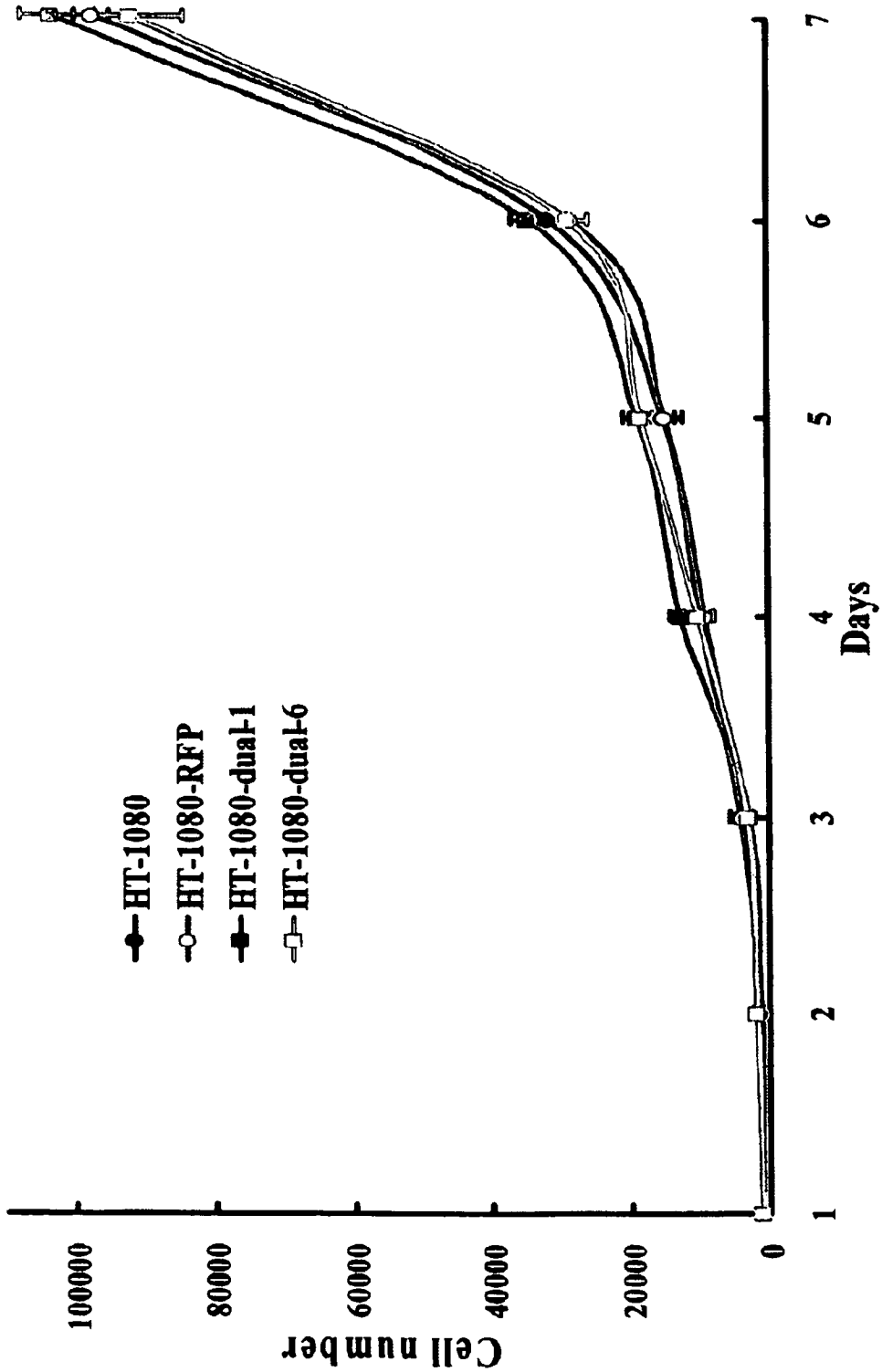

Cell number
100000
80000
60000
40000
20000
0
1
2
3
4
5
6
7
Days
HT-1080
HT-1080-RFP
HT-1080-dual-1
HT-1080-dual-6

