The University *of* Western Ontario
BIOLOGICAL AGENTS REGISTRY FORM
Approved Biohazards Subcommittee: July 8, 2011
Biosafety Website: www.uwo.ca/humanresources/biosafety/

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario (UWO) or in charge of a laboratory/facility where the use of Level 1, 2 or 3 biological agents is described in the laboratory or animal work proposed. The form must also be completed if any work is proposed involving animals carrying zoonotic agents infectious to humans or involving plants, fungi, or insects that require Public Health Agency of Canada (PHAC) or Canadian Food Inspection Agency (CFIA) permits.

This form must be updated at least every 3 years or when there are changes to the biological agents being used.

Containment Levels will be established in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Public Health Agency of Canada (PHAC) or Containment Standards for Veterinary Facilities, 1st edition 1996, Canadian Food Inspection Agency (CFIA).

Electronically completed forms are to be submitted to Occupational Health and Safety, (OHS), (Support Services Building, Room 4190 or to jstanle2@uwo.ca) for distribution to the Biohazards Subcommittee. For questions regarding this form, please contact the Biosafety Officer at extension 81135 or biosafety@uwo.ca. If there are changes to the information on this form (excluding grant title and funding agencies), contact Occupational Health and Safety for a modification form. See website: www.uwo.ca/humanresources/biosafety.

Please ensure that all questions are fully and clearly answered. Failure to do so will lead to the form being returned, which will cause delays in your approval and frustration for you and your colleagues on the Committee.

If you are re-submitting this form as requested by the Biohazards Subcommittee, please make modifications to the form in bold print, highlighted in yellow. Please re-submit forms electronically.

PRINCIPAL INVESTIGATOR:	**Andrew Leask**
DEPARTMENT:	**Dentistry**
ADDRESS:	**DSCI 0067**
PHONE NUMBER:	**81102**
EMERGENCY PHONE NUMBER(S):	
EMAIL:	**andrew.leask@schulich.uwo.ca**

Location of experimental work to be carried out :

Building : **dsci** Room(s): **0064**
Building : Room(s):
Building : Room(s):

***For work being performed at Institutions affiliated with the University of Western Ontario, the Safety Officer for the Institution where experiments will take place must sign the form prior to its being sent to the University of Western Ontario Biosafety Officer (See Section 15.0, Approvals).**

FUNDING AGENCY/AGENCIES: **CIHR**
GRANT TITLE(S): **The role of CTGF in tissue repair and fibrosis**
UNDERGRADUATE COURSE NAME(IF APPLICABLE): **N/A**

List all personnel working under Principal Investigators supervision in this location:

Name	UWO E-mail Address	Date of Biosafety Training
Fen Guo	**fguo9@uwo.ca**	**November 17, 2010**
Shangxi Liu	**shangxi.liu@schulich.uwo.ca**	**October 10, 2009**
Wei Sha	**wsha2@uwo.ca**	**June 16, 2010**
Katherine Thompson	**kthomp58@uwo.ca**	**May 11, 2010**
James Hutchenreuther	**jhutche2@uwo.ca**	**June 15, 2010**

Please explain how the biological agents are used in your project and how they are stored and disposed of. The BARF without this description will not be reviewed.

the cells are stored in a liquid nitrogen tank. the virus is stored in a -80 freezer. All are thawed and used entirely in our level 2 hood. These are disposed of via autoclaving in a biohazard bag, labeled and biosafety is called for pickup

Please include a ONE page research summary or teaching protocol in lay terms.
Forms with summaries more than one page will not be reviewed.

Our long-term objective is to uncover new treatments for fibrotic disease.
We want to study the effect of adhesive proteins such as CTGF/CCN2 to fibrosis and tissue repair. Fibroproliferative diseases can affect individual organs and tissues, such as the kidney, liver, lung, blood vessels and oral cavity, or be systemic, such as in diffuse systemic sclerosis (diffuse scleroderma, dSSc). As dSSc affects all organs, elucidating the molecular basis for active dSSc will be beneficial in understanding the nature of fibrotic disease. For example, dSSc patients exhibit fibrosis of the mouth causing gingival retraction and microstomia. Fibrotic fibroblasts, such as in dSSc, show increased proliferative capacity, and elevated extracellular matrix (ECM) production and remodeling. Although not normally expressed by post-natal fibroblasts, Connective tissue growth factor (CTGF) can be induced by the pro-fibrotic cytokine TGFb However, constitutive expression of CTGF is a characteristic feature of fibrotic fibroblasts, including those from active lesions of dSSc. CTGF is proposed to mediate the pro-fibrotic functions of TGFb indeed, CTGF works with TGFb to promote sustained fibrosis in vivo. As TGFb is pleitropic, anti-TGFb strategies might result in unwanted side-effects. I have proposed that in fibrotic disease CTGF may represent a more selective anti-fibrotic target than TGF□ (Leask et al., J. Invest. Dermatol. 122, 1-6, 2004). However, the precise physiological contribution of CTGF to fibroblast biology and the selectivity of CTGF action are not known. This knowledge is necessary to support anti-CTGF therapy as an anti-fibrotic approach. I hypothesize that CTGF selectively—either in the presence or absence of TGFb—mediates fibroblast proliferation, and ECM production and remodeling.

Objectives: To probe the role of CTGF in fibroblast biology, tissue repair and fibrosis, I will:
1) determine the biological processes altered by loss of CTGF in mouse embryonic fibroblasts (MEFs);
2) identify the gene expression and signal transduction cascades affected by loss of CTGF in MEFs; and
3) establish whether overexpression of CTGF in adult mice produces fibrotic responses in vivo.

Also, since CTGF is an adhesive protein we are more generally testing the effects of adhesive signaling on fibrosis and tissue repair, and use the information generated with the reagents below to test the mechanisms of action of the adhesive cascade in driving fibrosis

1.0 Microorganisms

1.1 Does your work involve the use of biological agents? ☒ YES ☐ NO
(non-pathogenic and pathogenic biological agents including but not limited to bacteria and other microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)? If no, please proceed to Section 2.0

Do you use microorganisms that require a permit from the CFIA? ☐ YES ☒ NO
If YES, please give the name of the species ______________________
What is the origin of the microorganism(s)? ______________________

Level 1 E.coli

Please describe the risk (if any) of escape and how this will be mitigated:

Please attach the CFIA permit.

Please describe any CFIA permit conditions:

1.2 Please complete the table below:

Full Scientific Name of Biological Agent(s)* (Be specific)	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/ Supplier	PHAC or CFIA Containment Level
DH5 alpha	☐ Yes ☒ No	☐ Yes ☒ No	☐ Yes ☒ No	**1**	**invitrogen**	☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3

***Please attach a Material Safety Data Sheet or equivalent from the supplier if the bacterium used is not on this link:**
http://www.uwo.ca/humanresources/docandform/docs/ohs/CFIA_Ecoli_list.pdf

Additional Comments: ______________________

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? ☒ YES ☐ NO
(If NO, please proceed to Section 3.0)

2.2 Please indicate the type of primary cells (i.e. derived from fresh tissue) that will be grown in culture:

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue	AUS Protocol Number
Human	☐ Yes ☐ No		Not applicable
Rodent	☒ Yes ☐ No	**skin, lung**	**2010-253**
Non-human primate	☐ Yes ☐ No		
Other (specify)	☐ Yes ☐ No		

2.3 Please indicate the type of established cells that will be grown in culture in:

Cell Type	Is this cell type used in your work?	Specific cell line(s)*	Containment Level of each cell line	Supplier / Source of cell line(s)
Human	☒ Yes ☐ No	**foreskin fibroblast**	**1**	**ATCC**
Rodent	☒ Yes ☐ No	**NIH 3t3, B16**	**1**	**ATCC**
Non-human primate	☐ Yes ☐ No			
Other (specify)	☐ Yes ☐ No			

Please attach a Material Safety Data Sheet or equivalent from the supplier. (For more information, see www.atcc.org)

2.4 For above named cell types(s) indicate PHAC or CFIA containment level required ☒ 1 ☐ 2 ☐ 2+ ☐ 3

Additional Comments: _____

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? ☐ YES ☒ NO
If no, please proceed to Section 4.0

3.2 Indicate in the table below the Human Source Material to be used.

Human Source Material	**Source/Supplier /Company Name**	**Is Human Source Material Infected With An Infectious Agent? YES/UNKNOWN**	**Name of Infectious Agent (If applicable)**	**PHAC or CFIA Containment Level (Select one)**
Human Blood (whole) or other Body Fluid		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Blood (fraction) or other Body Fluid		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Organs or Tissues (unpreserved)		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Organs or Tissues (preserved)		Not Applicable		Not Applicable

Additional Comments: _____

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents, or cells described in Sections 1.0 and 2.0? ☒ YES ☐ NO If **NO**, please proceed to Section 5.0

4.2 Will genetic modification(s) involving plasmids be done? ☒ YES, complete table below ☐ NO

Bacteria Used for Cloning *	Plasmid(s) **	Source of Plasmid	Gene Transformed or Transfected	Will there be a change due to transformation of the bacteria?	Will there be a change in the pathogenicity of the bacteria after the genetic modification?	What are the consequences due to the transformation of the bacteria?
DH5a	**pGL3BARL, pGL3fuBARL, pSL3, pSL4, pSL5, pBARLS, pfuBARLS, pSL9/rLuc**	**Moon lab http://www.springerlink.com/content/v062nq405r027772/fulltext.pdf.**	**these are reporter constructs to test b-catenin transcript-ional activity and have already been approved last year**	**no**	**none**	**none**

E. coli

** Please attach a Material Safety Data Sheet or equivalent if available.*
*** Please attach a plasmid map.*
****No Material Safety Data Sheet is required for the following strains of E. coli:*
http://www.uwo.ca/humanresources/docandform/docs/ohs/CFIA_Ecoli_list.pdf

4.3 Will genetic modification(s) of bacteria and/or cells involving viral vectors be made?
☒ YES, complete table below ☐ NO

Virus Used for Vector Construction	Vector(s) *	Source of Vector	Gene(s) Transduced	Describe the change that results from transduction
lentivirus sc-108080	**sc-39329-V** **sc-108080**	**Santa Cruz** **Santa Cruz**	**shRNA CTGF** **empty**	**Proliferation is suppressed** **none**

** Please attach a Material Safety Data Sheet or equivalent.*

4.3.1 Will virus be replication defective? ☒ YES ☐ NO

4.3.2 Will virus be infectious to humans or animals? ☐ YES ☒ NO

4.3.3 Will this be expected to increase the containment level required? ☐ YES ☒ NO

5.0 Will genetic sequences from the following be involved?

- HIV ☐ NO ☒ YES, specify **please see paper below**

- HTLV 1 or 2 or genes from any Level 1 or Level 2 pathogens ☒ NO ☐ YES, specify
- SV 40 Large T antigen ☒ NO ☐ YES
- E1A oncogene ☒ NO ☐ YES
- Known oncogenes ☒ NO ☐ YES, specify
- Other human or animal pathogen and or their toxins ☐ NO ☐ YES, specify

5.1 Is any work being conducted with prions or prion sequences? ☒ NO ☐ YES

Additional Comments: **we are purchasing the 3rd generation viruses directly, we will not be making them ourselves. see Dull et al J Virol 1998 Nov; 72(11) 8463-71. There is only the LTRs and the Psi-sequence of HIV**

Not answered (one question)

6.0 Human Gene Therapy Trials

6.1 Will human clinical trials be conducted involving a biological agent? ☐ YES ☒ NO
(including but not limited to microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)
If no, please proceed to Section 6.0

6.2 If YES, please specify which biological agent will be used:
Please attach a full description of the biological agent.

6.3 Will the biological agent be able to replicate in the host? ☐ YES ☐ NO

6.4 How will the biological agent be administered?

6.5 Please give the Health Care Facility where the clinical trial will be conducted:

6.6 Has human ethics approval been obtained? ☐ YES, number: ☐ NO ☐ PENDING

7.0 Animal Experiments

7.1 Will live animals be used? ☒ YES ☐ NO If **NO**, please proceed to section 7.0

7.2 Name of animal species to be used **mouse**

7.3 AUS protocol # **2010-053; 2009-097**

7.4 Will any of the agents listed in section 4.0 be used in live animals
☒ NO ☐ YES, specify:

7.5 Will the agent(s) be shed by the animal:
☒ YES ☐ NO, please justify:

7.4 + 7.5 do not make sense?