Figure 3A

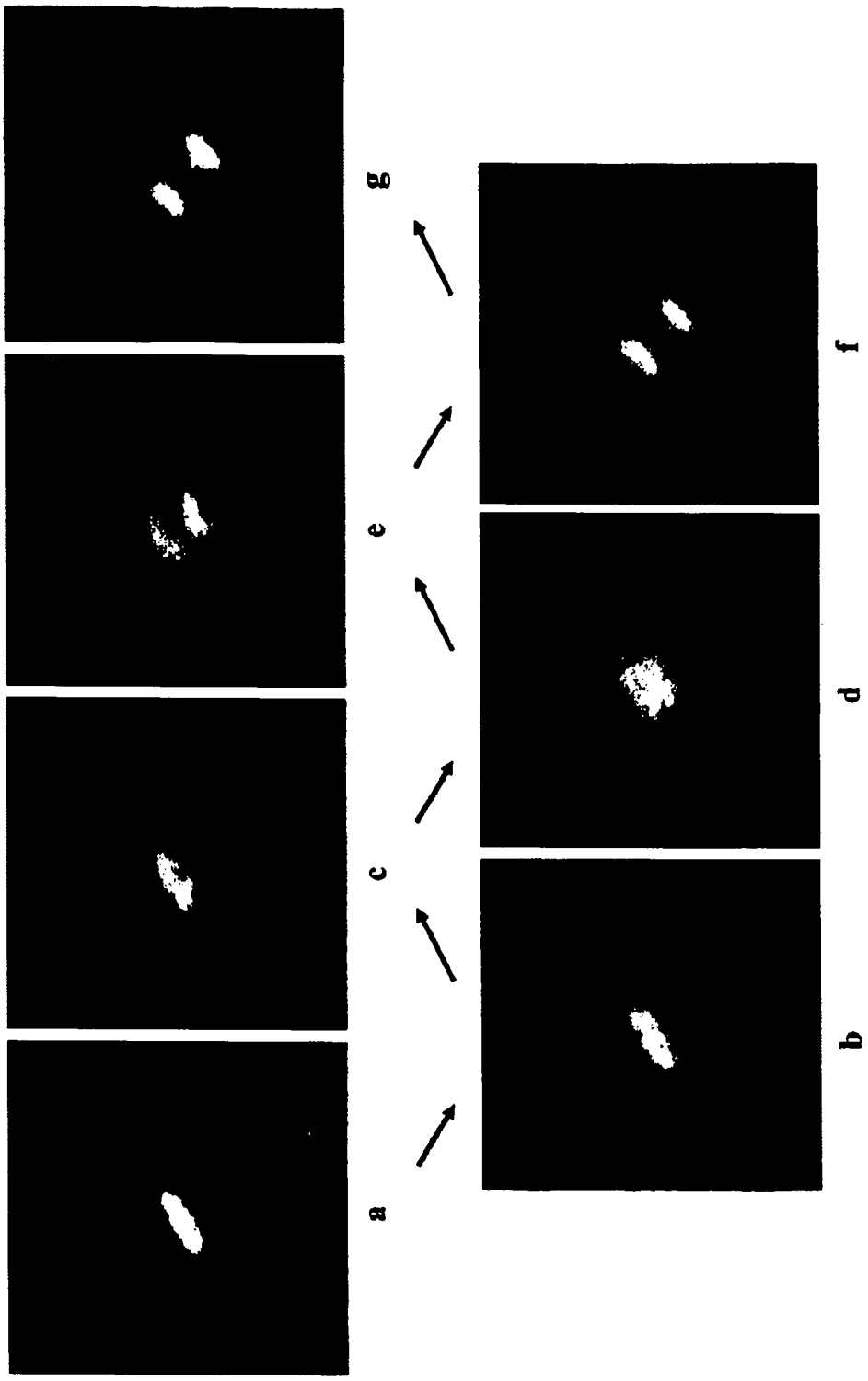


Figure 3B

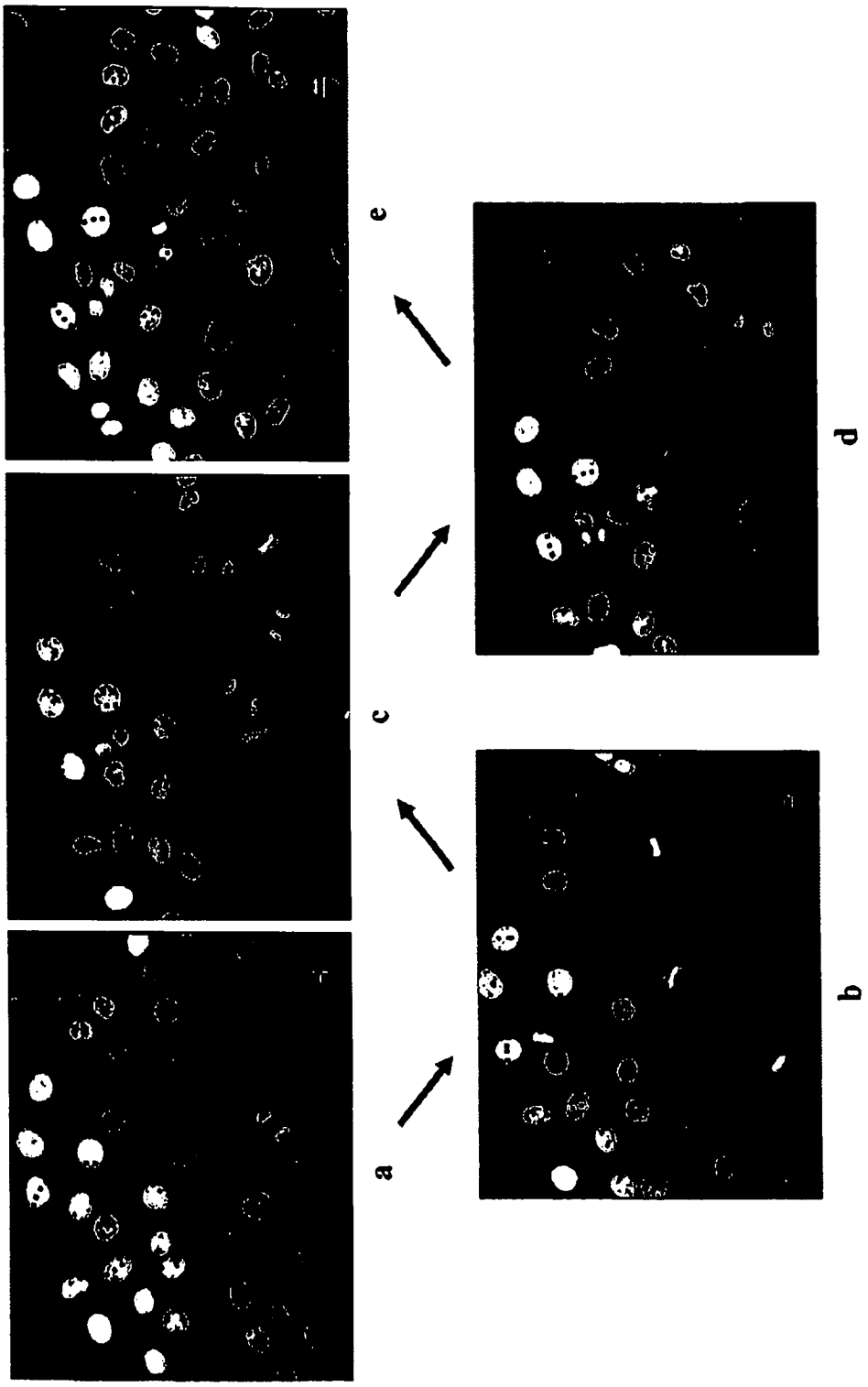


Figure 4A

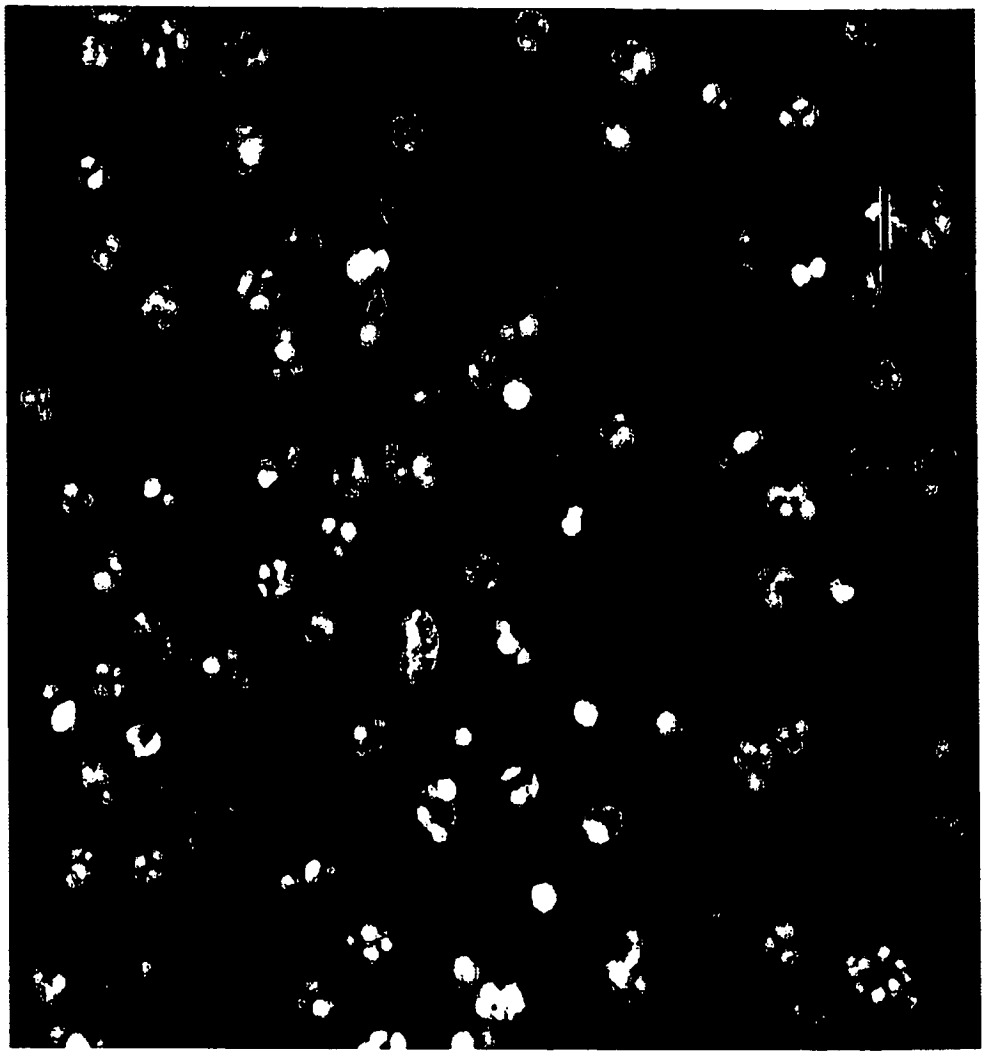


Figure 4B

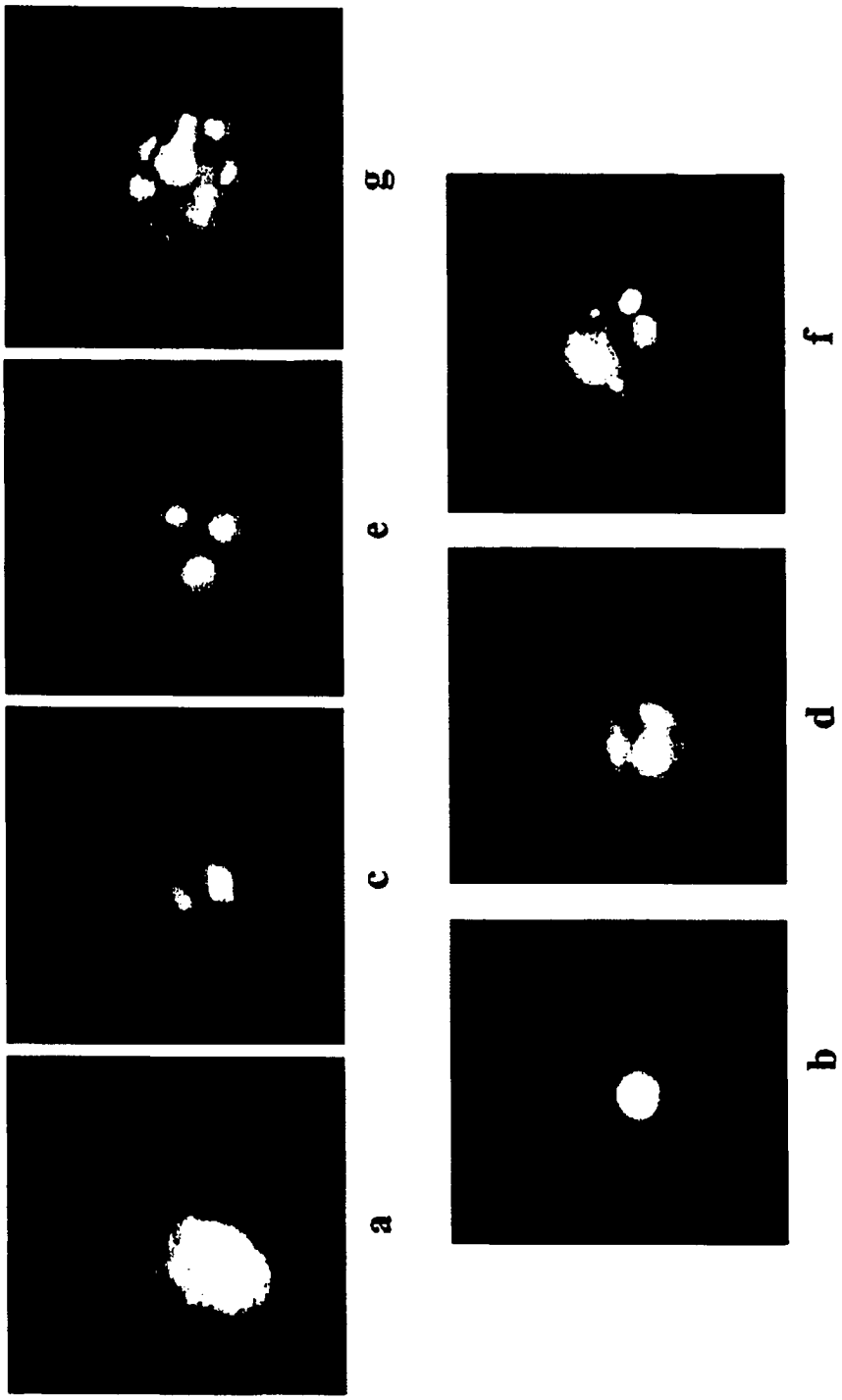


Figure 5

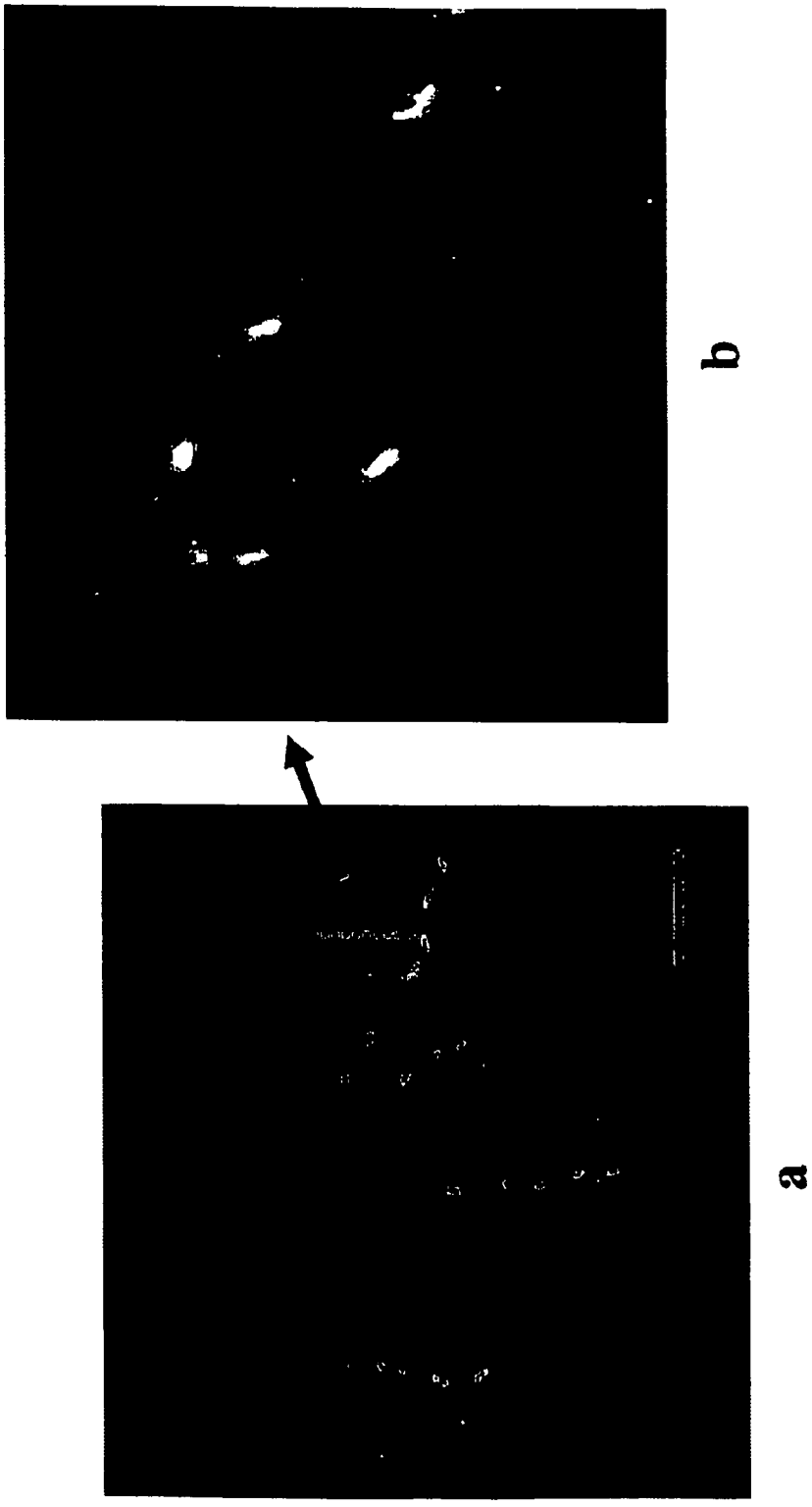
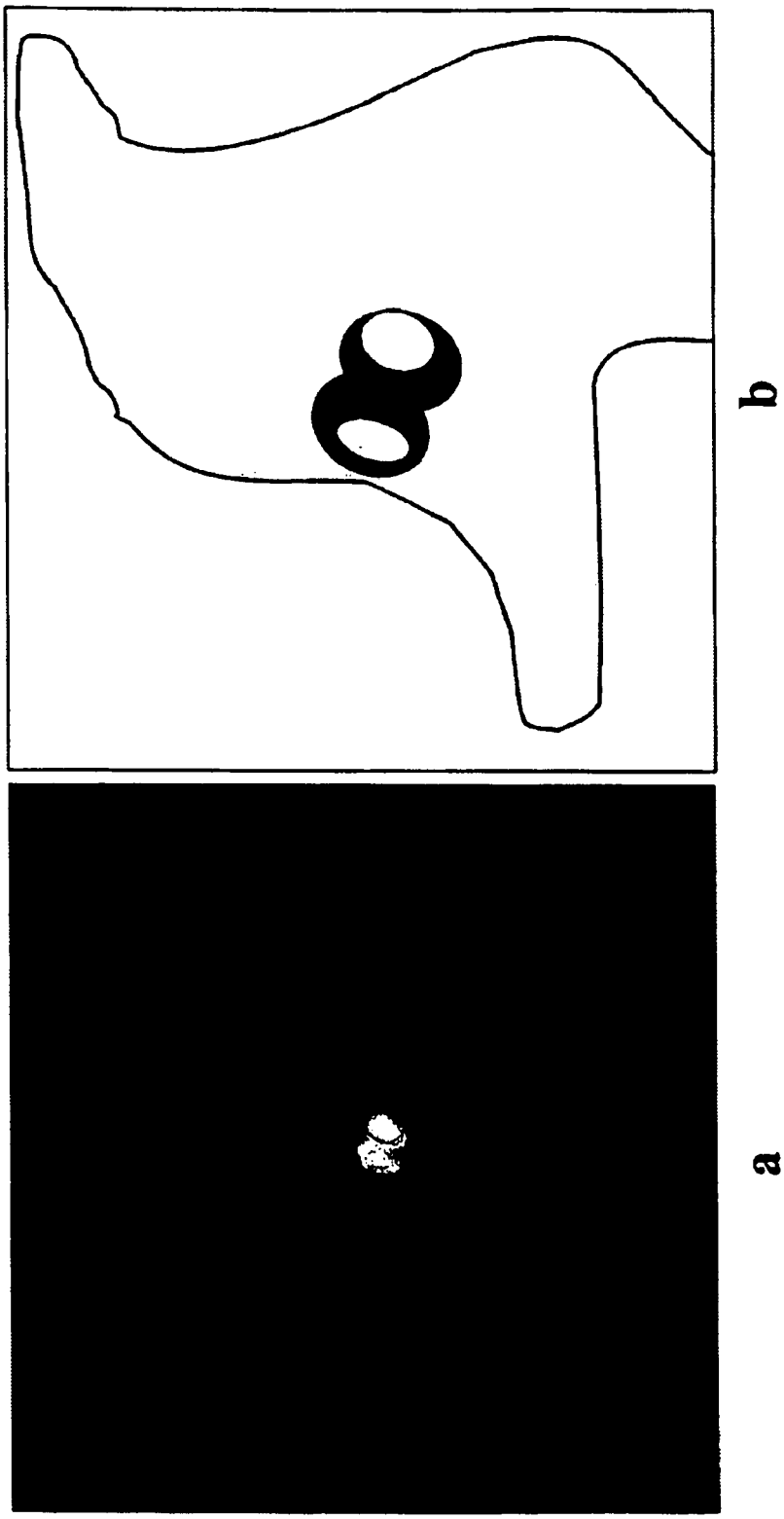