8.0 Use of Animal species with Zoonotic Hazards

8.1 Will any animals with zoonotic hazards or their organs, tissues, lavages or other body fluids including blood be used (see list below)? ☐ YES ☒ NO - If **NO**, please proceed to section 8.0

8.2 Will live animals be used? ☐ YES ☐ NO

8.3 If **YES**, please specify the animal(s) used:

♦ Pound source dogs	☐ YES	☐ NO
♦ Pound source cats	☐ YES	☐ NO
♦ Cattle, sheep or goats	☐ YES, species	☐ NO
♦ Non-human primates	☐ YES, species	☐ NO
♦ Wild caught animals	☐ YES, species & colony #	☐ NO
♦ Birds	☐ YES, species	☐ NO
♦ Others (wild or domestic)	☐ YES, specify	☐ NO

8.4 If no live animals are used, please specify the source of the specimens:

9.0 Biological Toxins and Hormones

9.1 Will toxins or hormones of biological origin be used? ☐ YES ☒ NO If no, please proceed to Section 9.0

9.2 If YES, please name the toxin(s) or hormones(s)
Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

9.3 What is the LD_{50} (specify species) of the toxin or hormone

9.4 How much of the toxin or hormone is handled at one time*?

9.5 How much of the toxin or hormone is stored*?

9.6 Will any biological toxins or hormones be used in live animals? ☐ YES ☐ NO
If **YES**, Please provide details:

*For information on biosecurity requirements, please see:
http://www.uwo.ca/humanresources/docandform/docs/healthandsafety/biosafety/Biosecurity_Requirements.pdf

Additional Comments: _____

10.0 Insects

10.1 Do you use insects? ☐ YES ☒ NO - If **NO**, please proceed to Section 10.0

10.2 If YES, please give the name of the species.

10.3 What is the origin of the insect?

10.4 What is the life stage of the insect?

10.5 What is your intention? ☐ Initiate and maintain colony, give location:
☐ "One-time" use, give location:

10.6 Please describe the risk (if any) of escape and how this will be mitigated:

10.7 Do you use insects that require a permit from the CFIA permit? ☐ YES ☐ NO
If **YES**, Please attach the CFIA permit & describe any CFIA permit conditions:

11.0 Plants

11.1 Do you use plants? ☐ YES ☒ NO - If **NO**, please proceed to Section 11.0

11.2 If YES, please give the name of the species.

11.3 What is the origin of the plant?

11.4 What is the form of the plant (seed, seedling, plant, tree…)?

11.5 What is your intention? ☐ Grow and maintain a crop ☐ "One-time" use

11.6 Do you do any modifications to the plant? ☐ YES ☐ NO
If yes, please describe:

11.7 Please describe the risk (if any) of loss of the material from the lab and how this will be mitigated:

11.8 Is the CFIA permit attached? ☐ YES ☐ NO
If **YES**, Please attach the CFIA permit & describe any CFIA permit conditions:

12.0 Import Requirements

12.1 Will any of the above agents be imported? ☐ YES, country of origin ☒ NO
If **NO**, please proceed to Section 12.0

12.2 Has an Import Permit been obtained from HC for human pathogens? ☐ YES ☐ NO

12.3 Has an import permit been obtained from CFIA for animal or plant pathogens? ☐ YES ☐ NO

12.4 Has the import permit been sent to OHS? ☐ YES, please provide permit # ☐ NO

13.0 Training Requirements for Personnel Named on Form

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by OHS:

- Biosafety
- Laboratory and Environmental/Waste Management Safety
- WHMIS (Western or equivalent)
- Employee Health and Safety Orientation

As the Principal Investigator, I have ensured that all of the personnel named on the form who will be using any of the biological agents in Sections 1.0 to 9.0 have been trained.

An X in the check box indicates you agree with the above statement... ☒
Enter Your Name Andrew Leask ***Date:*** November 16, 2011

14.0 Containment Levels

14.1 For the work described in sections 1.0 to 9.0, please indicate the highest HC or CFIA Containment Level required. ☐ 1 ☒ 2 ☐ 2+ ☐ 3

14.2 Has the facility been certified by OHS for this level of containment?
☒ YES, location and date of most recent biosafety inspection: **11/15/2010**
☐ NO, please certify
☐ NOT REQUIRED for Level 1 containment

14.3 Please indicate permit number (not applicable for first time applicants): **BIO-UWO-0218**

15.0 Procedures to be Followed

15.1 Are additional risk reduction measures necessary beyond containment level 1, 2, 2+ or 3 measures that are unique to these agents? ☐ YES ☒ NO
If **YES** please describe:

15.2 Please outline what will be done if there is an exposure to the biological agents listed such as a needlestick injury or an accidental splash:
Post-Exposure Treatment:

- **Skin exposure / Percutaneous: Wash affected area and apply antiseptic (3% H2O2), report to the Health Center.**
- **Mucous membrane exposure (splash to eye): flush eyes for 15 minutes using eyewash, then report to Health Center.**
- **Ingestion: Report to the Health Center.**

15.3 As the Principal Investigator, I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.shs.uwo.ca/workplace/newposition.htm

An X in the check box indicates you agree with the above statement... ☒
Enter Your Name **Andrew Leask** ***Date:*** **Nov 16, 2011**

15.4 Additional Comments: _____

16.0 Approvals

1) UWO Biohazards Subcommittee:
SIGNATURE: _____
Date: _____

2) Safety Officer for the University of Western Ontario
SIGNATURE: _____
Date: _____

3) Safety Officer for Institution where experiments will take place (if not UWO):
SIGNATURE: _____
Date: _____

Approval Number: _____ Expiry Date (3 years from Approval): _____

Special Conditions of Approval:

-------- Original Message --------
Subject:please see attached
Date: Tue, 15 Nov 2011 16:10:40 -0500
From: Andrew Leask <Andrew.Leask@schulich.uwo.ca>
To: Jennifer Stanley <jstanle2@uwo.ca>

Jennifer:
http://datasheets.scbt.com/sc-108080.pdf
They do not give out the maps as they are proprietary.
Other than that, I hope all questions have been asked.

Dear Dr. Leask,

Thank you for inquiring with Santa Cruz Biotechnology regarding Control shRNA Lentiviral Particles-A: sc-108080.

Here is some information about our lentiviral products, which are packaged using 3rd generation lentiviral technology. I've also included a reference for your records.

Lentivirus expression systems that are third generation of HIV-based lentivectors were developed originally for gene therapy applications and due to their biosafety, are in growing use in research labs, and offered through commercial vendors, including Santa Cruz Biotechnology, Inc. RCV testing is not required for third-generation, commercially available Lentiviral vector systems.

1. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, and Naldini L. A third-generation lentivirus vector with a conditional packaging system. J Virol 1998 Nov; 72(11) 8463-71. PMID: 9765382.

Please feel free to contact me with any future questions.

Madeline Baker
Technical Service Representative
Santa Cruz Biotechnology, Inc.
1.800.457.3801 ext. 131
bakerm@scbt.com

Canadian Food Inspection Agency | Agence canadienne d'inspection des aliments

Office of Biohazard Containment and Safety
Science Branch, CFIA
59 Camelot Drive, Ottawa, Ontario K1A 0Y9
Tel: (613) 221-7068 Fax: (613) 228-6129
Email: ImportZoopath@inspection.gc.ca

Bureau du confinement des biorisques et sécurité
Direction générale des sciences, ACIA
59 promenade Camelot, Ottawa, Ontario K1A 0Y9
Tél: (613) 221-7068 Téléc: (613) 228-6129
Courriel: ImportZoopath@inspection.gc.ca

October 20th, 2009

Ms. Shamila Survery / Mr. Michael Decosimo
Cedarlane Laboratories Ltd
4410 Paletta Court
Burlington, Ontario L7L 5R2

By Facsimile: (289) 288-0020

SUBJECT: **Importation of *Escherichia coli* strains**

Dear Ms. Survery / Mr. Decosimo:

Our office received your query about the importation of *Escherichia coli* from the American Type Culture Collection (ATCC) located in Manassas, Virginia, United States. The following *Escherichia coli* strains are consider to be level 1 animal pathogens:

- 5K
- 58
- 58-161
- 679
- 1532
- AB284
- AB311
- AB1157
- AB1206
- AG1
- B
- BB4
- BD792
- BL21
- BL21 (DE3)
- BM25.8
- C
- C-1a
- C-3000
- C25
- C41 (DE3)
- C43 (DE3)
- C600
- Cavalli Hfr
- CIE85
- DH1
- DH10 GOLD
- DH10B
- DH5
- DH5-alpha
- DP50
- DY145
- DY380
- E11
- EJ183
- EL250
- EMG2
- EPI 300
- EZ10
- FDA Seattle 1946
- Fusion-Blue
- H1443
- HF4714
- HB101
- HS(PFAMP)R
- Hfr3000
- Hfr3000 X74
- HMS174
- J52
- J53
- JC3272
- JC7661
- JC9387
- JF1504
- JF1508
- JF1509
- JJ055
- JM83
- JM101
- JM109
- K12
- KC8
- KA802
- KAM32
- KAM33
- KAM43
- LE450
- LE451
- LE452
- MB408
- MBX1928
- MC1061
- MC4100 (MuLac)
- MG1655
- MM294
- MS101
- NC-7
- Nissle 1917
- One Shot STBL3
- OP50
- P678
- PA309
- PK-5
- PMC103
- PR13
- Rri
- RV308
- S17-1λ -PIR
- SCS1
- SMR10
- SOLR
- SuperchargeEZ10
- SURE
- TOP10
- TG1
- U5/41
- W208
- W945
- W1485
- W3104
- W3110
- WA704
- WP2
- X1854
- X2160T
- X2541
- X2547T
- XL1-BLUE
- XL1-BLUE-MRF
- XLOLR
- Y10
- Y1090 (1090)
- YN2980
- W3110
- WG1
- WG439
- WG443
- WG445

The Office of Biohazard Containment and Safety (BCS) of the Canadian Food Inspection Agency (CFIA) only issues import permits for microorganisms that are pathogenic to animals, or parts of microorganisms that are pathogenic to animals. As the products listed above are not considered pathogenic to animals, the Office of BCS does not have any regulatory requirements for their importation.

Please note that other legislation may apply. You may wish to contact the Public Health Agency of Canada's (PHAC) Office of Laboratory Security at (613) 957-1779.

Note: Microorganisms pathogenic to animals and veterinary biologics require an import permit from the CFIA.

Sincerely,

Cinthia Labrie
Head, Animal Pathogen Importation Program
Office of Biohazard Containment & Safety

Canada

Cancer Immunol Immunother (2008) 57:1–17
DOI 10.1007/s00262-007-0365-5

REVIEW

Anti-cancer therapies targeting the tumor stroma

Valeska Hofmeister · David Schrama · Jürgen C. Becker

Received: 22 December 2006 / Accepted: 5 February 2007 / Published online: 27 July 2007

Abstract For anti-tumor therapy different strategies have been employed, e.g., radiotherapy, chemotherapy, or immunotherapy. Notably, these approaches do not only address the tumor cells themselves, but also the tumor stroma cells, e.g., endothelial cells, fibroblasts, and macrophages. This is of advantage, since these cells actively contribute to the proliferative and invasive behavior of the tumor cells via secretion of growth factors, angiogenic factors, cytokines, and proteolytic enzymes. In addition, tumor stroma cells take part in immune evasion mechanisms of cancer. Thus, approaches targeting the tumor stroma attract increasing attention as anti-cancer therapy. Several molecules including growth factors (e.g., VEGF, CTGF), growth factor receptors (CD105, VEGFRs), adhesion molecules ($\alpha v\beta 3$ integrin), and enzymes (CAIX, FAPα, MMPs, PSMA, uPA) are induced or upregulated in the tumor microenvironment which are otherwise characterized by a restricted expression pattern in differentiated tissues. Consequently, these molecules can be targeted by inhibitors as well as by active and passive immunotherapy to treat cancer. Here we discuss the results of these approaches tested in preclinical models and clinical trials.