Figure 6



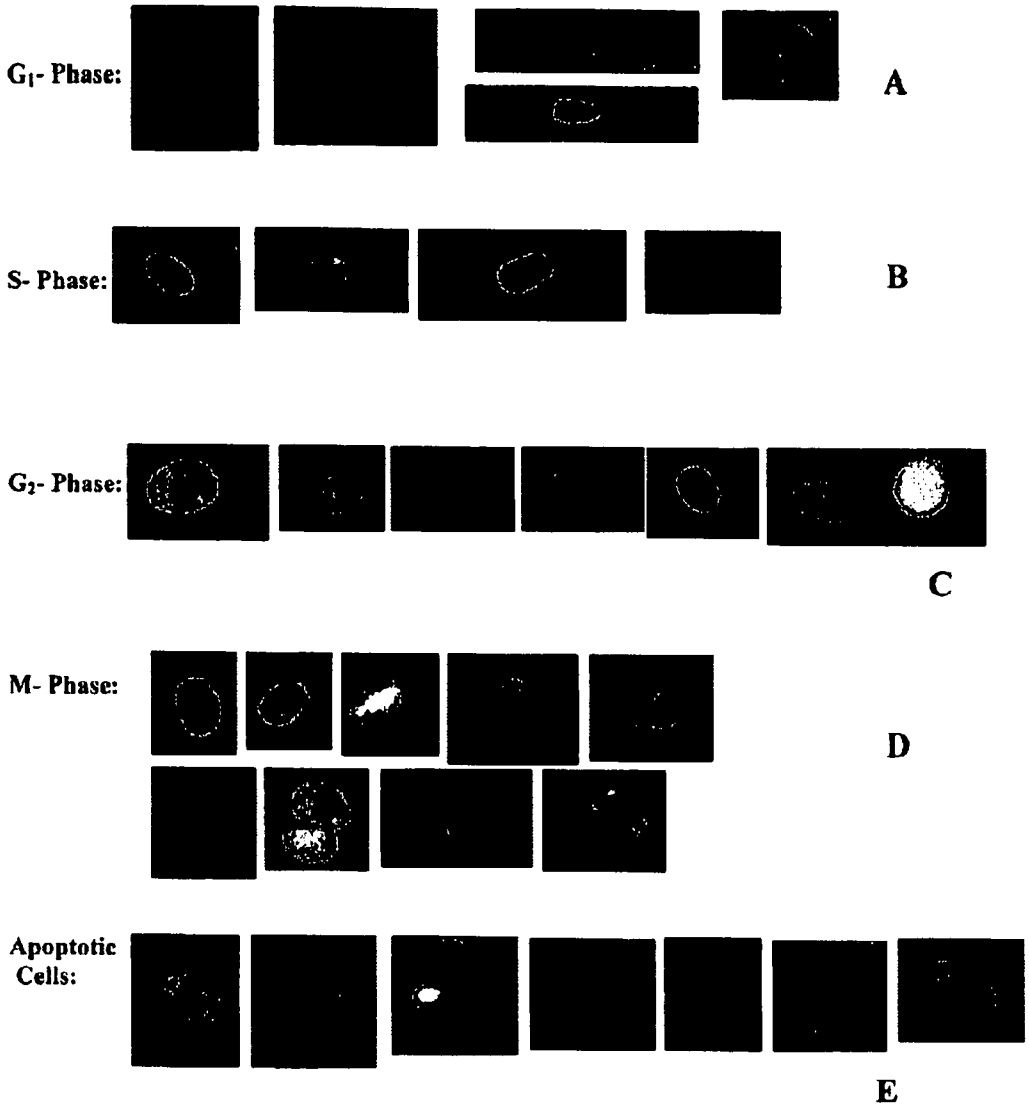


Figure 7

Figure 8

PC-3 Dual Cells were treated by Taxol (0.8 μg/ml)

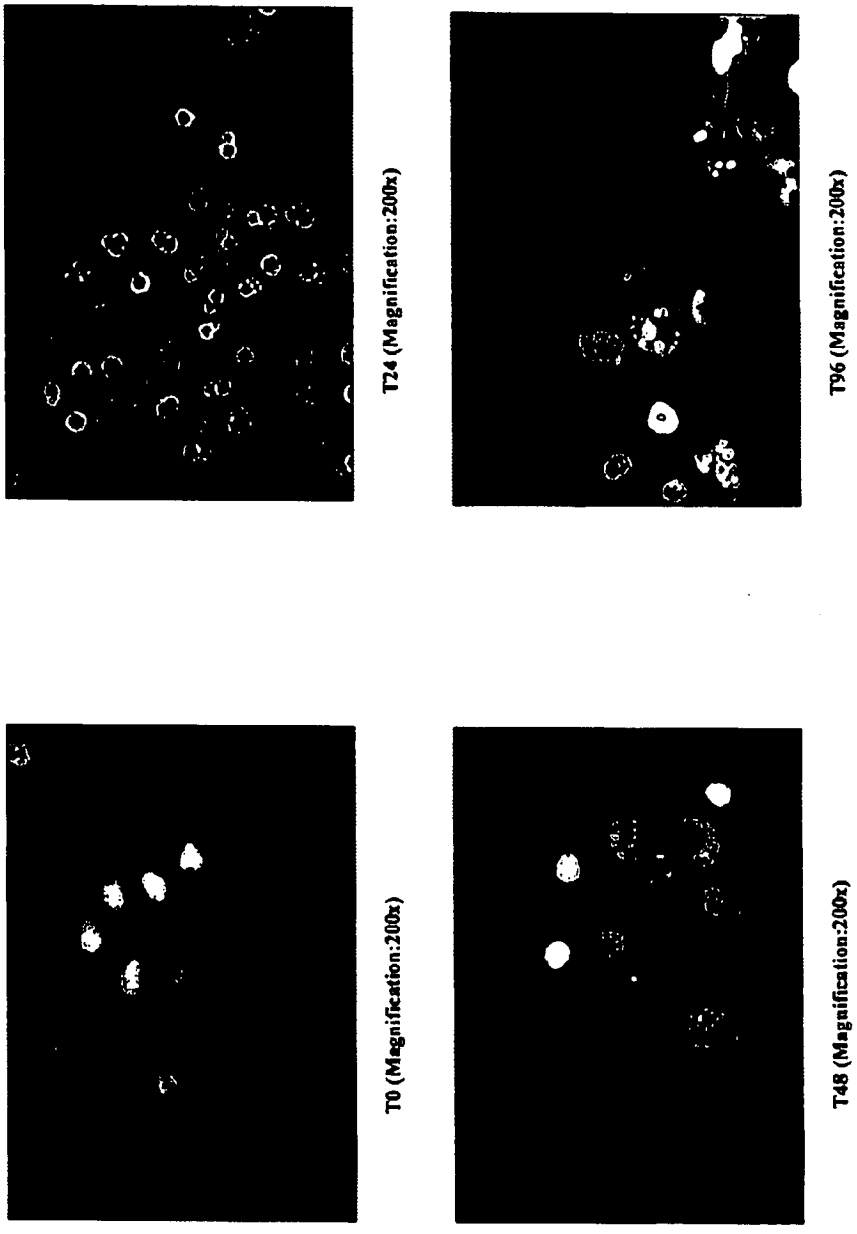
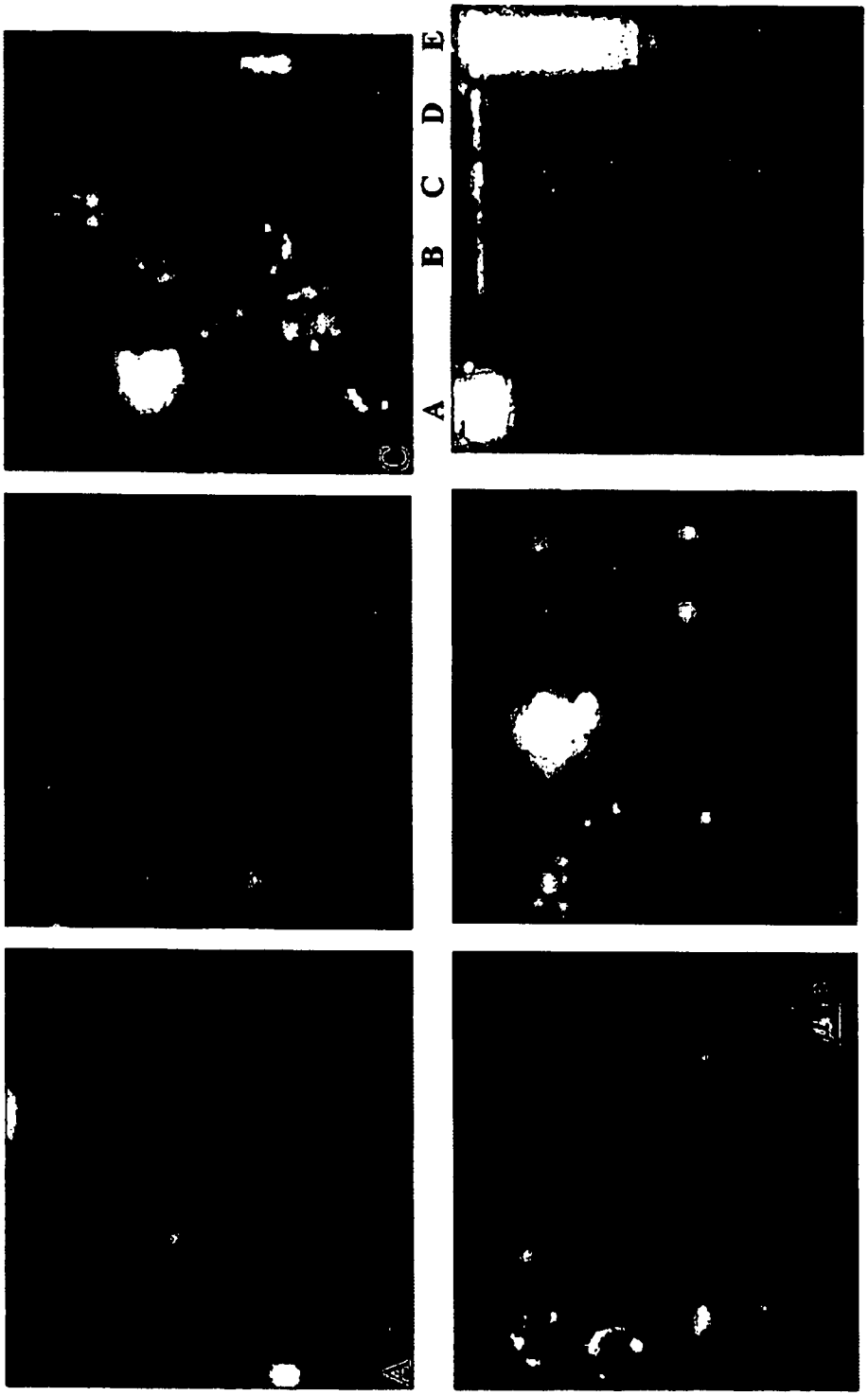


Figure 9

Visualizing taxol induced apoptotic cells in vitro in real time



Real-time apoptoses were observed under fluorescence microscopy and verified by DNA ladder analysis when PC-3-dual cells were incubated with 0. 8 μg Taxol. (A) control; (B) 12 hours after treatment; (C) 24 hours after treatment; (D) 36 hours after treatment; (E) 48 hours after treatment.

Figure 10

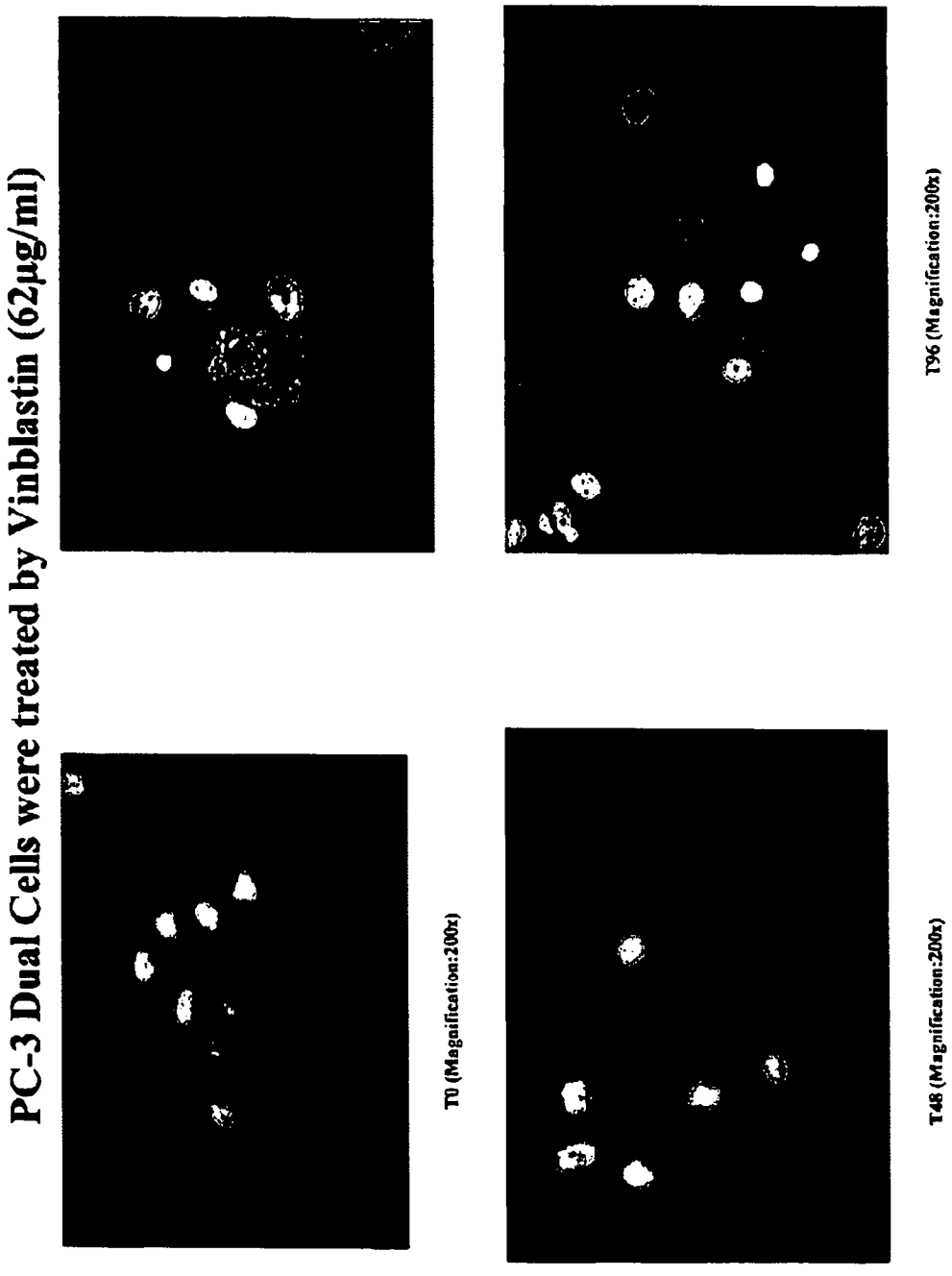
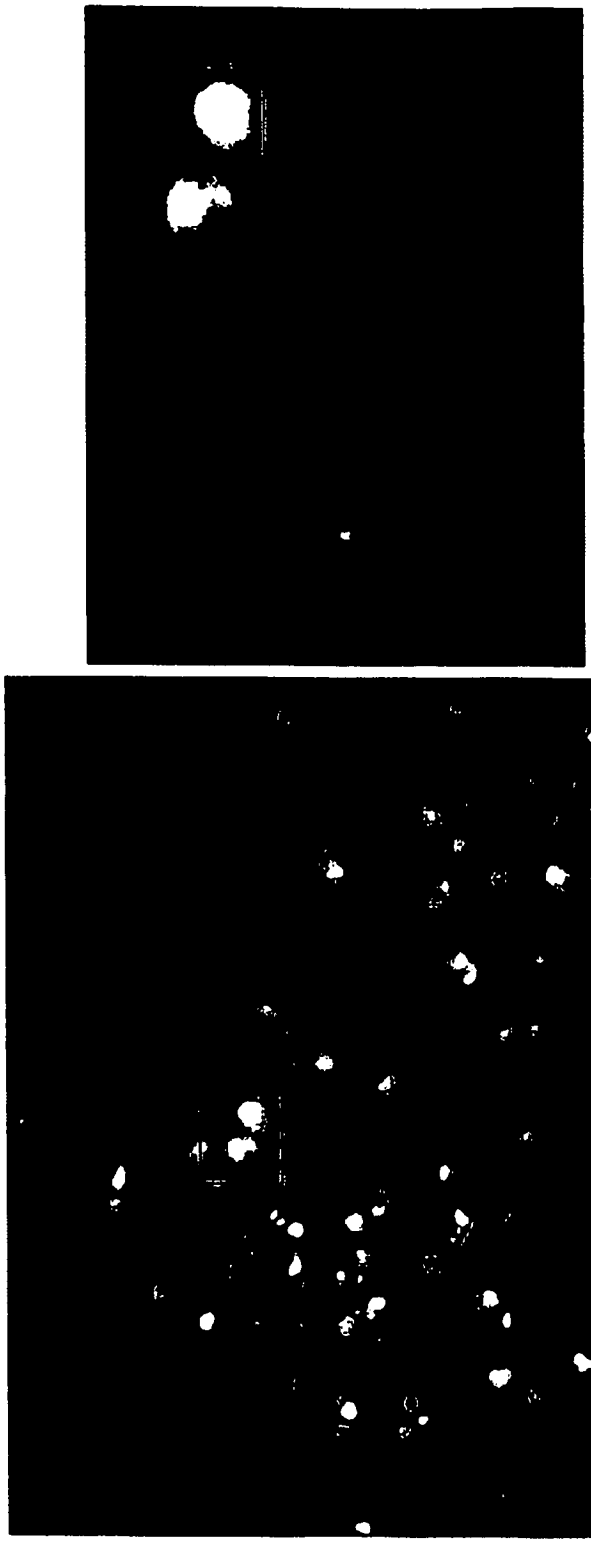

PC-3 Dual Cells were treated by Vinblastin (62μg/ml)
T0 (Magnification:200x)
T96 (Magnification:200x)
T48 (Magnification:200x)

Figure 11

Visualizing apoptotic cancer cells in vivo



PC-3 Dual colored human prostate cancer cells were injected in the foot pat of nude mice. The mice were sacrificed after 20 days. Apoptotic PC-3 dual colored cell was observed in the lymph node under fluorescence microscopy

REAL TIME MEASUREMENT OF CELLULAR RESPONSES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119(e) from U.S. Ser. Nos. 60/427,604 filed 18 Nov. 2002 and 60/404,005 filed 16 Aug. 2002, the contents of which are incorporated herein by reference.

TECHNICAL FIELD

The invention relates to real time observation of cellular proliferation, drug sensitivity, cell cycle position, and other cellular responses in vitro. The invention relates to direct observation of cells, especially living cells, by use of fluorescent proteins as markers. The status of cells in a cell cycle can be monitored by separately labeling the nucleus and cytoplasm.