Keywords Angiogenesis · Endothelial cells · Extra-cellular matrix · Fibroblasts · Invasion · Therapy

Abbreviations

CAIX	Carbonic anhydrase IX
CAF	Cancer-associated fibroblast
CTGF	Connective tissue growth factor
DPPIV	Dipeptidyl peptidase IV
ECM	Extra-cellular matrix
FAPα	Fibroblast activation protein α
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
(N)SCL(C)	(Non) small cell lung (cancer)
PAGRIT	Pretargeted antibody guided radioimmunotherapy
PSMA	Prostate-specific membrane antigen
SIP	Small immunoprotein format
TAM	Tumor-associated macrophage
TEC	Tumor endothelial cell
TEM	Tumor endothelial marker
TIMP	Tissue inhibitor of metalloproteinases
uPA(R)	Urokinase plasminogen activator (receptor)
VEGF(R)	Vascular endohelial growth factor (receptor)

V. Hofmeister · D. Schrama · J. C. Becker (✉)
Department of Dermatology, Julius-Maximilians-University of Wuerzburg, Josef-Schneider-Str. 2, 97080 Wuerzburg, Germany
e-mail: Becker_JC@klinik.uni-wuerzburg.de

Introduction

The development and progression of cancer depends on genetic and epigenetic alterations in the transformed cells. However, many steps in cancerogenesis, e.g., proliferation, invasion, angiogenesis, and metastasis are promoted by microenvironmental factors such as growth factors and proteolytic enzymes produced by stromal cells (Fig. 1). Indeed, the reciprocal interactions between tumor and tumor stroma cells, i.e., cancer-associated fibroblasts (CAFs), tumor endothelial cells (TECs), and tumor-associated macrophages (TAMs), result in tumor progression. CAFs are reactive fibroblasts with a distinctive phenotype as compared to quiescent fibroblasts in differentiated adult

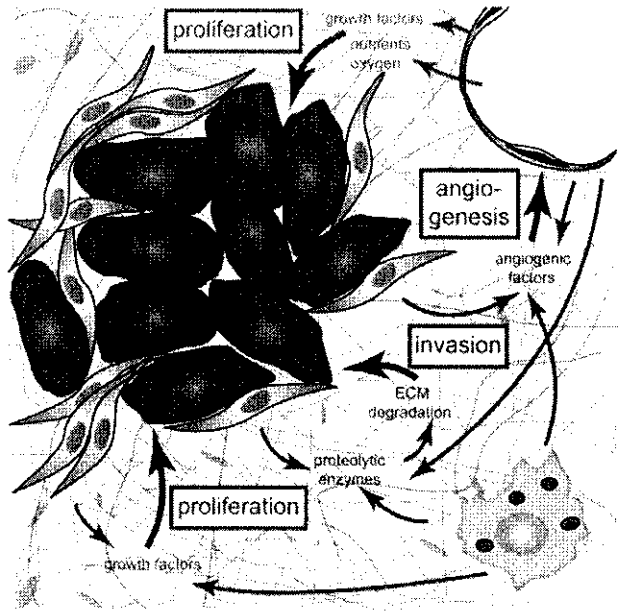

Fig. 1 Influence of the tumor microenvironment on tumor development. Tumor stromal cells, e.g., cancer associated fibroblasts (CAFs), tumor endothelial cells (TECs), and tumor-associated macrophages (TAMs) express growth factors sustaining tumor growth, angiogenic factors promoting angiogenesis, and proteolytic enzymes catalyzing the degradation of the ECM facilitating tumor cell invasion and finally metastasis. Tumor cells are depicted in *brown*, CAFs in *orange*, TECs in *red*, and TAMs in *yellow*

tissue. They are present in the close vicinity of tumor cells and enhance tumor growth by secreting growth factors such as transforming growth factor β (TGF-β), matrix degrading enzymes, e.g., matrix metalloproteinases (MMPs), and angiogenic factors, like vascular endothelial growth factor (VEGF). TECs also support the neoplastic cells by production of growth factors, but more importantly they are mandatory for tumor hem- and lymphangiogenesis. TAMs represent a major component of the leukocyte infiltrate in solid tumors. They secrete both growth factors and proteolytic enzymes, moreover, they generate an immune privileged state of the tumor microenvironment. Thus, both tumor stromal cells and their products are promising targets for cancer therapy.

Tumor stroma cells differ from their normal counterparts by upregulation or induction of various molecules (Table 1). Their upregulation is selective for the tumor microenvironment and occurs in a broad spectrum of solid tumors. Corresponding to the various interactions of tumor and stroma cells anti-stromal therapies fall into different categories. To date primarily molecules contributing to angiogenesis, e.g., vascular endothelial growth factor (VEGF) and its receptors have been targeted. Moreover, proteins involved in remodeling of the extra-cellular matrix (ECM) (e.g., MMPs, uPA/PAR system) have been silenced or functionally inhibited. However, due to redundancy of signals the inhibition of one factor or even a group of molecules is often not sufficient to significantly influence tumor growth and progression. Therefore, alternative strategies aim at modulating the tumor microenvironment by eliminating the stromal cells, e.g., by antibodies or by cytotoxic effector cells. Besides targeting stroma cells immunotherapeutically [41], new strategies are currently in preclinical or clinical testing. In the following sections we describe a selection of these approaches for the treatment of cancer (Table 2).

Strategies to prevent tumor angiogenesis

Angiogenesis, the development of new blood vessels, plays a central role in the pathogenesis of cancer. It is crucial for maintaining the supply of oxygen and nutrients and the removal of waste products to support tumor growth beyond a few mm^3 (Fig. 1). Consequently, TECs play a major role in tumor progression. Their growth is sustained by autocrine and paracrine secretion of growth factors by stroma and tumor cells, respectively. It should be further noted, that TECs are essential to maintain the oncogenic phenotype of tumor cells by production of several soluble factors. Consequently, growth and angiogenic factors, e.g., VEGF or TGF-β, and their receptors as well as TECs themselves have been targeted for cancer therapy (Fig. 2).

The potent pro-angiogenic growth factor VEGF and its tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) play a fundamental role in tumor vessel formation. VEGF is abundantly expressed in most tumors due to hypoxic conditions, acidic pH, hypoglycemia and several inducing cytokines, as well as genetic and epigenetic changes in tumor cells. Its expression, however, is not restricted to the tumor cells. VEGF is also expressed in CAFs, TECs, TAMs, and the inflammatory infiltrate (Table 1). Its expression is associated with increased angiogenesis, tumor growth, invasion, metastasis, and a greater risk of recurrence. Hypoxia and increased VEGF levels enhance expression of VEGFR-1 and VEGFR-2 on the vascular endothelium of the tumor, but both receptors are also expressed on tumor cells such as melanoma, thyroid, ovarian, breast carcinoma or cutaneous T cell lymphoma. In consequence, several VEGF- and VEGFR-antagonists have been developed and several of these inhibitors have already entered the clinic (Table 2; Fig. 2a).

In this regard the most advanced therapy consists of the recombinant humanized anti-VEGF mab Bevacizumab. It neutralizes VEGF, thereby blocking signal transduction mediated by VEGFR-1 and -2. Indeed, more then 200 clinical studies with Bevacizumab are currently being initiated or in progress (http://www.clinicaltrials.gov).

Table 1 Target molecules expressed by tumor stroma cells

Target	Expression		Function
	Tumors	Cells	
VEGF	Universal (e.g., breast, colorectal, renal cell, NSCL, hepatocellular cancer)	CAFs, TAMs, inflammatory infiltrate, tumor cells	Angiogenic factor
VEGFR-1 (Flt-1), VEGFR-2 (KDR, Flk-1)	Melanoma, thyroid, ovarian, breast carcinoma, cutaneous T cell lymphoma	TECs, tumor cells	VEGF receptors
CD105 (endoglin)	Breast, prostate, gastric, colorectal, renal cell, cervix, and endometrial carcinoma, melanoma, glioblastoma	TECs, activated monocytes, differentiated macrophages, early B cells, erythroid precursor cells, follicular DCs, fibroblasts, melanocytes, heart smooth muscle cells, trophoblasts	Accessory TGF-β superfamily receptor
PSMA	Prostate, breast, renal cell, bladder, NSCL, and rectal carcinoma, glioblastoma multiforme, melanoma, soft tissue sarcoma	TECs, tumor cells (prostate cancer), epithelia of prostate, urinary bladder, esophagus, stomach, large and small intestine, colon, kidney tubules, and liver	Glutamate carboxypeptidase
TEM8 (ATR)	Breast, esophagus, lung, bladder, and colorectal cancer	TECs, colorectal cancer cells	Adhesion molecule
CTGF (CCN2)	Glioblastoma, prostate, mammary carcinoma, pancreatic adenocarcinoma, lung adenocarcinoma, NSCL cancer, esophageal cancer, breast carcinoma, melanoma, chondrosarcoma, oral squamous cell cancer, acute lymphoblastic leukemia, rhabdomyosarcoma, hepatocellular carcinoma/hepatoma, colorectal cancer	CAFs, TECs, vascular smooth muscle cells, cancer cells, neural, and some epithelial cell types in diverse tissues, pancreatic stellate cells	Growth factor
$\alpha v\beta 3$ integrin	Breast, prostate, renal cell cancer, glioma, and melanoma	TECs	Adhesion molecule
MMPs	Breast, prostate, gastric, colorectal, pancreatic, NSCL, SCL, ovarian cancer, melanoma	CAFs, TECs, TAMs, tumor cells	Endopeptidases
uPa	Breast, gastrointestinal, renal, prostate, ovarian cancer	CAFs, TAMs, (TECs, tumor cells)	Serine protease
uPAR (CD87)	Breast, gastrointestinal, renal, prostate, ovarian cancer	Tumor cells, (TAMs)	uPa receptor
Tenascin-C	Breast, uterus, ovaries, lung, prostate, pancreas, colon, stomach, oral squamous cell, liver, and Merkel cell carcinoma, glioma, astrocytoma, lymphoma, sarcoma, melanoma	CAFs, tumor cells	Binds to cell surface receptors (integrins, proteoglycans, cell adhesion molecules of the immunoglobulin family) and ECM components (heparin, fibronectin, and collagen)
FAPα (seprase)	Breast, gastric, colon, colorectal, gastric, pancreas, prostate, bladder, renal, head and neck, ovarial carcinoma, NSCLC melanoma	CAFs, TECs, tumor cells	Serine protease, dipeptidyl peptidase
CAIX (MN, G250)	Renal cell, colorectal, cervix, NSCL, bladder, kidney cancer	CAFs, tumor cells, normal gastric epithelium	Carbonic anhydrase

Antigens expressed on tumor stroma cells, i.e., CAFs, TECs, and TAMs, are listed with their expression pattern and function