BACKGROUND ART

The green fluorescent protein (GFP) from *Aequorea victoria* was discovered in 1962. Knowledge of the structure, mechanism and applications of GFP developed very rapidly after cloning of the GFP gene in 1992. After demonstration of the heterologous expression of the GFP gene in other organisms, GFP became one of the most widely used reporter genes. In 1999, another family of fluorescent proteins, including *Discosoma* Red (DsRed), was cloned from corals followed by *Anemonia* asRed in 2000 and HcRed in 2001. The biological role of these proteins extends from a pure signal function as in GFP to photoprotection of photosynthetic symbionts by the novel proteins isolated from corals. For two of these colored, water-soluble proteins, GFP and DsRed, with a molecular weight of 25–27 kD, X-ray structure analysis has demonstrated a homologous β-barrel structure. A common feature of the primary structure of these proteins is that the amino acids, tyrosine and glycine, which occupy GFP positions 66 and 67, are conserved and participate in the formation of the chromophore during a post-translational autocatalytic modification.

These proteins (wild type and mutants) can be used as multicolor reporters. The spectral range of their fluorescence spans almost 180 nm extending from the "blue" peak position of 460 nm to 640 nm in the red region of the spectrum. GFP is one of the most widely-used genetic markers in cell biology (Gerdes, H.-H., and Kaether, C., *FEBS Letters* (1996) 389:44–47), in immunology (Kawakami, N., et al., *Immunology Lett* (1999) 70:165–171), as well as in studies of infectious disease, e.g.—of host-pathogen interaction on model animals (Zhao, M., et al., *Proc. Natl. Acad. Sci.* (2001) 98:9814–9818). GFP has been used for whole-body imaging of tumor growth, metastasis, and angiogenesis (Yang, M., et al., *Proc. Natl. Acad. Sci.* (2002) 99:3824–3829; Yang, M., et al., *Proc. Natl. Acad. Sci.* USA (2000) 97:1206–1211; and Yang, M., et al., *Proc. Natl. Acad. Sci.* USA (2001) 98:2616–2621), gene expression (Yang, M., et al., *Proc. Natl. Acad. Sci.* USA (2000) 97:12278–12282), and bacteria infection (Zhao, M., et al, supra).

In all these applications, the red emission is of special importance with respect of minimization of background emission and in vivo scattering as well as for FRET (fluorescence resonance energy transfer) analysis. High extinction coefficients, quantum yield, and the monomeric state of fluorescent proteins are very important parameters for their use as reporters including in vivo applications. In contrast to GFP, which has only a small tendency to dimerize, the related proteins have a pronounced tendency to form oligomers, e.g.—tetramers as observed for DsRed, or even higher aggregates. Extinction coefficients and quantum yields are also relatively low for red proteins and for the newly developed monomeric DsRed.

The human histone H2B gene has been fused to the gene encoding the GFP of *Aequorea victoria* and transfected into human cells to generate stable lines constitutively expressing H2B-GFP. The H2B-GFP fusion protein was incorporated into nucleosomes without affecting cell cycle progression. H2B-GFP allowed high-resolution imaging of nuclei including both mitotic chromosomes and interphase chromatin, and the latter revealed various chromatin condensation states in live cells (Kanda, T., et al., *Curr. Biol.* (1998) 8:377–385).

The disclosures of the cited documents are incorporated herein by reference.

These various proteins have been used to monitor tumor metastases, and as reporter genes to monitor expression. See, for example, Kanda, T., et al., *Curr. Biol.* (1998) 8:377–385; Yang, M, et al., *Clin. Exp. Metastasis* (1999) 17:417–422; Yang, M, et al., *Proc. Natl. Acad. Sci. USA* (2001) 98:2616–2621; and Yang, M, et al., *Proc. Natl. Acad. Sci. USA* (2002) 99:3824–3829. However, to applicants' knowledge, these proteins have not been used to monitor cellular proliferation, cell cycle status, or drug sensitivity of cells in vitro, nor have dual labeled cells been used either in vitro or in vivo.

Most in vitro techniques for monitoring proliferation involve observation techniques which results in killing the cells. For example, cells that proliferate attached to a plastic surface may be released from the plastic by enzymes, such as trypsin, and then counted using a particle counter. Also commonly employed are stains such as tetrazolium dyes which are reduced by electrons derived from mitochondrial enzyme activity and negatively affect the viability of the cells. In addition, the lacZ gene may be introduced into the cells and used as a marker, but in order to visualize activity, the cells must be stained.

Thus, traditional methods of monitoring proliferation involve disruption of normal cellular metabolism, even resulting in cell death.

The present invention offers an opportunity to observe proliferation and other cellular activity in vitro or in vivo in real time. These observations may also extend to observing the cell cycle by taking advantage of changes in nuclear/cytoplastic ratios at various stages.

To applicants knowledge, although the H2B gene fused to GFP has been used to label the nucleus, no suggestion has been made to separately label the nucleus and cytoplasm of living cells with two different color proteins. The present invention provides the capability to observe such living cells.

DISCLOSURE OF THE INVENTION

The present invention is directed to methods for observing proliferation and sensitivity of cells to various compounds in vitro and in vivo. In addition, the invention is directed to dual labeled cells where one color is used to label the nucleus and a different color to label the cytoplasm.

Thus, in one aspect, the invention is directed to a method to measure cellular proliferation in vitro which method

comprises the step of observing, as a function of time, changes in intensity of fluorescence of cells in culture labeled with a fluorescent protein.

In another aspect, the invention is directed to monitoring the response of cells to compounds such as drugs in vitro by measuring, as a function of time, the fluorescence emitted by cell cultures in the presence and absence of test compound, wherein the cells in said cultures have been modified to express a fluorescent protein.

In still another aspect, the invention is directed to a method to label living cells by providing said cells with an expression system for a first fluorescent protein of a first color which remains in the cytoplasm, and an expression system for a second fluorescent protein coupled to a nuclear targeting protein wherein upon expression of said first protein and said second fusion protein, the nucleus of said cells is labeled with one color and the cytoplasm with a different color.

In another aspect, the invention is directed to cells thus prepared, which are stably labeled in the cytoplasm with a first fluorescent protein and in the nucleus with a second fluorescent protein of a different color from the first.

In other aspects, the invention is directed to methods of using the dual-labeled living cells obtained by the method of the invention either in culture or in a living plant or animal. The effect of various agents on the cell cycle can be determined using these cells as well.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows stable high GFP and RFP expressing human fibrosarcoma cells (HT-1080-dual-1) in vitro. Human fibrosarcoma cells (HT-1080) were transduced with RFP and the neomycin resistance gene, histone H2B-GFP and the hygromycin resistance gene in a retrovirus vector. Double transformants were selected with G418 and hygromycin and stable clones were established. Bar, 50 μm.

FIG. **2** is a comparison of cell proliferation rates of parental and fluorescent protein expressing clones. Three dishes for each clone (parental HT-1080, HT-1080-RFP, HT-1080-dual-1 and HT-1080-dual-6) were used at each time-point to count the cell number for one week. Cells were trypsinized, stained with trypan blue, and counted in a hemocytometer. Filled circle shows averaged cell number for each group.

FIGS. **3A–3B** show time-course observation of cell proliferation process in vitro using fluorescent labeled cells. HT-1080-dual-1 cells were cultured in PRMI 1640 supplemented with 10% FBS and time-course images were captured from the same living cells under fluorescence microscopy at various time points. FIG. **3A** is taken at five-minute intervals. a: 0 minute. b: 5 minutes. c: 10 minutes. d: 15 minutes. e: 20 minutes. f: 25 minutes. g: 30 minutes. Bars; 20 μm. FIG. **3B** is taken at thirty-minute intervals. a: 0 minute. b: 30 minute. c: 60 minutes. d: 90 minutes. e: 120 minutes. Bars; 75 μm. Green and white circles indicate representative areas where proliferating cells were observed. Even if mitotic cells changed their relative positions, they were easily visualized.

FIGS. **4A–4B** show images of HT-1080-dual-6 apoptosis induced by staurosporine. HT-1080-dual-6 cells were incubated with 2 μM staurosporine for apoptosis induction. The cells were visualized under blue light and images were captured with a CCD camera and fluorescence microscope. FIG. **4A** shows an image twelve hours after 2 μM staurosporine treatment. Apoptosis was well induced to HT-1080-dual cells at a high rate. Bar, 50 μm. FIG. **4B** shows real-time high-magnification images of HT-1080-dual-6 cells apoptotic processes induced by 2 μM staurosporine as follows: a: no treatment. b: 2 hours after staurosporine treatment. c: 4 hours. d: 6 hours. e: 8 hours. f: 10 hours. g: 12 hours. The condensation of cytoplasm and nucleus and fragmentation of nucleus were well visualized. Bar, 10 μm.

FIGS. **5A–5B** show brain experimental metastases immediately after injection of HT-1080-dual-1 cells into the common carotid artery. Emboli of dual-coded cells in micro arteries were observed at the single cell level through the skull with skin flap window. The cell morphology and the morphology of the nuclei of each cell are visualized. FIG. **5A** is a low magnification view. Bar, 400 μm, the white square indicates the area whose high magnification view is shown in FIG. **5B**. FIG. **5B** shows a high magnification view. Bar, 100 μm.

FIGS. **6A–6B** show real time image of tumor cell proliferation on living mouse. Real time image of mitotic tumor cells in live mouse was captured 12 hours after the cells injection. FIG. **6A** shows a high magnification image. Bar, 50 μm. FIG. **6B** is a schema of FIG. **6A**.

FIGS. **7A–7D** show images of the cell cycle of dual labeled PC-3 human prostate cancer cells, including G_1-phase, S-phase, G_2-phase and M-phase. FIG. **7E** shows PC-3 dual labeled cells undergoing apoptosis.

FIG. **8** shows the time course of effect of Taxol™ on PC-3 dual labeled cells.

FIG. **9** shows alternative imaging of the effect of Taxol™, including the results shown in gel electrophoresis.

FIG. **10** shows the effect of the treatment of vinblastin on dual labeled PC-3 cells.

FIG. **11** shows an in vivo image of apoptotic PC-3 dual labeled cells.

MODES OF CARRYING OUT THE INVENTION

Enhancement of information obtainable by cellular labeling can be obtained by separately labeling the nucleus and cytoplasm. Such dual labeling permits not only monitoring of cell proliferation, but also monitoring of events in cellular life. Particularly convenient are the labels of the invention wherein the nucleus is labeled with one color emitted as fluorescence and the cytoplasm is labeled with another color.

Also a feature of the invention are methods for monitoring proliferation of cells in vitro or in vivo in real time simply by monitoring fluorescence. While the dual labeled cells may be used in this method, dual labeling is not strictly speaking necessary. Cells labeled with only a single fluorescent label whose intensity can be measured while the cells remain alive can be used. The response to various treatments or drugs can also be monitored in this way by ascertaining the effect of the drug or agent on cellular proliferation.

Suitable cells for use in the invention are cells of any eukaryotic organism, such as yeast, fungi, plants, vertebrate and invertebrate animals, including human cells. For observation of unicellular organisms such as yeast and, for example, molds or fungi, direct observation in culture is preferred. Observation in cell culture may also be employed for cells of higher plants and animal cells. Suitable animals include, typically, laboratory animals such as rats, mice and other rodents, domestic animals such as livestock, fish, and human cells.

For methods of the invention which do not require dual labeling of the cytoplasm and nucleus, procaryotic cells may be used in addition to the eukaryotes discussed above.

The cells are labeled, according to the methods of the present invention, with fluorescent proteins. As used herein, a "fluorescent protein" refers to a protein that, upon appropriate stimulation, will emit light. Typically, a fluorescent protein emits light in the visible range—i.e., in the range of about 400 nm–800 nm, when illuminated with an excitation wavelength. When instrumentation is employed a wider range may be used. Other mechanisms for evoking fluorescence can also be used; a convenient example is that of luciferase, where metabolic energy is used to effect signal generation. Thus, a "fluorescent protein" refers to a protein which will emit light in the "visible" range when appropriate energy is supplied, whether by excitation radiation, chemiluminescence, or other mechanisms of supplying the necessary energy to effect light emission.