Table 2 Targeting the tumor stroma for anti cancer therapy

Substance	Target	Structure	Mechanism of action	State	References
VEGF/VEGFR					
Bevacizumab	VEGF	Recombinant humanized anti-VEGF mab	Neutralization of VEGF	Phase II, approved by the FDA for metastatic colorectal cancer	[42, 44–46, 107]
IMC-1C11	VEGFR-2	Antibody		Clinical (phase I)	[81]
Adsflt	VEGF	Soluble VEGFR-1 receptor	Interception of VEGF	Preclinical	[53]
ExFlk.6His	VEGF	Soluble VEGFR-2	Interception of VEGF	Preclinical	[61]
VEGF-Trap	VEGF	Decoy receptor based on VEGFR-1 and VEGFR-2 fused to an Fc segment of IgG1	Interception of VEGF	Preclinical	[21]
Flt1-intraceptors	VEGF	Transfection with plasmids encoding VEGFR-1 coupled with an endoplasmatic retention signal	Intracellular interception of VEGF (reduction of secretion)	Preclinical	[96]
RPI.4610 (angiozyme)	VEGFR-1	Anti-VEGFR-1 ribozyme	downregulation of expression	Clinical (phase II)	[49] NCT00021021
CD105 (endoglin)					
SN6j	CD105	Antibody		Preclinical	[99]
c-SN6j	CD105	Humanized SN6j antibody		Preclinical	[95]
scDb EDGCD3	CD105	CD3/CD105-bispecific single-chain diabody	Recruitment of CTL to TEC	Preclinical	[54]
PSMA					
MDX-070	PSMA	Fully human antibody		Clinical (phase II)	Medarex
MLN2704	PSMA	De-immunized antibody conjugated to maytansinoid 1 (DM1)	Depolymerization of mirotubuli	Clinical (phase I/II)	[37] Millennium Pharmaceuticals Inc., NCT00052000, NCT00070837, NCT00058409
A5-PE40	PSMA	Recombinant Pseudomonas exotoxin A (PE40) single chain antibody	ADP ribosylation of eukaryotic elongation factor 2	Preclinical	[103]
PSMA ADA	PSMA	Monomethylauristatin E conjugated to a human monoclonal antibody to PSMA	Inhibition of tubulin polymerization	Peclinical	[66] Progenics Pharmaceuticals, Inc
HuJ591	PSMA	Humanized antibody, partly labeled with radioactive isotopes (e.g., 177Lu-HuJ591)	ADCC radioimmunotherapy	Clinical (phase I and II)	[71] NCT00195039
	PSMA	Nanoparticle-aptamer bioconjugate	Targeting of docetaxel to PSMA expressing cells	Preclinical	[29]
TEM8 (ATR = the anthrax-toxin-receptor)					
LeTx	TEM8 positive cells	Anthrax toxin	Targeting anthrax toxin to tumor endothelial and tumor cells	Preclinical	[15]
	TEM8 positive cells	Anthrax toxin fusion proteins	Active toxin released by cleavage by MMPs	Preclinical	[62]
CTGF					
FG-3019	CTGF	Fully human antibody	Blocking antibody	Preclinical	[5, 27]

Table 2 continued

Substance	Target	Structure	Mechanism of action	State	References
DN-9693	CTGF	Inhibitor	Prevents stabilization of CTGF mRNA	Preclinical	[51]
αvβ3 integrin					
Cilengitide (EMD 121974)	αv integrins	Cyclic RGD peptide	Inhibition	Clinical phase II	[6] Merck KgaA, NCT00112866, NCT00085254, NCT00121238, NCT00103337, NCT00077155
EMD270179 + SU5416	αv integrins	Cyclic RGD peptide + VEGFR2 antagonist	Inhibition of αv integrin and VEGFR2 signaling	Preclincial	[98]
S247	αv-integrins	Peptidomimetic	Antagonist	Preclincial	[1, 86]
HPMA-RGD4C, HPMA-RGDfK	αvβ3 integrin positive cells	^{111}In, ^{99m}Tc or ^{90}Y-labeled peptide HPMA polymer	Radiotherapy	Preclinical	[72, 73]
	αvβ3 integrin positive cells	RGD peptide coupled to immunoliposome	Delivery of combretastatin	Preclinical	[79]
αv-siRNA	αv integrins	Liposome encapsuled siRNA	Downregulation of expression	Preclinical	[10]
tTF-RGD	αv integrins positive cells	Truncated tissue factor fused to GRGDSP	Induction of thrombosis in the tumor	Preclinical	[47]
RGD-4C AAVP-HSVtk	αv integrins positive cells	Adeno-associated virus bacteriophage hybrid vector encoding HSVtk (herpex simplex virus thymidin kinase)	Targeting of HSVtk to αv-integrin positive cells, activation of prodrug GCV	Preclinical	[36]
Vitaxin MEDI-523	αvβ3 integrin	Humanized antibody	Blocking antibody	Clinical (phase I/II)	[35,78] MedImmune, Inc
Abergrin MEDI-522	αvβ3 integrin	Humanized antibody	Blocking antibody	Clinical (phase II)	[69] NCT00049712, NCT00111696, NCT00066196, NCT00284817, NCT00072930, MedImmune, Inc
MMPs					
Marimastat (BB-2516)	Broad spectrum (MMP-1, -2, -3, -7, -9, and -12)	Collagen-based, peptidomimetic hydroxamate	Inhibition of activity (mimics the structure of collagen and reversibly chelates the zinc atom in the MMP's active site)	Clinical (phase III)	[16, 17, 94, 97]
Tanomastat (BAY 12-9566)	MMP-2, -3, and -9	Non-peptidic biphenyl MMP inhibitor	Inhibition of activity	Clinical (phase III)	[39, 74, 75]
Rebimastat (BMS-275291)	Broad spectrum	Non-peptidic MMP inhibitor	Inhibition of activity	Clinical (phase III)	[59, 70, 88]
Prinomastat (AG3340)	MMP-2, -3, -9, -13, -14 collagenase III	Non-peptidic MMP inhibitor	Inhibition of activity	Clinical (phase III)	[11] Agouron Pharmaceuticals Inc
Metastat (CMT-3, Col-3)	MMP-1, -2, -8, -9, and -13	Tetracycline	Inhibition of activity	Clinical (phase II)	[26] NCT00020683
TIMPs	MMPs	Adenoviral expression of TIMPs	Inhibition of activity	preclinical	[4, 18]
Ro-28-2653	MMP-2, -9 and -14	Pyrimidine-trione derivative	Inhibition of activity	Preclinical	[2, 67]

Table 2 continued

Substance	Target	Structure	Mechanism of action	State	References
SB-3CT	MMP-2, -9	Mechanism-based inhibitor	Inhibition of activity	Preclinical	[56]
uPA/uPAR					
PAI-2	uPA	213Bi labeled PAI-2	Local radiotherapy	Preclinical	[83]
	uPA or uPAR	siRNA constructs	Downregulation of expression	Preclinical	[52, 82]
mhATF-BPTI	uPAR	Receptor binding part of uPA linked to the plasmin inhibitor BPTI (aprotinin)	Inhibition of plasmin activity	Preclinical	[58]
uPA-UT1	uPAR	uPAR binding part linked to urinary trypsin inhibitor (UT1)	Inhibition of plasmin activity	Preclinical	[50]
DTAT	uPAR positive cells	Catalytic portion of diphtheria toxin (DT) fused to amino-terminal (AT) fragment of uPA	Diphteria toxin binding to uPAR	Preclincial	[101]
	uPA/uPAR positive cells	TNFα prodrugs comprise either an uPA-selective or a dual uPA-MMP-2-specific linker	TNFα activation by uPA	Preclinical	[32]
PrAg-U2 + FP59 or PrAg-U2-R200A + PrAg-L1-I210A + FP59	uPA/MMP expressing cells	Anthrax toxin lethal factor	Toxin activation by uPA or uPA and MMP	Preclinical	[63, 90]
Å6	uPA/uPAR interaction	uPA-derived peptide	Noncompetitive antagonist	Clinical (phase II)	[9] Ångstrom Pharmaceuticals, NCT00083928
Tenascin-C					
81C6	Tenascin-C	Murine ^{131}I-labeled antibody	Radioimmunotherapy	Clinical (phase I, phase II)	[85, 89] NCT00002753, NCT00002752
ST2146 and/or ST2485	Tenascin-C	^{125}I-labeled biotinylated antibody	PAGRIT (Pretargeted Antibody Guided Radioimmunotherapy)	Preclinical	[80]
TTA1	Tenascin-C	Aptamer		Preclinical	[38]
SIP (F16)	Tenascin-C	1251 labeled human SIP antibody		Preclinical	[14]
ATN-RNA	Tenascin-C	siRNA	Inhibition of expression	Clinical	[108]
FAPα (seprase)					
PT-100 (Val-boro-Pro)	FAPα, DPPIV	Small molecule inhibitor (amino boronic dipeptide)	Inhibition of activity	Clinical (phase I)	[3, 76]
Gly-Pro^{P}-$(OPh)_2$, Tyr-Pro^{P}-$(OPh)_2$	FAPα, DPPIV	Small molecule inhibitors	Inhibition of activity	Preclinical	[33]
Ac-Gly-BoroPro	FAPα	Small molecule inhibitor	Inhibition of activity	Preclinical	[28]
	FAPα	Antibody	Inhibition of activity	Preclinical	[24]
Sibrotuzumab (BIBH 1)	FAPα	Antibody (humanized F19 antibody)		Clinical (phase II)	[40, 93]
	FAPα	FAPα-CD3-bispecific single chain antibody	Recruitment of CTLs to the tumor stroma	Preclinical	[104]
	FAPα	Anti-FAPα-TNFα fusion protein	Binding of the soluble construct to the cell surface mimicks membrane-integrated TNFα signaling	Preclinical	[8]

Table 2 continued

Substance	Target	Structure	Mechanism of action	State	References
	FAPα	Human tissue factor fused with a single chain anti-FAPα antibody	Coagulation	Preclinical	[87]
sc40-FasL	FAPα	FAPα single chain antibody FasL fusion protein	Induced apoptosis of FAPα positive cells	Preclinical	[91]
CAIX (MN, G250)	CAIX (and CAXII)	Sulfonamides	Inhibition of activity	Preclinical	[7, 22, 77, 100]
Rencarex WX-G250	CAIX	Antibody	ADCC	Clinical (phase III)	[12, 13] NCT00209183, NCT00087022
cG250-131I, cG250-In111, cG250-Lu177	CAIX	Radioactive labeled anti CAIX antibody	Radioimmunotherapy	Clinical (phase I/II)	[19, 20] NCT00003102, NCT00142415
	CAIX expressing cells	Adoptive transfer of autologous T lymphocytes expressing a single-chain antibody directed to CAIX	Recruitment of T cells to CAIX expressing cells	Clinical (phase I)	[57]

A selection of cancer therapeutica targeting the tumor stroma in clinical or preclinical development is listed with their names, the substance class, their mechanism of action, and their current state of development

Bevacizumab significantly inhibits growth of different tumor types (e.g., colorectal, renal cell, NSCL cancer) already as single-agent therapy, which can be additively or synergistically enhanced by chemo- or radiotherapy [42, 107]. Hence, a combined regimen has been approved by the FDA [46]. Alternatively VEGF signaling may also be inhibited by receptor-specific antibodies. Anti-VEGFR-2 antibodies act synergistically with chemo- or radiation therapy [48, 55]. The growth of established human tumor xenografts of different origin was inhibited due to decreased angiogenesis, reduced tumor cell proliferation and enhanced tumor cell apoptosis by treatment with antibodies. The VEGFR-2 antibody IMC-1C11 is currently being tested in clinical studies [81]. Soluble receptors intercept the growth factor before it can bind to the cell surface receptor. To this end, an adenoviral vector encoding the extra-cellular domain of VEGFR-1 showed promising activity in different tumor models and a reduction of vascular density was also reached by soluble VEGFR-2 [53, 61]. A soluble receptor comprising portions of VEGFR-1 and -2 demonstrated an even higher efficacy [21]. Another approach to reduce VEGF level in vivo has been tested in corneal neovascularization but may be used in anti-tumor therapy as well; transfection with plasmids encoding VEGFR-1 coupled with an endoplasmatic retention signal reduced injury-induced VEGF secretion [96]. Other approaches investigated include VEGF antisense oligonucleotides or ribozymes. In this regard, VEGFR-1 ribozymes were well tolerated in patients and objective tumor responses were reached in combination with carboplatin and paclitaxel [49].

An endothelial cell surface molecule of particular interest for vascular targeting is CD105 (endoglin), a 95 kDa cell surface protein expressed as a homodimer. CD105 functions as an accessory protein for kinase receptor complexes of the TGF-β superfamily and modulates TGF-β signaling, i.e., it antagonizes the inhibitory effects of TGF-β1, e.g., the suppression of growth, migration and capillary tube formation. In addition, CD105 has anti-apoptotic effects under hypoxic conditions. CD105 expression is not restricted to TECs (Table 1). However, only on TECs CD105 is expressed in significant amounts. CD105 is upregulated by TGF-β2 and hypoxia. Its expression has been described for a multitude of solid tumors (Table 1). Expression of CD105 is correlated with vascular density and poor prognosis. These properties render CD105 an attractive target for therapeutic interventions (Table 2; Fig. 2a). In preclinical studies, CD105-specific antibodies—either alone or conjugated with different effector molecules—react only weakly or not at all with quiescent endothelium but specifically bind to proliferating endothelial cells during tumor angiogenesis [31]. Correspondingly, side effects were low [68]. To test

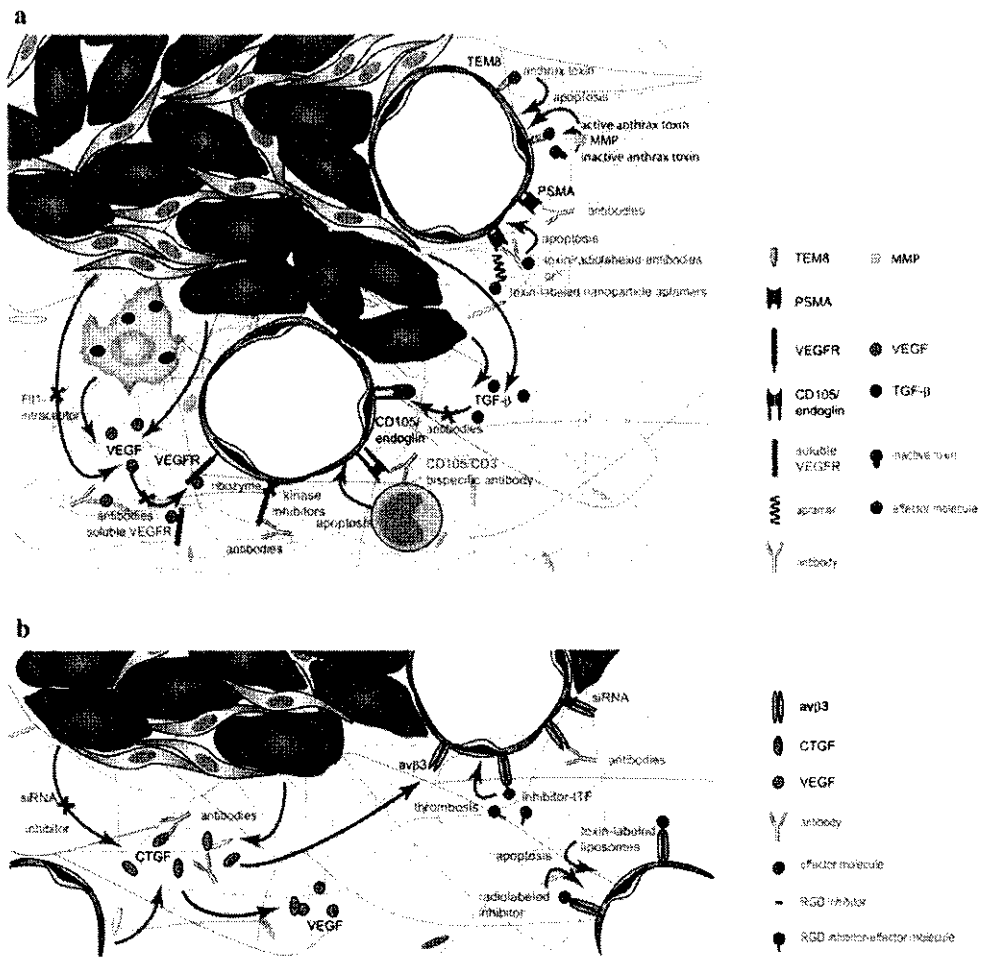

Fig. 2 Targeting endothelial cells for anti cancer therapy. A variety of proteins are targeted to prevent angiogenesis. **a** The function of molecules expressed on endothelial cells, e.g., VEGFRs, PSMA, TEM8, and CD105 is inhibited either directly by inhibitors and antibodies or alternatively their expression is prevented by ribozymes. In addition, they are exploited to eliminate tumor endothelial cells, e.g., by bispecific antibodies or specific expression or accumulation of toxins at endothelial cells. **b** CTGF is targeted by siRNA, inhibitors or antibodies. $\alpha v\beta 3$ integrin expression is prevented by siRNA, its function is inhibited by antibodies, and effector molecules are targeted to $\alpha v\beta 3$ integrin positive cells using inhibitors binding to $\alpha v\beta 3$ integrin. Expression/secretion of target molecules is indicated by *green arrows*, binding to receptors by *brown arrows*, and their effects by *black arrows*. Therapeutical substances are depicted in red and their effects by *blue arrows*

the effect of CD105-specific antibodies on human blood vessels, chimeric vasculature of human and murine origin was generated by transplantation of human foreskin onto immunodeficient mice: The growth of tumors induced in these tissues is reduced by anti-CD105 antibodies [99]. Recently, the safety of a humanized anti-CD105-antibody has been demonstrated in a primate model [95]. Lysis of endothelial cells is also achieved by retargeting CTL to CD105-positive cells by means of CD3/CD105-bispecific single-chain diabodies in vitro [54].

Prostate-specific membrane antigen (PSMA) is a 110 kDa glycoprotein with glutamate carboxypeptidase activity. PSMA is the prototype cell surface marker of prostate cancer as it is expressed on malignant prostate cancer cells. In addition, PSMA is expressed on some normal tissues (Table 1). Nevertheless, it has been widely used as therapeutic target in preclinical prostate cancer models [29, 37, 66]. PSMA is also abundantly expressed on TECs of many solid tumors (Table 1). Thus, targeting PSMA seems to be a feasible therapeutic option for different kinds of tumors apart from prostate carcinoma (Table 2; Fig. 2a). Currently, the fully human anti-PSMA antibody MDX-070 is tested in phase II trial for prostate cancer (Medarex).[1] While PSMA-specific antibodies alone often did not inhibit tumor growth, immunotoxins significantly delayed tumor progression in preclinical models [37]. A single-chain antibody fused to Pseudomonas exotoxin A specifically binds to PSMA-positive prostate cancer cells and reduces their viability [103]. Auristatin conjugated to a human monoclonal antibody to PSMA enhanced survival in a murine xenograft prostate model [66]. Furthermore, in clinical studies PSMA-specific antibodies labeled with radioactive isotopes induced objective responses [71]. Similarly, docetaxel is targeted to PSMA expressing cells by nanoparticle-aptamer bioconjugates which exhibited anticancer efficiency in a xenograft model [29].

TEM8 (ATR = the anthrax-toxin-receptor) is a transmembrane receptor belonging to the tumor endothelial markers (TEMs). It binds to the collagen subunit $\alpha 3$ (VI) but its cellular function is still unknown. TEM8 is strongly

[1] http://library.corporate-ir.net/library/63/639/63952/items/176589/6_MEDX120905_Nichol1.pdf.

expressed on vascular endothelial cells during embryo- and carcinogenesis. Its expression on endothelial cells is associated with enhanced cell–matrix-interaction and migration. TEM8 protein has been found on TECs of different cancer types but also on cancer cells themselves (Table 1). Recently, TEM8 mRNA has been detected in a cell population, coexpressing DC and endothelial markers, capable of generating functional blood vessels; thus, indicating that TEM8 has a function during vasculogenesis [25]. Since TEM8 functions as a docking protein for the Bacillus anthracis toxin, its upregulation on tumor endothelial cells has been exploited to target anthrax toxin to tumor endothelial and tumor cells [15] (Table 2). Fusion proteins that have been designed to allow the active toxin to be released by cleavage by metalloproteinases which are present in the tumor microenvironment, have demonstrated therapeutic activity, thereby illustrating the feasibility of an anti-TEM8 therapy (Fig. 2a) [62].

The connective tissue growth factor (CTGF, CCN2) is a 38 kDa extra-cellular matricellular protein that belongs to the CCN cysteine-rich family of proteins. CTGF has multiple functions. It interacts with integrin receptors, including $\alpha v\beta 3$, and several growth factors, e.g., TGF-β. In addition, it serves as biostore for angiogenic factors such as VEGF. The VEGF signaling can be restored by cleavage of CTGF by MMP. CTGF is upregulated by estrogen, TGF-β, EGF (epidermal growth factor), PDGF (platelet-derived growth factor), VEGF and hypoxia, for example. CTGF modulates cell adhesion, migration, proliferation, chemotaxis, apoptosis, ECM deposition, and angiogenesis and is important in reproduction, embryonic development, wound repair, inflammation, fibrosis disorders, and tumorigenesis. CTGF expression levels are elevated in a multitude of cancers where it is produced by several types of stromal cells including TECs, vascular smooth muscle cells, and CAFs, and can also be expressed in cancer cells (Table 1). This over-expression correlates with tumor stage and/or prognosis [105, 106]. Moreover, addition of CTGF to the medium increases proliferation and invasiveness of pancreatic cancer cells in vitro [5]. Nevertheless in some cancers such as esophageal, NSCL, and colorectal carcinoma high CTGF expression has been associated with improved survival, reduced angiogenesis, and metastasis. To this end, transfection of lung adenocarcinoma cell lines with CTGF-over-expressing vectors reduced VEGF expression, microvessel density, tumor growth, and metastasis in xenograft models [23]. As CTGF frequently is upregulated in fibrosis, CTGF mainly has been targeted in fibrotic diseases [60]. However, these approaches may also be effective in cancer. A CTGF-specific antibody, FG-3019, is currently being tested in a preclinical pancreatic cancer model (FibroGen, Fig. 2b), where it decreases tumor growth, metastasis, as well as angiogenesis and enhances the therapeutic effect of gemcitabine [5, 27]. Alternatively, stabilization of CTGF mRNA induced by VEGF can be inhibited by the angiogenesis inhibitor DN-9693 (Table 2) [51].

On TECs several integrins, e.g., $\alpha v\beta 3$ and $\alpha 5\beta 1$, are upregulated. These cell surface adhesion molecules are involved in cell growth, migration, tumor invasion, proliferation, metastasis, angiogenesis, and survival. The integrin $\alpha v\beta 3$, is minimally expressed in mature vasculature and found on new blood vessels as well as the surface of many solid tumors (Table 1). Its expression is associated with an aggressive tumor phenotype. In consequence, it represents a target for anti-cancer therapy (Table 2; Fig. 2b). Several antagonists, including antibodies, Arg–Gly–Asp (RGD) peptides, and recombinant proteins induce tumor regression by reduction of angiogenesis and enhanced apoptosis of TEC [6, 84]. The RGD peptide cilengitide, for example, is tested in several clinical phase II studies for the treatment of glioblastoma (NCT00112866, NCT00085254) and prostate cancer (NCT00121238, NCT00103337) alone or in combination with radiation therapy. Similarly, the RGD peptidomimetic S247 and the cyclic peptides RGD4C and RGDfK decrease angiogenesis, tumor growth, and metastasis and improve survival in mice [86, 98]. In addition, radiosensitivity of endothelial cells is synergistically enhanced [1]. Recently, efforts were made to improve the affinity and pharmacological properties of these antagonists, e.g., by binding them to HPMA [*N*-2-hydroxypropyl) methacrylamide] polymers thereby enhancing tumor to background ratios of the antagonist's distribution in the body [72, 73]. The RGD4C peptide has also been chosen to target αv integrin positive cells by integration of this peptide into virus capsids or liposomes. An adeno-associated virus bacteriophage hybrid vector encoding the herpex simplex thymidine kinase that activates the prodrug ganciclovir proved effective in preclinical models [36]. Alternatively, combretastatin has been targeted to tumors by liposomes linked to RGD peptides which delayed tumor growth in a melanoma model [79]. Such liposomes have also been used to deliver siRNAs resulting in reduced prostate cancer bone metastases [10]. Fusion of truncated tissue factor to an αv-targeting peptide induced thrombosis in tumor vessels, thereby inhibiting growth or inducing even regression of tumors in adenocarcinoma, melanoma and fibrosarcoma models [47].