In many embodiments of the invention, fluorescent proteins, such as those described in the Background section above, are employed. As is apparent, these fluorescent proteins come in a variety of colors; in some notations, the color is included in the acronym for the protein—such as GFP for green fluorescent protein and RFP for red fluorescent protein. However, since the originally isolated fluorescent protein was green, in some instances the acronym GFP is used generically to describe these fluorescent proteins regardless of the wavelength emitted. Thus, in one sense, GFP can emit light in the yellow, red or blue wavelength range, for example. It will be clear from the context whether the generic meaning of GFP is used or whether it is intended that the emission be in the green wavelengths. For example, in illustrative embodiments, a red fluorescing protein is used to label the cytoplasm and a green fluorescing protein to label the nucleus. In notating these cells, GFP really means emission of green wavelengths and RFP emission of red wavelengths.

The invention provides methods for observing proliferation of, and determining proliferation rates of, cells in vitro or in vivo by means of observing the intensity of emitted fluorescence as a function of time. Clonal heterogeneity can also be determined using this technique.

Methods for obtaining transformed cells that produce fluorescent proteins are by now well known in the art. A wide variety of colors of fluorescence is available and stable cell lines have been produced as described, for example, in U.S. Pat. No. 6,232,523, incorporated herein by reference. In one embodiment of the present invention, these cells, rather than being observed in vivo, are employed in a real time in vitro assay.

In one embodiment, cancer cell lines stably expressing a fluorescent protein, such as GFP, are plated into 96-well dishes. At periodic time points, the plates are measured for fluorescence in each well in a fluorescence plate reader such as Molecular Devices Gemini. In each particular well, as the cells proliferate, the intensity of the fluorescence increases. Thus, the rate of proliferation can be read directly by plotting or otherwise manipulating the data of fluorescence intensity versus time.

For use to test the effects of various agents on the proliferation of cells, the wells of the plates are marked such that one set of wells serves as a control and other sets are incubated with drugs of interest. Plates are read for fluorescence intensity in each well at appropriate time intervals, typically either every day or every two days to obtain growth curves for control and drug-treated wells. The cultures need no treatment or additions for these measurements. Since GFP does not fluoresce after being hydrolyzed in dying apoptotic or necrotic cells, the GFP is an instant marker of

cell viability. Total fluorescence in each well correlates with the number of living cells present allowing quantification. Accordingly, the ability of the test compounds to inhibit or to enhance proliferation can readily be determined.

In one application of the foregoing procedures, each well may be supplied with a minimal number of cells, or even a single cell, derived from a tumor. In this manner, tumor heterogeneity may be accounted for by employing the invention methods. The heterogeneity is observed by differences obtained among the wells, each representing the proliferation of one or a small quantity of cells obtained from the tumor.

The invention also provides stably transformed cells which express markers for the cytoplasm and nucleus. By supplying living cells with an expression system for a fluorescent protein lacking a nuclear targeting signal, and with an expression vector comprising a nucleotide sequence encoding a fusion protein wherein a second fluorescent protein of a different color is coupled to a nuclear targeting sequence, both the nucleus and the cytoplasm will be separately visible under microscopic observation. By thus dual labeling the cells, events in the cell cycle can conveniently be monitored and the effect of various agents on the cell cycle can also be evaluated. The cells may be directly observed under a microscope when in culture, or can be observed in an intact animal, even a living animal.

By "color" of the emitted light is meant the wavelength at which the light is emitted. The nucleus and the cytoplasm must emit light at different wavelengths which can be separately determined, and are thus designated as "different colors." The difference in color need not necessarily be detectable by the naked eye; although it is preferable that this level of difference in wavelength be present, it is also possible by using filters and/or detectors with different wavelength sensitivities to observe, with the aid of suitable software, even small differences in wavelength. Thus, for example, by the use of a wide field microscope, such as those described in U.S. Pat. No. 6,444,992, and incorporated herein by reference, differing wavelengths which are closely related can also be used.

It is, however, preferred to simplify observation by using fluorescent proteins whose color differences are detectable by the eye. Instrumentation is also simplified if wavelength differences are sufficient to permit visual differentiation.

Because the cells are stably transformed to produce the two fluorescent proteins, they can be observed while they are alive and undergoing the various stages of the cell cycle. The cells can be observed in culture, or can be observed while they are present in a living organism. For example, whole body observation in real time of living animals using cells stably transformed with green fluorescent protein is described in U.S. Pat. Nos. 6,251,384, 6,235,968 and 6,235,967 incorporated herein by reference . For use in whole organisms, proteins that are highly fluorescent are preferred; thus, only mildly fluorescent proteins such as luciferase are not practical when whole body observation is employed.

Of course, if foreign cells are implanted into laboratory animals, these animals must be sufficiently immunocompromised that the cells are not rejected. Techniques for immunocompromising a variety of animals are known in the art; convenient subjects which are already immunocompromised include nude or SCID mice and similarly modified other rodents such as rats.

The fluorescent proteins are any of those generally available in the art such as those described in the Background section hereinabove. A multiplicity of modifications of the

originally isolated *A. victoria* green fluorescent protein has led to proteins with a variety of colors, and to proteins that are readily expressed in a wide variety of cell types. The options among fluorescent proteins of various emission wavelengths are many and are well known.

Any method of modifying the cells to be studied to contain the expression systems is suitable. The methods of transformation will depend on the nature of the cells and would include, for example, lipofection, electroporation, and viral infection, as a none-limiting list. For plant cells, *Agrobacterium*-mediated transformation can also be used, as well as modification of protoplasts. The choice of control sequences for the expression systems containing the nucleotide sequences encoding the proteins can also be varied and the choice of the appropriate controls and vectors will depend on the nature of the cells and the mode of cell modification.

Any suitable nucleus targeting signal can be used; exemplified below is the histone H2B; however, other sequences targeting the nucleus are known and could be substituted therefor.

Thus, the cells to be modified are transfected with a suitable vector comprising an expression system for each of the fluorescent proteins, one and only one of the fluorescent proteins being coupled to an additional amino acid sequence which will target that protein to the nucleus. The vectors used for transformation may be separate vectors for the fluorescent protein destined for the cytoplasm and the fluorescent protein of a different color destined for the nucleus or both expression systems can be contained on the same expression vector. The nuclear targeting sequence may be employed first, followed by transfection so that the cells contain the expression system for the fluorescent protein that will label the cytoplasm, preferably assuring the stability of the cell line between transfection events in order to assure stability. The order of transfection could also be reversed with the expression system for the cytoplasmic protein administered first. Alternatively, both expression vectors might be contacted with the cell simultaneously, preferably using different selection markers to assure co-transfection. It would also be possible to use a bicistronic expression system for both proteins.

In order to assure stable modification, including instances where the relevant expression systems may be integrated into the genome, the cells are subjected to selection pressure. Suitable selection markers will depend on the nature of the cells; G418 or hygromycin resistance is a convenient marker for a wide variety of cells; other alternative methods of selection include the use of a toxin such as methotrexate with respect to DHFR based systems. Those of ordinary skill will understand the type of selection to be employed.

Thus, in one approach, a suitable cell line is infected with a retroviral vector comprising an expression system wherein a nucleotide sequence encoding a fluorescent protein which emits blue light fused to an amino acid sequence encoding a nuclear targeting signal. The viral vector further contains hygromycin resistance as a selectable marker. The treated cells are then subjected to selection pressure in the presence of hygromycin and after several rounds of selection, stable transformants are obtained. The stably transformed cells are then treated with DNA using electroporation wherein the vector comprises green emitting fluorescent protein coupled to DHFR. The cells are subjected then to rounds of selection with both hygromycin and methotrexate to obtain a cell line wherein the nucleus is stained blue and the cytoplasm green.

The differential staining obtainable by the method of the invention is useful in view of the fact that various portions of the cell cycle give rise to different distributions and/or intensities of radiation emitted from the nucleus and the cytoplasm. Thus, for example, the ratio of intensities will permit determination of cell cycle position. In addition, the morphology of the nucleus is altered when apoptosis occurs and this can readily be detected. The effect of various agents, including various small molecule drugs, proteins, antisense or triplex forming nucleic acids or inhibitor RNA can be tested by observing the effects of these agents on the cellular cycle or morphology. Differential targeting of various agents to the cytoplasm or to the nucleus can also be observed using the methods of the invention. The characteristics of the cells that can be evaluated include dormancy, apoptosis, stage of cell cycle, location of targeting agents, and a multiplicity of other characteristics that will familiar to the artisan. If desired, agents used to treat the cells may themselves be labeled.

Thus, if the cells are to observed through a microscope in culture, the agent may be added directly to the culture. If the cells are to observed in a living animal or plant, the agent is typically administered directly to the animal or plant.

In one embodiment, cancer cells are doubly labeled with histone H2B-GFP expressed only in the nucleus and DsRed-2 introduced with a retroviral vector that is expressed solely in cytoplasm. This allows nuclear-cytoplastic ratios to be determined by the ratio of green to red fluorescence. Such measurements will enable the determination of the relative number of cells in the proliferation state of the cell cycle. The S and G_2 phases are determined by a higher nuclear cytoplastic ratio or higher ratios of green to red fluorescence due to DNA synthesis in the nucleus during the cell doubling process than in non-proliferating cells. The dual-color cells are plated in 96-well dishes and the intensity and ratio of red and green fluorescence determined at various times. By means of this ratio, the status in the cell cycle may be determined.

As with observation of proliferation per se, the assay can be adapted to test the results of treating these cells with various compounds. In this application, control cells are plated in one set of wells and test agents in other sets. Plates are then put in a fluorescence reader capable of dual wavelength measurements to measure the relative increase in green and red fluorescence and their ratio. This will enable the determination of relative proliferation rates as well as on cell cycle position and effects of drugs on these processes.

The heterogeneity of any tissue from which the cells are derived can also be accounted for in the present method by utilizing minimal number of cells per well.

The following examples are intended to illustrate but not to limit the invention. In all cases, images were captured directly with a Hamamatsu C5810 3CCD camera (Hamamatsu Photonics, Bridgewater, N.J.). For macro-imaging, a fluorescence light box (Lightools Research, Encinitas, Calif.) was used. For micro-imaging, a Leica fluorescence stereo microscope model LZ12 was coupled with the CCD camera. This microscope was equipped with a GFP filter set and a mercury lamp with a 50-W power supply. Images were processed for contrast and brightness and analyzed with the use of Image ProPlus 3.1 software. 1024×724 pixel high-resolution images were captured directly on an IBM PC 40).

Preparation A

Production of RFP Retrovirus

For RFP retrovirus production 22), The Hind III/Not I fragment from pDsRed2 (CLONTECH Laboratories, Inc.,

Palo Alto, Calif.), containing the full-length red fluorescent protein cDNA, was inserted into the Hind III/Not I site of pLNCX2 (Clontech) that has the neomycin resistance gene to establish the pLNCX2-DsRed2 plasmid. PT67, an NIH3 T3-derived packaging cell line, expressing the 10 Al viral envelope, was purchased from CLONTECH Laboratories, Inc. PT67 cells were cultured in DME medium (Irvine Scientific, Santa Ana, Calif.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gemini Bio-products, Calabasas, Calif.). For vector production, PT67 cells, at 70% confluence, were incubated with a precipitated mixture of LipofectAMINE™ reagent (Life Technologies, Grand Island, N.Y.), and saturating amounts of pLNCX2-DsRed2 plasmid for 18 hours. Fresh medium was replenished at this time. The cells were examined by fluorescence microscopy 48 hours post-transduction. For selection of a clone producing high amounts of a RFP retroviral vector (PT67-DsRed2), the cells were cultured in the presence of 200–1,000 μg/ml of G418 (Life Technologies) for seven days. In some cases, after 15 days of drug selection, surviving colonies were checked under fluorescence microscopy and GFP or RFP-positive colonies were isolated. Several clones were selected and expanded into cell lines after virus titer test by use of 3T3 cell line.