In murine models, antibodies to $\alpha v\beta 3$ integrin block angiogenesis, tumor formation, and metastasis. In clinical studies the $\alpha v\beta 3$ inegrin-specific antibody Vitaxin (MEDI-523) proved to be safe, however, only limited efficacy was observed, e.g., disease stabilization in a leiomyosarcoma patient [35, 78]. Vitaxin is now being investigated in phase II trials in melanoma and prostate cancer (MedImmune, Inc.). The antibody Abergrin (MEDI-522), that has a

greater affinity to $\alpha v\beta 3$ integrin, was similarly well tolerated in patients and is currently being tested in prostate (NCT00072930) and colorectal cancer (NCT00284817) as well as metastatic melanoma alone or in combination therapy (NCT00111696, NCT00066196, MedImmune, Inc.) [69].

Interference with the remodeling of the extra-cellular matrix

Malignant progression of tumors is a complex process in which cells need to gain the ability to invade into surrounding tissues. For this purpose, cancer cells need to attach to, degrade and invade the ECM. These events are followed by invasion of the wall of blood or lymphatic vessels; after transport within the blood or lymph circulation, extravasation of cancer cells again involves degradation of the basement membrane and the ECM. For all these steps structural changes of the ECM are essential. Several proteases are involved in these processes including the MMPs and the urokinase plasminogen activator (uPA).

MMPs are a family of membrane-anchored and secreted zinc-dependent endopeptidases. Collectively, they are capable of degrading all ECM and basement membrane components. Their activity is important for matrix remodeling in biological processes such as embryonic development, tissue regeneration, and wound healing. In the tumor micromilieu MMPs are frequently upregulated in response to growth factors, cytokines and membrane-anchored molecules. They are produced by both tumor and tumor stroma cells, predominantly at the invasive front of the tumor (Table 1). Indeed, MMP expression correlates with an invasive phenotype of tumor cells. MMPs have been implicated in tumor growth, invasion, metastasis, angiogenesis, and cancer cell survival/apoptosis as well as the clinical course in a variety of cancer types. Thus, several approaches to inhibit MMP expression and function have been tested for cancer therapy (Table 2; Fig. 3a). A wide variety of substances has been used to block MMP activity, e.g., hydroxamates, bisphosphonates, tetracyclines, and tissue inhibitors of metalloproteinases (TIMPs). The therapeutic efficacy of synthetic MMP inhibitors has been demonstrated in vitro and in preclinical studies. Moreover, several drugs have proceeded into the clinic and even advanced to phase III clinical trials [102]. Pseudopeptidic hydroxamate inhibitors, for example, bind the zinc atom in the catalytic domain of MMPs and have broad specificities. Batimastat (BB-94), illomastat (Galardin, GM-6001) and the orally bioavailable derivative marimastat (BB-2516) inhibit the activity of many MMPs. Marimastat clinically improved progression free and overall survival of patients with advanced gastric cancer [16]. However, neither progression free nor overall survival was prolonged in metastatic breast cancer or small cell lung (SCL) cancer patients after first line chemotherapy or in pancreatic carcinoma patients in combination with gemcitabine [17, 94, 97]. The dose-limiting side effect of marimastat treatment is musculoskeletal pain. The non-peptidic biphenyl MMP inhibitor tanomastat (BAY 12-9566) has higher specificity towards MMP-2, -3, and -9. It demonstrated anti-invasive, anti-metastatic, and anti-angiogenic activity in a variety of tumor models and has also been evaluated in clinical studies. Although clinical effects were seen in combination with chemotherapy toxicity limits its usage [74]. Moreover, phase III trials revealed that tanomastat had a negative impact on progression free and overall survival in ovarian, pancreatic, and SCL cancer [39, 75]. A small molecule, broad spectrum inhibitor that lacks musculoskeletal side effects is rebimastat (BMS-275291) [88]. However, it could not demonstrate any beneficial effect if used as monotherapy for treatment of patients with breast and colorectal cancer or in combination with paclitaxel and carboplatin in non small cell lung (NSCL) cancer [59, 70, 88]. A nonpeptidic inhibitor that has also been tested in phase III clinical studies is prinomastat (AG3340). It was examined in prostate and NSCL cancer in combination with chemotherapy, but it also lacked efficacy [11]. Another group of MMP inhibitors are chemically modified tetracyclines, like metastat (CMT-3, Col-3) which inhibits several MMPs, e.g., MMP-1, -2, -8, -9, and -13. In rodent models, it inhibits malignant cell invason and angiogenesis [65]. Metastat has been examined in a phase II clinical trial in patients with Kaposi sarcoma where it reduced MMP-2 and MMP-9 plasma levels [26]. Apart from these synthetic inhibitors endogenous MMP inhibitors have also been evaluated. Under physiological conditions these TIMPs are essential for the regulation of MMP activity. TIMPs have antitumoral function since they can inhibit cell invasion, tumor growth, metastasis and angiogenesis. This notion has been confirmed by adenoviral expression of TIMPs in tumor tissues [4, 18]. However, TIMPs do not act selectively on the MMPs promoting tumor growth. Furthermore, TIMPs are associated with the upregulation of the antiapoptotic protein Bcl-X_L [43].

As described above, despite some clinical effects of MMP inhibitors, the results of clinical trials have been rather disappointing. In most studies the MMP inhibitors had no beneficial and sometimes even adverse effects [11, 17, 94, 97]. This may partly be explained by the usage of broad spectrum inhibitors and the treatment of patients without knowledge about the expression of the respective MMPs in the individual tumors. MMPs are over-expressed in most tumors, however, their expression pattern varies in each tumor type and even in metastases of the same tumor

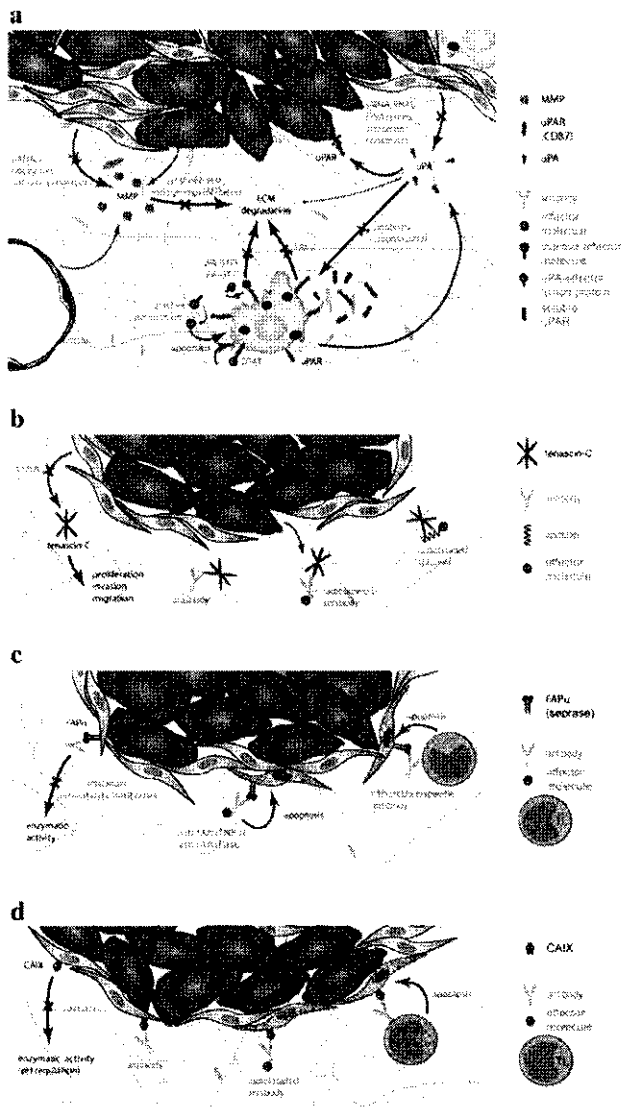

Fig. 3 Therapy approaches influencing the ECM degradation and targeting CAFs. **a** MMPs and the uPA/uPAR system influence the ECM degradation in the tumor microenvironment and promote invasion and metastasis of tumors. They are targeted by inhibiting their expression, e.g., by siRNA, antisense constructs, ribozymes or DNAzymes or their function by inhibitors, antibodies or soluble receptors, for example. In addition the uPA/uPAR system is used to induce apoptosis by recruitment or activation of toxins. **b** Tenascin expression is downregulated by siRNA approaches or targeted by antibodies or aptamers labeled with radioisotopes. Molecules expressed on the cell surface of CAFs, e.g., **c** FAPα and **d** CAIX, are also used to directly destroy these stroma cells mediated by antibodies (labeled with radioactive isotopes or different effector molecules) or cellular immune responses. Expression/secretion of target molecules is indicated by green arrows, binding to receptors by *brown arrows*, and their effects by *black arrows*. Therapeutical substances are depicted in *red* and their effects by *blue arrows*

to different organs. As broad spectrum inhibitors may have both inhibitory and promoting effects on tumor development and progression, more selective inhibitors for tumor-progression-associated MMPs have been developed. Ro-28-2653 inhibitis MMP-2, -9 and -14 and shows high anti-invasive activity in vitro. In preclinical models, it inhibits growth of MMP producing tumor cells as well as the tumor growth promoting effect of stromal cells [67]. The anti tumor effect is enhanced by combination with chemotherapy [2]. SB-3CT, a mechanism-based inhibitor that is formed only within the active site of the targeted enzymes, i.e., MMP-2 and MMP-9, inhibits liver metastases and increases survival in an aggressive murine model of T-cell lymphoma [56].

Several approaches to selectively inhibit the expression of a specific MMP have been examined, e.g., antisense constructs, RNA interference, and antisense ribozymes. However, even if single MMPs are targeted, the results are not always predictable as some MMPs may have a dual effect on tumor progression. Notably, MMPs cleave a diverse variety of substrates, resulting in opposing effects on tumor growth [30]. Indeed, activation of growth and angiogenic factors, and cleavage of FasL promote tumor development whereas the generation of angiogenesis inhibitors, e.g., angiostatin, arrestin, canstatin, endostatin, tumstatin, and ADAMTSs may inhibit tumor progression.

The serine protease urokinase plasminogen activator (uPA) converts plasminogen to plasmin which is able to degrade many ECM proteins such as collagen IV, laminin, and fibronectin either directly or through activation of other proteases. uPA, its inhibitor plasminogen activator inhibitor-1 (PAI-1), and its receptor uPAR (CD87) are involved in cell migration, tissue degradation, and angiogenesis under normal and pathological conditions. They do not only regulate ECM degradation but also cell adhesion and migration mediated by the interaction between uPAR and integrins, as well as ECM components, such as vitronectin. Over-expression of uPA and uPAR is a characteristic of various human cancers (Table 1). Whereas uPAR is particularly detected on tumor cells, uPA is mainly expressed by TAMs and CAFs, and to lesser extent on TECs. Studies in uPA knock out mice have confirmed the role of stromal uPA expression for tumor progression. Consequently, the uPA system has been addressed for anti-cancer therapy. Several approaches to interfere with the uPA system, ranging from neutralizing antibodies, soluble receptors, catalytically inactive uPA fragments, and synthetic peptides/peptidomimetics to antisense approaches, RNAi vectors, and DNAzymes have been tested to date (Table 2; Fig. 3a). The inhibitory effects of these substances have been conclusively demonstrated in preclinical models. Treatment with 213Bi labeled PAI-2 inhibits tumor growth in a pancreatic cancer xenograft model [83]. In murine tumor models intratumoral injection of small interfering RNA constructs for uPA and uPAR abrogated growth of established tumors [52, 82]. Adenoviral delivery of a chimeric protein composed of the receptor binding part of

uPA linked to the plasmin inhibitor BPTI (aprotinin) or the treatment with hybrid proteins consisting of the uPAR binding part and urinary trypsin inhibitor (UTI) also reduced tumor growth and metastases in tumor models [50, 58]. In addition, toxins or bioactive molecules binding to components of the uPA system or being activated after cleavage by uPA in the tumor microenvironment have been developed [32, 63, 90, 101]. In all these systems the selective targeting of cells expressing uPA/uPAR, was not associated with major side effects. Recently, an uPA-derived peptide, Å6, which in animal models reduced tumor growth, metastasis, and angiogenesis alone or in combination with other therapies was evaluated in a phase I clinical study in patients with gynaecologic, especially ovarian, cancer. This study demonstrated the safety of Å6. Moreover, this trial suggested some clinical potential [9]. Thus, Å6 is currently being tested in a phase II study (NCT00083928, Ångstrom Pharmaceuticals).