Preparation B

Production of Histone H2B-GFP Vector

For histone H2B-GFP retrovirus production, the histone H2B gene was kindly provided by professor Geoff Wahl (Salk Institute). This gene has no stop codon, enabling the ligation of the H2B gene to the 5' coding region of the *Aequoria victoria* EGFP gene (Clontech) 24). Then this histone H2B-GFP fusion gene was inserted at the Hind III/Cal I site of the pLHCX (Clontech) that has the hygromycin resistant gene. To establish a clone producing high amounts of a histone H2B-GFP retroviral vector, the pLHCX histone H2B-GFP plasmid was transfected to PT67 cells by same method for PT67-DsRed2. The transfected cells were cultured in the presence of 200–400 μg/ml of hygromycin (Life Technologies) for fifteen days and finally PT67-histone H2B-GFP cells were established. In some cases, after 15 days of drug selection, surviving colonies were checked under fluorescence microscopy and GFP or RFP-positive colonies were isolated. Several clones were selected and expanded into cell lines after virus titer test by use of 3T3 cell line.

EXAMPLE 1

Human Prostate Cancer Cells-Expressing Histone H2B-GFP in the Nucleus and pLNC DsRed in the Cytoplasm

Dual color PC-3 cells were isolated that express GFP exclusively in the nucleus due to fusion of GFP with histone H2B (21) and express RFP exclusively in the cytoplasm. These cells demonstrate the feasibility of dual color imaging of live prostate cancer cells.

Step I: Preparation of DsRed-Expressing Cells.

PC-3 cells were transformed with pLNC DsRed-2 which is produced from PT67 packaging cells. The DsRed-s expression in the PC-3 cells was monitored under fluorescence microscopy. Selection was with increasing amounts of G418.

Step II: Preparation of Dual Labeled H2B GFP and DsRed-2 Cells.

The DsRed-2 PC-3 cells were transfected with pLHC H2B-GFP DNA using LipofectAMINE Plus™. After 24 hours incubation, the H2B GFP and DsRed-2-expressing cells were selected by increasing amounts of both hygromycin and G418.

Cell-cycle position in living cells is analyzed by the area ratio of the green nucleus to the red cytoplasm. Apoptosis is determined by nuclear morphology in living cells.

EXAMPLE 2

RFP and Histone H2B-GFP Gene Transduction of Fibrosarcoma Cells

For RFP and histone H2B-GFP gene transduction, 70% confluent HT-1080 cells, derived from human fibrosarcoma and were purchased from American Type Culture Collection (Rockville, Md.). To establish dual-color cell, clones of HT-1080 expressing RFP in the cytoplasm (HT-1080-RFP), was established initially. Briefly, HT-1080 cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67-RFP cells and RPMI 1640 (Mediatech, Inc., Herndon, Va.) containing 10% fetal bovine serum for 72 hours. Fresh medium was replenished at this time. Cells were harvested by trypsin/EDTA 72 hours post-transduction and subcultured at a ratio of 1:15 into selective medium, which contained 200 μg/ml of G418. The level of G418 was increased stepwise up to 800 μg/ml. HT-1080-RFP were isolated with cloning cylinders (Bel-Art Products, Pequannock, N.J.) using trypsin/EDTA and amplified by conventional culture methods.

Two cloned sub-lines (HT-1080-dual-1 and HT-1080-dual-6) stably expressed GFP in the nucleus and RFP in cytoplasm. Additional clones containing only RFP or GFP were obtained as controls (HT-1080-RFP, HT-1080-GFP).

EXAMPLE 3

Cell Proliferation Rates of Parental HT-1080-RFP and HT-1080-Dual Clones

Each fluorescent-tagged HT-1080 clone (HT-1080-RFP, HT-1080-dual-1 or HT-1080-dual-6) and parental clone (HT-1080) was seeded at a density of 1×10^3 cells/dish in 100 mm dishes with RPMI with 10% FBS medium (day 1). The dishes were kept in an incubator at 37° C. and 5% CO_2. Every other day (days 2–7), three dishes for each clone were used for cell counts. Briefly, resuspended cells collected after trypsinization were stained with trypan blue (Sigma). Only viable cells were counted with a hemocytometer (Reichert Scientific Instruments, Buffalo, N.Y.) subsequently.

FIG. **1** shows the selected HT-1080-dual-color cells have bright GFP and RFP fluorescence in vitro. Green fluorescence is well localized in the nuclei. All cells in the population expressed GFP and RFP, indicating stability of the transgene. FIG. **2** shows there was no difference in the proliferation rates of parental HT-1080, HT-1080-RFP, HT-1080-dual-1 or HT-1080-dual-6 clones determined in monolayer culture.

The HT-1080-dual-1 cells were cultured in 150 mm dishes with RPMI 1640 medium with 10% FBS. Every 5–30 minutes, the dishes were set under fluorescence stereo microscope and time-course images were captured from the same living cells.

FIG. **3**A shows a time-course series of images of HT-1080 dual-1 during mitosis at 5-minute intervals. FIG. **3**B shows mitotic cells at 30-minute intervals, where mitotic cells could readily be followed even if they changed position in a large population.

EXAMPLE 4

Real Time Observation of Apoptotic Process In Vitro

To capture the images of real time apoptotic processes, the HT-1080-dual-6 clone was used. Staurosporine (Alexis, San Diego, Calif.), dissolved in DMSO and stored at −80 degrees, was used for induction of apoptosis. 3×10^5 cells were seeded in a 25 cm^2 flask with RPMI 1640 with 10% FBS. Staurosporine was added the next day at a concentration of 2 μM. Every 2 hours, the flask was set under the fluorescent stereo microscope and real time images were captured at each time point.

Staurosporine 2 μM induced apoptosis in HT-1080-dual-6 cells. (FIGS. **4**A and **4**B). Progressive fragmentation of nucleus could be observed every two hours.

EXAMPLE 5

Real Time Observation of Dual-Labeled Cells In Vivo

All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals under assurance of number A3873-1. Animals were kept in a barrier facility under HEPA filtration. Mice were fed with autoclaved laboratory rodent diet (Teckland LM-485, Western Research Products, Orange, Calif.).

For cell injection in the common carotid artery, nude mice were anesthetized with a ketamine mixture (ketamine HCl 10 μl, xylazine 7.6 μl, acepromazine maleate 2.4 μl, H_2O 10 μl) via subcutaneous injection. At first, a longitudinal skin incision was made on the neck. After the detecting submandibular gland, it was cut at the middle part and retracted to each side. The right sternohyoid muscle and right sternomastoideus muscle and connective tissue were separated with a blunt instrument. After detection of the right common carotid artery, the artery was gently released from surrounding connective tissue. Light tension was put on the proximal site of artery with a blunt-end hook (Fine Science Tools, Inc., Foster City, Calif.). A total of 200 μl of medium containing 2×10^5 HT-1080-dual-1 cells was injected in the artery by using 33G needle (Fine Science Tools). Immediately after the injection, the injected site was pressed with a swab to prevent bleeding or leakage of injected tumor cells for a while. After confirmation of arrest of bleeding, the skin was closed with a 6-0 suture. All procedures of the operation described above were performed with a 7× dissection microscope (MZ6, Leica, Deerfield, Ill.).

After common carotid-artery injection of HT-1080-dual-1, embolic cells in a brain micro-artery were visualized even through the skull via a scalp flap (FIGS. **5**A and **5**B). The skull of the mouse is relatively transparent. The tumor cells were observed to have elongated to adapt to the microvessels similar to leukocytes. The nuclei of tumor cells were very elongated in the tumor cells visualized in the vessels.

EXAMPLE 6

Visualizing Nucleus-Cytoplasmic Dynamics of Tumor Cells In Vivo

To visualize tumor cells nuclear cytoplasmic dynamics in vivo, mice were anesthetized with ketamine mixture. The surfaces of ears were directly observed by fluorescence stereo microscope under blue light.

To observe nucleus cytoplasmic dynamics in lung metastasis, six nude mice were injected with 1×10^6 HT-1080-dual-1 cells at the volume of 300 μl in the tail veins. At various time points, mice were sacrificed and lungs were removed. The metastatic colonies on the lung were visualized directly under blue light. For histological analysis, the excised lungs were embedded with tissue frozen medium (Triangle Biomedical Sciences, Durham N.C.) and stored at −80 degrees. Frozen sections were prepared by cryostat LEICA model CM-1850 at the thickness of 4 μm. The prepared sections were directly observed under fluorescence stereo microscope with GFP filter set.

Micrometastases on excised lungs were visualized with each nucleus in close proximity to each other (data not shown). The cells appear to be distorted to enable contact between nuclei.

This study opens up the possibility of real time observation of tumor cell nuclear-cytoplasmic dynamics including apoptotic process at the cellular level in vivo as well as in vitro. Mitotic cells were readily visualized after injection in the mouse ear in live mice. After tail-vein injection, dual-color micrometastases were visualized in excised lungs.

EXAMPLE 7

Whole Body Fluorescent Optical Imaging

Real time images of mitotic cells could be captured in the living mouse 12 hours after the injection of HT-1080-dual-1 (FIGS. **6**A and **6**B). The cells shown seemed to be extravasated and appear rounded, similar to dividing cells in culture. The conditions of each nucleus and the boundary of the cells were visualized in the living animal without any fixation or staining.

EXAMPLE 8

Preparation of a Dual-Colored Human Prostate Cancer Cell Line

PC-3 cells were grown in RPMI1640 medium supplemented with 10% FCS. Exponentially growing cells (in 10 cm dishes) were incubated with the viral supernatants from PT67/pLHCX H2B-EGFP in the presence of 8 μg ml^{-1} Polybrene. After overnight incubation, medium was changed, and the infected cells were expanded for step-wise Hygromycin selection. After 15 days of drug selection, surviving colonies were visualized under fluorescence microscopy, and GFP-positive colonies were isolated. A clone with uniform, high-level expression of H2B-EGFP was selected.

Exponentially growing H2B-EGFP expression cells were then incubated with the viral containing supernatants from PT67/pLNCX DsRed-2 in the presence of 8 μg/ml Polybrene. After overnight incubation medium was changed and the infected cells were expanded for step-wise G418 selection after 48 hours infection. After 15 days of drug selection, surviving colonies were visualized under fluorescence microscopy and RFP-positive colonies were isolated. A clone with uniform, high-level expression of both H2B-EGFP and RFP (PC3-dual) was selected.

For growth rate determination, 1×10^5 cells were cultured in 60 mm Petri dishes and counted every day for one week. The number of viable cells/well was determined at the indicated times in triplicate, excluding dead cells by trypan blue staining.

The high expression of H2B-EGFP and cytoplasm RFP in this cell line was stable for more than three months in the absence of Hygromycin B and G418 selection culture. The proliferation rate of the dual-colored cells is same as its

parental cell PC-3 in vitro. These results indicate that dual-colored PC-3 could be a useful model for in vivo and in vitro studies.