Tenascin-C is an extra-cellular hexameric glycoprotein expressed during embryonic development and adult tissue remodeling. Alternative splicing results in monomers of different sizes and the large isoform is virtually undetectable in differentiated tissues but is abundantly expressed in the stroma of most solid tumors (Table 1). Tenascin-C is expressed by both cancer cells and CAFs, especially at the invasive front. Tenascin-C is upregulated by hypoxia, mechanical stress, and various cytokines including TGF-β and CTGF. Tenascin-C promotes tumor growth by several mechanisms including enhanced proliferation, invasion, and migration, as well as escape from immune surveillance, and it is associated with a poor prognosis. Antibodies to tenascin-C delay tumor growth and induce apparent cures in xenograft models (Table 2; Fig. 3b). Treatment of patients with recurrent brain tumors, i.e., glioma and astrocytoma, with the I131 labeled antibody 81C6 demonstrated limited toxicity and induced a prolonged survival [85]. This antibody has also induced clinical responses in patients with non-Hodgkin lymphoma [89]. Additive tumor targeting was obtained by combining two different antitenascin antibodies [80]. In another approach, fluorescence and radiolabeled aptamers to tenascin-C were used in xenograft models for tumor imaging, suggesting that labeling of aptamer conjugates may be used to deliver radioisotopes and chemotherapeutics [38]. Affinity matured human antibodies to tenascin-C in a small immunoprotein format (scFv disulfide linked homodimer) have been generated and their specific accumulation in glioblastoma of a murine model has been demonstrated [14]. In glioblastoma multiforme and astrocytoma patients following brain resection tenascin-C specific RNA interference was applied to suppress tumor growth [108].

Fibroblast activation protein α (FAPα, seprase) is another enzyme participating in ECM degradation. FAPα is a serine protease with dual function, i.e., gelatinase/collagenase and dipeptidyl peptidase (N-terminal, post-prolyl amino peptidase) activity. Its natural substrate has not yet been identified. FAPα is expressed during embryonic development and wound healing. While it is hardly present in differentiated adult tissues, it is selectively induced on reactive stromal fibroblasts of more than 90% of common solid tumors. Besides its primary localization in fibroblasts, FAPα mRNA has also been detected in endothelial cells undergoing reorganization and capillary morphogenesis. Endothelial expression of FAPα protein in complexes with dipeptidyl peptidase IV (DPPIV) has been described in capillary-like blood vessels in breast ductal carcinoma. The biological function of FAPα in the tumor microenvironment still remains elusive. It has been suggested that FAPα functions via degradation of the ECM and/or processing of soluble factors (such as chemokines, hormones or bioactive peptides). FAPα over-expression is associated with reduced dependency on exogenous growth factors, enhanced tumor growth, invasion, angiogenesis, and metastasis. However, the role of FAPα in cancerogenesis is still controversial as in some studies a beneficial effect of FAPα expression has been demonstrated: (i) expression of FAPα in a mouse melanoma model decreased tumorigenicity, restored contact inhibition, induced cell cycle arrest and growth factor dependence; (ii) in human breast carcinoma expression of FAPα in fibroblasts is associated with longer overall and disease-free survival. Nevertheless, the differential expression of FAPα in cancer versus normal tissues makes it a promising therapeutic target (Fig. 3c). PT-100, an inhibitor of FAPα and DPPIV dipeptidyl peptidase activity, upregulates cytokine and chemokine production by stroma cells, and thereby augments the anti-tumor immune response, and it was well tolerated in a phase I clinical study [3, 76]. Other peptide inhibitors are also in preclinical development. Dipeptide proline diphenyl phosphonates exert an anti-invasive effect on the FAPα positive melanoma cell line LOX [33]. Recently, an FAPα-specific small molecule inhibitor has been developed which specifically targets FAPα but not DPPIV [28]. FAPα-directed anti-catalytic antibodies have been demonstrated to reduce the growth of FAP$^+$ tumor cells [24]. However, some of the biological effects of FAPα are mediated independently from its catalytic function as catalytic mutants still influence tumor growth. In consequence, several antibody constructs have been tested not only to inhibit the enzymatic activity of FAPα but also to mediate the destruction of FAPα^+ cells. The humanized version of the mab F19 to FAPα, Sibrotuzumab (BIBH 1) has been demonstrated to be safe and well tolerated in clinical studies [40, 93]. The ^{131}I-labeled BIBH 1 was used to characterize its biodistribution in humans, demonstrating that it selectively accumulated in primary and metastatic colorectal as well as

NSCL cancer [93]. However, the FAPα-specific antibody alone lacked therapeutic efficacy. Thus, FAPα-specific antibodies have been used to target bioactive molecules to the tumor stroma (reviewed in [92]): (i) An FAPα-CD3-bispecific single chain antibody was used to recruit CTLs to the tumor stroma [104]; (ii) TNFα was directed to FAPα expressing cells by an anti-FAPα-TNFα fusion protein; binding of the construct mimicks membrane-integrated TNFα signaling and leads to apoptosis and tissue factor production in vitro and reduced tumor growth in vivo [8]; (iii) the function of tissue factor in the coagulation cascade was also used in another approach where the human tissue factor was fused with a single chain anti-FAPα antibody; after binding to the cell surface coagulation was triggered in vitro [87]; finally (iv) a FasL fusion protein regained full activity upon cell-surface binding and induced apoptosis of FAPα positive cells in vitro and prevented growth of FAPα-positive tumor cells without systemic toxicity in vivo [91].

For the selective destruction of CAFs antigens not directly implicated in the ECM degradation may also be considered (Fig. 3d). The carbonic anhydrase IX (CAIX, MN, G250) is a marker for tumor hypoxia and is important for pH regulation. CAIX is over-expressed in CAFs of renal cell, colorectal, cervix, NSCL, bladder, and kidney cancer as well as on some malignant cells themselves (Table 1). High CAIX expression in tumors is associated with an unfavorable prognosis. It should be noted, however, that CAIX is also expressed in normal gastric epithelium. Efforts are in progress to design carbonic anhydrase inhibitors specifically binding to CAIX [77, 100]. Sulfonamide derivatives, for example show efficacy at inhibiting hypoxia-induced acidosis in vitro [7, 22]. With respect to passive immune therapy, the CAIX-specific antibody WX-G250 (Rencarex) efficiently mediated ADCC in vitro [64]. Moreover, treatment of renal cell carcinoma patients with this antibody resulted in clinical responses [12, 13]. Conjugates of this antibody with radioactive isotopes improved survival in preclinical studies, and currently are being tested in the clinic [19, 20]. Spontaneous T cell responses against CAIX are only rarely detected in cancer patients even if TIL are used for analysis [34]. Thus, in a recently initiated clinical trial the adoptive transfer of autologous T lymphocytes transduced to express a single-chain antibody to CAIX is being investigated [57].

Conclusion

Stroma cells influence tumor initiation and progression. They allow vasculo- and angiogenesis, as well as recruitment of additional stromal cells; secrete growth factors and proteolytic enzymes, and modify the ECM to make it more suitable for the tumor cells. A variety of methods to inhibit these tumor-promoting interactions of tumor and tumor stroma cells are currently being tested in preclinical and clinical studies. These approaches target one or multiple molecules to inhibit their expression, to interfere with their function or to destroy the tumor stroma cells. Several of these strategies achieved promising results in early clinical trials demonstrating that they may become an effective tool to treat cancer. In consequence, efforts are in progress to advance tumor stroma-directed therapies both to be applied by themselves or to complement conventional treatments.

Acknowledgments This work was supported by the DFG (Klinische Forschergruppe 124).

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ATCC® **Product Information**

Cell line info

Cell Line Designation: BJ
ATCC® Catalog No. CRL-2522™

Table of Contents:

Cell Line Description

Organism: *Homo sapiens* (human)
Tissue: skin; foreskin
Age: newborn
Gender: male
Morphology: fibroblast
Growth Properties: adherent
DNA profile (STR analysis)
Amelogenin: X,Y
CSF1PO: 10,12
D13S317: 8,9
D16S539: 9,13
D5S818: 12
D7S820: 11,12
TH01: 7,8
TPOX: 10,11
vWA: 16,18
Depositors: J.R. Smith
Comments: The line was established from skin taken from normal foreskin. A frozen ampule at population doubling 2.3 was received at the ATCC in April, 2000. The BJ cell line has a long lifespan in comparison with other normal human fibroblast cell lines. They have been reported to have a maximal life span of 85 to 90 population doublings [PubMed: 9454332]. These cells have a reported normal diploid karyotype at population doubling 61 but an abnormal karyotype at population doubling 82 [PubMed: 9916803]. They are telomerase negative [PubMed: 10330139]. The cells may be used for stable transfection studies.
Cells from ATCC ampules have the capacity to proliferate to a maximum of 72 population doublings before the onset of senescence.

Biosafety Level: 1

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: *Biosafety in Microbiological and Biomedical Laboratories,* 4th ed. HHS Publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office, 1999. The entire text is available online at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

Use Restrictions

These cells are distributed for research purposes only. ATCC recommends that individuals contemplating commercial use of any cell line first contact the originating investigator to negotiate an agreement. Third party distribution of this cell line is discouraged, since this practice has resulted in the unintentional spreading of cell lines contaminated with inappropriate animal cells or microbes.

Handling Procedure for Frozen Cells

To insure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at –70°C. Storage at –70°C will result in loss of viability.

SAFETY PRECAUTION: ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. *It is important to note that some vials leak when submersed in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.*

1. Thaw the vial by gentle agitation in a **37°C** water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).

2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. *All of the operations from this point on should be carried out under strict aseptic conditions.*

3. It is recommended that the cryoprotective agent be removed immediately. Centrifuge the cell suspension at approximately 125 xg for 5 to 10 minutes. Discard the supernatant and resuspend the cell pellet in an appropriate amount of fresh growth medium. to an appropriate size vessel.

4. Incubate the culture at 37°C in a suitable incubator. A 5% CO_2 in air atmosphere is recommended if using the medium described on this product sheet.

Handling Procedure for Flask Cultures

The flask was seeded with cells (see specific batch information) grown and completely filled with medium at ATCC to prevent loss of cells during shipping.

1. Upon receipt visually examine the culture for macroscopic evidence of any microbial contamination. Using an inverted microscope (preferably equipped with phase-contrast optics), carefully check for any evidence

of microbial contamination. Also check to determine if the majority of cells are still attached to the bottom of the flask; during shipping the cultures are sometimes handled roughly and many of the cells often detach and become suspended in the culture medium (but are still viable).

2. **If the cells are still attached,** aseptically remove all but 5 to 10 ml of the shipping medium. The shipping medium can be saved for reuse. Incubate the cells at 37°C in a 5% CO_2 in air atmosphere until they are ready to be subcultured.

3. **If the cells are not attached,** aseptically remove the entire contents of the flask and centrifuge at 125 xg for 5 to 10 minutes. Remove shipping medium and save. Resuspend the pelleted cells in 10 ml of this medium and add to 25 cm^2 flask. Incubate at 37°C in a 5% CO_2 in air atmosphere until cells are ready to be subcultured.

Subculturing Procedure

Volumes used in this protocol are for 75 cm2 flask; proportionally reduce or increase amount of dissociation medium for culture vessels of other sizes.

1. Remove and discard culture medium.

2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53mM EDTA solution to remove all traces of serum which contains trypsin inhibitor.

3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).

   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.

4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.

5. Add appropriate aliquots of the cell suspension to new culture vessels.
   Subcultivation Ratio: 1:2 to 1:9

6. Incubate cultures at 37°C.

Note: For more information on enzymatic dissociation and subculturing of cell lines consult Chapter 10 in ***Culture of Animal Cells, a manual of Basic Technique*** by R. Ian Freshney, 3rd edition, published by Alan R. Liss, N.Y., 1994.