EXAMPLE 9

Observation of Cell Cycle and Apoptosis

The dual-color cells could be easily placed in their cell cycle position by the "nuclear-cytoplasmic" ratio as observed in living cells. For fluorescence microscopy of these cells, a Leica fluorescence stereo microscope model LZ12 equipped with a mercury 50W lamp power supply was used. To visualize both GFP and RFP fluorescence at the same time, excitation was produced through a D425/60 band pass filter, 470 DCXR dichroic mirror, and emitted fluorescence was collected through a long pass filter GG475 (Chroma Technology, Brattleboro, Vt.).

FIGS. **7A–7D** show the cell cycle phases; FIG. **7E** shows apoptosis.

The large nuclear size compared to the cytoplasm readily identified G_2-phase cells compared to G_1-phase cells with much smaller nuclear-cytoplasm ratios. The S-phase cells had nuclear-cytoplasmic ratios larger than G_1 but smaller than G_2. Prophase cells about to enter mitosis were identified by chromosome condensation. Cells with the metaphase plate lined up were readily observed and could be visualized as they entered anaphase. Apoptotic cells were identified by an aberrant nuclear morphology including fragmentation.

Thus, sequential cell cycle progression of individual cells could be followed in real time with photo micrographs taken at frequent intervals.

EXAMPLE 10

Real-Time Visualization of Drug-Induced Apoptosis

PC-3 dual cells were treated with Taxol™ (0.8 ug/ml); thymidine (2 mM), and vinblastine (60 nM). Real-time imaging was done at 0, 12, 24, 36, and 48 hours. Cells were collected for DNA extraction and agarose gel electrophoresis (1.8%) analysis. Paclitaxel (Taxol™) (0.8 μg/ml) had very specific effects on the nuclei causing them to form a ring-shaped structure apparently due to the attachment of the chromatin to the nuclear membrane. FIG. **8** shows this was readily visible with the GFP-labeled nuclei against the background of the RFP-labeled cytoplasm. The nuclear ring structures were viable by 24 hours. By 48 hours, the cells could be seen entering apoptosis with very aberrant nuclear structures and fragmentation.

FIG. **9** shows that the ring structures induced by Taxol™ observed at 24 hours after initiation of treatment was before DNA fragmentation occurred and could be observed by gel electrophoresis. By 48 hours, when the cells were well into apoptosis, DNA fragmentation could be observed.

Another tubulin agent, vinblastine (60 nM), had different effects on the cell nuclei as compared to Taxol™, wherein the nuclei became more condensed and the cytoplasm appeared to expand as shown in FIG. **10**.

EXAMPLE 11

In Vivo Observation

All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health (NIH) Guide for the care and use of animals under NIH assurance number A3873-1. Male nude mice (NCr-nu) between 5 and 6 weeks of age were maintained in a barrier facility on HEPA-filtered racks. The dual colored PC-3 human prostate cancer cells were injected (2×10^6) into the nude-mouse foot pad. Mice were euthanized after 20 days. Tumor and lymph nodes and lungs were processed for fluorescence microscopy.

FIG. **11** shows mitotic dual-color PC-3 cells were observed in the lymph node under fluorescence microscopy.

What is claimed is:

1. A method to prepare living cells which cells comprise a first fluorescent protein localized to the nucleus and a second fluorescent protein localized to the cytoplasm

wherein said first and second fluorescent proteins emit light of different wavelengths which method comprises

modifying living cells to contain either

(a) a first expression system for expression of said first fluorescent protein wherein said first fluorescent protein is fused to an amino acid sequence which targets said fusion protein to the nucleus and a second expression system for expression of a second fluorescent protein lacking a nucleus targeting sequence; or

(b) an expression system that expresses both said first fluorescent protein and second fluorescent protein as described; and

selecting said modified cells for cells that have been stably modified.

2. The method of claim **1**, wherein in step (a), said cells are first modified with said first expression system and then modified with said second expression system or vice versa.

3. The method of claim **1**, wherein in step (a), the cells are modified with both expression systems simultaneously.

4. The method of claim **1**, wherein said selecting is by culturing in the presence of an antibiotic or a toxin.

5. A colony of living cells stably modified to produce a first fluorescent protein fused to an amino acid sequence targeting the nucleus and a second fluorescent protein lacking an amino acid sequence targeting the nucleus;

wherein said first and second fluorescent proteins emit visible light at different wavelengths.

6. A colony of living cells which are modified to contain a first fluorescent protein localized to the nucleus and a second fluorescent protein localized to the cytoplasm wherein said first fluorescent protein and second fluorescent protein are of different colors.

7. The colony of claim **6**, wherein said first fluorescent protein is green and said second fluorescent protein is red.

8. The colony of claim **5**, wherein said amino acid sequence targeting the nucleus is histone H2B.

9. A method to determine the cell cycle position of living cells which method comprises assessing the ratio of nuclear area to cytoplasmic area of the cells of the colony of claim **6**.

10. The method of claim **9**, wherein said assessing is performed as a function of time.

11. The method of claim **9**, wherein said cells of said colony are observed in a living animal.

12. A method to determine the effect of an agent on cells, which method comprises

treating a first sample of the colony of claim **6** with said agent and observing the effect of said treating on the distribution and/or intensity of radiation emitted from said colony.

13. The method of claim **12**, which further comprises observing the distribution and/or intensity of radiation emitted from a second sample of said colony that has not been treated with said agent, and

comparing the observations made on the first sample with those on the second sample.

14. The method of claim **12**, wherein the distribution and/or intensity are evaluated for being characteristic of dormancy.

15. The method of claim **12**, wherein said distribution and/or intensity are evaluated for being characteristic of apoptosis.

16. The method of claim **12**, wherein said distribution and/or intensity are evaluated for being characteristic of stages in the cell cycle.

17. A method to determine the location of an agent as the cytoplasm or nucleus which method comprises treating the colony of claim **6** with said agent and observing the distribution and/or intensity of radiation emitted from the cytoplasm and nucleus.

18. The method of claim **17**, wherein said agent itself is labeled, and said method further comprises directly observing the location of the label.

19. A method to determine the proliferation rate of a cell culture which method comprises culturing cells which have been modified to contain a fluorescent protein; and

measuring the fluorescence emitted by said cells as a function of time,

whereby the rate of proliferation of said cells is determined, as correlated to the rate of increase of intensity of emitted fluorescence.

20. The method of claim **19**, wherein said fluorescent protein is a green fluorescent protein (GFP) or a red fluorescent protein (RFP).

21. The method of claim **19**, wherein said culture is grown from a single cell.

22. A method to determine the effect of a test compound on cell proliferation which method comprises

culturing cells in the presence and absence of said test compound, wherein said cells have been modified to contain a fluorescent protein;

measuring the intensity of fluorescence as a function of time in the presence and absence of said compound so as to determine the rate of proliferation in the presence and absence of said compound, as correlated to the rate of increase of intensity of emitted fluorescence; and

comparing the rate of proliferation in the presence and absence of said compound;

wherein a change in the rate of proliferation in the presence as opposed to the absence of said compound identifies said compound as a modulator of cellular proliferation.

23. The method of claim **22**, wherein said fluorescent protein is a green fluorescent protein (GFP) or a red fluorescent protein (RFP).

24. The method of claim **22**, wherein said culturing is commenced from a single cell.

25. A method to determine the heterogeneity of a tumor, which method comprises culturing a multiplicity of colonies from individual cells or individual groups of cells contained in said tumor; and

determining the rates of proliferation of said cell cultures;

whereby cultures exhibiting different rates of proliferation indicate heterogeneity of said tumor.

26. The method of claim **25**, wherein said cells have been modified to contain a fluorescent protein and the rates of proliferation are determined, as correlated to the rate of increase of intensity of emitted fluorescence, by measuring the intensity of emitted fluorescence as a function of time.

27. The method of claim **26**, wherein said cells have been modified to contain a first fluorescent protein localized to the nucleus and a second fluorescent protein localized to the cytoplasm wherein said first fluorescent protein and second fluorescent protein are of different colors.

* * * * *

INSTRUCTIONS FOR ATTORNEYS COMPLETING CIVIL COVER SHEET FORM JS-44

Authority For Civil Cover Sheet

The JS-44 civil cover sheet and the information contained herein neither replaces nor supplements the filings and service of pleading or other papers as required by law, except as provided by local rules of court. This form, approved by the Judicial Conference of the United States in September 1974, is required for the use of the Clerk of Court for the purpose of initiating the civil docket sheet. Consequently a civil cover sheet is submitted to the Clerk of Court for each civil complaint filed. The attorney filing a case should complete the form as follows:

I. (a) Plaintiffs - Defendants. Enter names (last, first, middle initial) of plaintiff and defendant. If the plaintiff or defendant is a government agency, use only the full name or standard abbreviations. If the plaintiff or defendant is an official within a government agency, identify first the agency and then the official, giving both name and title.

(b) County of Residence. For each civil case filed, except U.S. plaintiff cases, enter the name of the county where the first listed plaintiff resides at the time of filing. In U.S. plaintiff cases, enter the name of the county in which the first listed defendant resides at the time of filing. (NOTE: In land condemnation ca: :ation of the tract of land involved.)

(c) Attorneys. Enter the firm name, addre: e several attorneys, list them on an attachment, noting in this section "(see attachmen

II. Jurisdiction. The basis of jurisdiction is se irisdictions be shown in pleadings. Place an "X" in one of the boxes. If there is mor he order shown below.

United States plaintiff. (1) Jurisdiction based officers of the United States are included here.

United States defendant. (2) When the plaintiff i in "X" in this box.

Federal question. (3) This refers to suits under nstitution of the United States, an amendment to the Constitution, an act of Congr : U.S. is a party, the U.S. plaintiff or defendant code takes precedence, and box 1 or

Diversity of citizenship. (4) This refers to suit different states. When Box 4 is checked, the citizenship of the different partie question actions take precedence over diversity cases.)

III. Residence (citizenship) of Principal Partie: ersity of citizenship was indicated above. Mark this section for each principal party

IV. Cause of Action. Report the civil statute dir iption of the cause.

V. Nature of Suit. Place an "X" in the app ed, be sure the cause of action, in Section IV above, is sufficient to enable the d ve Office to determine the nature of suit. If the cause fits more than one nature of s

VI. Origin. Place an "X" in one of the seven b

Original Proceedings. (1) Cases which originate

Removed from State Court. (2) Proceeding: rict courts under Title 28 U.S.C., Section 1441. When the petition for removal is g

Remanded from Appellate Court. (3) Check th er action. Use the date of remand as the filing date.

Reinstated or Reopened. (4) Check this box t. Use the reopening date as the filing date.

Transferred from Another District. (5) For cas Do not use this for within district transfers or multidistrict litigation transfers.

Multidistrict Litigation. (6) Check this box when a multidistrict case is transferred into the district under authority of Title 28 U.S.C. Section 1407. When this box is checked, do not check (5) above.

Appeal to District Judge from Magistrate Judgment. (7) Check this box for an appeal from a magistrate's decision.

VII. Requested in Complaint. Class Action. Place an "X" in this box if you are filing a class action under Rule 23, F.R.Cv.P.

Demand. In this space enter the dollar amount (in thousands of dollars) being demanded or indicate other demand such as a preliminary injunction.

Jury Demand. Check the appropriate box to indicate whether or not a jury is being demanded.

VIII. Related Cases. This section of the JS-44 is used to reference relating pending cases if any. If there are related pending cases, insert the docket numbers and the corresponding judge names for such cases.

Date and Attorney Signature. Date and sign the civil cover sheet.

(rev. 07/89)