Medium Renewal

Every 2 to 3 days

Complete Growth Medium

The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium:

- fetal bovine serum to a final concentration of 10%

This medium is formulated for use with a 5% CO_2 in air atmosphere.
ATCC tested fetal bovine serum is available as ATCC Catalog No. 30-2020 (500ml) and ATCC Catalog No. 30-2021 (100ml).

Cryoprotectant Medium

Complete growth medium described above supplemented with 5% (v/v) DMSO. Cell culture tested DMSO is available as ATCC Catalog No. 4-X.

Additional Information

Additional product and technical information can be obtained from the catalog references and the ATCC Web site at www.atcc.org, or by e-mail at tech@atcc.org.

References

(additional references may be available in the catalog description at **www.atcc.org**)

Yi X et al. **Both transcriptional and posttranscriptional mechanisms regulate human telomerase template RNA levels.** Mol. Cell. Biol. 19: 3989-3997, 1999 PubMed: 99262970

Bodnar AG et al. **Extension of life-span by introduction of telomerase into normal human cells.** Science 279: 349-352, 1998 PubMed: 98113956

Huffman KE et al. **Telomere shortening is proportional to the size of the G-rich telomeric 3'-overhang.** J. Biol. Chem. 275: 19712-19722, 2000 PubMed: 10787419

Morales CP et al. **Absence of cancer-associated changes in human fibroblasts immortalized with telomerase.** Nat. Genet. 21: 115-118, 1999

Hay, R. J., Caputo, J. L., and Macy, M. L., Eds. (1992), **ATCC Quality Control Methods for Cell Lines**. 2nd edition, Published by ATCC.

Caputo, J. L., **Biosafety procedures in cell culture**. J. Tissue Culture Methods 11:223-227, 1988.

Fleming, D.O., Richardson, J. H., Tulis, J.J. and Vesley, D., (1995) **Laboratory Safety: Principles and Practice**. Second edition, ASM press, Washington, DC.

ATCC Warranty

The viability of ATCC products is warranted for 30 days from the date of shipment. If you feel there is a problem with this product, contact Technical Services by phone at 800-638-6597 (U.S., Canada, and Puerto Rico) or 703-365-2700 (elsewhere) or by e-mail at tech@atcc.org.

Disclaimers

This product is intended for laboratory research purposes only. It is not intended for use in humans.

While ATCC uses reasonable efforts to include accurate and up-to-date information on this product sheet, ATCC makes no warranties or representations as to its accuracy. Citations from scientific literature and patents are provided for informational purposes only. ATCC does not warrant that such information has been confirmed to be accurate.

This product is sent with the condition that you are responsible for its safe storage, handling, and use. ATCC is not liable for any damages or injuries arising from receipt and/or use of this product. While reasonable effort is made to insure authenticity and reliability of strains on deposit, ATCC is not liable for damages arising from the misidentification or misrepresentation of cultures.

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Or contact your local distributor.

Cell Line Designation: NIH/ 3T3
ATCC Catalog No. CRL-1658™

Table of Contents:

Cell Line Description

Organism: *Mus musculus* (mouse)
Strain: NIH/Swiss
Tissue: embryo
Morphology: fibroblast
Growth properties: adherent
VirusSuscept: murine sarcoma viruses; murine leukemia viruses
Depositors: S.A. Aaronson
Comments: The NIH/3T3, a continuous cell line of highly contact-inhibited cells was established from NIH Swiss mouse embryo cultures in the same manner as the original random bred 3T3 (ATCC CCL-92™) and the inbred BALB/c 3T3 (ATCC CCL-163™). The established NIH/3T3 line was subjected to more than 5 serial cycles of subcloning in order to develop a subclone with morphologic characteristics best suited for transformation assays. These cells are useful for DNA transfection and transformation studies.
Tested and found negative for ectromelia virus (mousepox).

Biosafety Level: 1

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: *Biosafety in Microbiological and Biomedical Laboratories,* 4th ed. HHS Publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 1999. The entire text is available online at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

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1. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).

2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. *All of the operations from this point on should be carried out under strict aseptic conditions.*

3. Transfer the vial contents to a centrifuge tube containing 9.0 ml complete growth medium and spin at approximately 125 xg for 5 to 7 minutes.

4. Resuspend cell pellet with the recommended complete growth medium (see the specific batch information for the culture recommended dilution ratio) and dispense into a 25 cm^2 or a 75 cm^2 culture flask. *It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition of the vial contents, the culture vessel containing the complete growth medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0 to 7.6).*

5. Incubate the culture at 37°C in a suitable incubator. A 5% CO_2 in air atmosphere is recommended if using the medium described on this product.

Handling Procedure For Flask Cultures

The flask was seeded with cells (see specific batch information) grown and completely filled with medium at ATCC to prevent loss of cells during shipping.

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handled roughly and many of the cells often detach and become suspended in the culture medium (but are still viable).

2. **If the cells are still attached,** aseptically remove all but 5 to 10 ml of the shipping medium. The shipping medium can be saved for reuse. Incubate the cells at 37°C in a 5% CO_2 in air atmosphere until they are ready to be subcultured.

3. **If the cells are not attached,** aseptically remove the entire contents of the flask and centrifuge at 125 xg for 5 to 10 minutes. Remove shipping medium and save. Resuspend the pelleted cells in 10 ml of this medium and add to 25 cm^2 flask. Incubate at 37°C in a 5% CO_2 in air atmosphere until cells are ready to be subcultured.

Subculturing Procedure

Never allow the culture to become completely confluent. Subculture at 80% confluency or less.
Volumes used in this protocol are for 75 cm2 flask; proportionally reduce or increase amount of dissociation medium for culture vessels of other sizes.

1. Remove and discard culture medium.

2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53mM EDTA solution to remove all traces of serum which contains trypsin inhibitor.

3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 10 minutes).

Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.

4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.

5. Add appropriate aliquots of the cell supension to new culture vessels. Use 3-5 x 10(3) cells/cm^2 and subculture about every 3 days.

Note: In order to maintain this property of **high contact inhibition** it is necessary to transfer routinely at only high dilutions, otherwise variants tend to be selected having reduced contact inhibition. Such low density make culture vessels appear sparse and cell growth sensitive to sub-optimal temperature and media conditions.

6. Incubate cultures at 37°C.

Note: For more information on enzymatic dissociation and subculturing of cell lines consult Chapter 10 in ***Culture of Animal Cells, a manual of Basic Technique*** by R. Ian Freshney, 3rd edition, published by Alan R. Liss, N.Y., 1994.

Medium Renewal

Two times per week.

Complete Growth Medium

The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium:

- bovine calf serum to a final concentration of 10%

This medium is formulated for use with a 5% CO_2 in air atmosphere. (Standard DMEM formulations contain 3.7 g/L sodium bicarbonate and a 10% CO_2 in air atmosphere is then recommended).
The calf serum initially employed and found to be satisfactory was from the Colorado Serum Co. Denver.

Cryoprotectant Medium

Complete growth medium described above supplemented with 5% (v/v) DMSO.
Cell culture tested DMSO is available as ATCC Catalog No. 4-X.

Additional Information

Additional product and technical information can be obtained from the catalog references and the ATCC Web site at www.atcc.org, or by e-mail at tech@atcc.org.

References

(additional references are available in the catalog at www.atcc.org)

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ATCC®

Product Information Sheet for ATCC® CRL-6475™

Cell Line Designation: B16-F10
ATCC® Catalog No. CRL-6475™

Table of Contents:

Cell Line Description

Organism: *Mus musculus* (mouse)
Strain: C57BL/6J
Tissue: melanoma; skin
Growth properties: adherent
Morphology: mixture of spindle-shaped and epithelial-like cells
Depositors: Naval Biosciences Laboratory
Comments: Confirmed as a murine cell line by ATCC. Tests for microbial contamination were negative.

DISCLAIMER: This cell line was deposited by the Naval Biosciences Laboratory. Researchers should be aware that one purpose of the NBL bank was to produce and distribute early passage cultures of cells from various clinical materials. Many of these lines, unlike most others available and published in the ATCC catalog, are primary and mixed. Those from tumors, for example, may consist of mixtures of stromal and cancer cells in which the former cell types predominate.

Biosafety Level: 1

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: *Biosafety in Microbiological and Biomedical Laboratories,* 5th ed. HHS Publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 2007. The entire text is available online at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

Use Restrictions

These cells are distributed for research purposes only. ATCC recommends that individuals contemplating commercial use of any cell line first contact the originating investigator to negotiate an agreement. Third party distribution of this cell line is discouraged, since this practice has resulted in the unintentional spreading of cell lines contaminated with inappropriate animal cells or microbes.

Handling Procedure for Frozen Cells

To insure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at –70°C. Storage at –70°C will result in loss of viability.

SAFETY PRECAUTION: ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. *It is important to note that some vials leak when submersed in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.*

1. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).

2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. *All of the operations from this point on should be carried out under strict aseptic conditions.*

3. Transfer the vial contents to a centrifuge tube containing 9.0 ml complete culture medium and spin at approximately 125 x g for 5 to 7 minutes. Discard supernatant.

4. Resuspend the cell pellet with the recommended complete medium and dispense into a 25 cm^2 culture flask. It is important to avoid excessive alkalinity of the medium during recovery of the cells. *It is suggested that, prior to the addition of the vial contents, the culture vessel containing the complete growth medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0 to 7.6).*

5. Incubate the culture at 37°C in a suitable incubator. A 5% CO_2 in air atmosphere is recommended if using the medium described on this product sheet.

Handling Procedure for Flask Cultures

The flask was seeded with cells, grown and completely filled with medium at ATCC to prevent loss of cells during shipping.

1. Upon receipt visually examine the culture for macroscopic evidence of any microbial contamination. Using an inverted microscope (preferably equipped with phase-contrast optics), carefully check for any evidence of microbial contamination. Also check to determine if the majority of cells are still attached to the bottom of the flask; during shipping the cultures are sometimes

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handled roughly and many of the cells often detach and become suspended in the culture medium (but are still viable).

2. **If the cells are still attached,** aseptically remove all but 5 to 10 ml of the shipping medium. The shipping medium can be saved for reuse. Incubate the cells at 37°C in a 5% CO_2 in air atmosphere until they are ready to be subcultured.

3. **If the cells are not attached,** aseptically remove the entire contents of the flask and centrifuge at 125 x g for 5 to 10 minutes. Remove shipping medium and save. Resuspend the pelleted cells in 10 ml of this medium and add to 25 cm^2 flask. Incubate at 37°C in a 5% CO_2 in air atmosphere until cells are ready to be subcultured.

Subculturing Procedure

Volumes used in this protocol are for 75 cm^2 flasks; proportionally reduce or increase amount of dissociation medium for culture vessels of other sizes.

1. Remove and discard culture medium.

2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53mM EDTA solution to remove all traces of serum which contains trypsin inhibitor.

3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).

   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.

4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.

5. Add appropriate aliquots of the cell suspension to new culture vessels.
   Subculture ratio: 1:6 to 1:10

6. Incubate cultures at 37°C.

Note: For more information on enzymatic dissociation and subculturing of cell lines consult Chapter 13 in **Culture Of Animal Cells: A Manual Of Basic Technique** by R. Ian Freshney, 5th edition, published by Wiley-Liss, N.Y., 2005.

Medium Renewal

Every 2 to 3 days

Complete Growth Medium

The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium:

- fetal bovine serum to a final concentration of 10%

This medium is formulated for use with a 5% CO_2 in air atmosphere. (Standard DMEM formulations contain 3.7 g/L sodium bicarbonate and a 10% CO_2 in air atmosphere is then recommended).
ATCC tested fetal bovine serum is available as ATCC® Catalog No. 30-2020 (500ml) and ATCC® Catalog No. 30-2021 (100ml).

Cryoprotectant Medium

Complete culture medium described above supplemented with 5% (v/v) DMSO. Cell culture tested DMSO is available as ATCC® Catalog No. 4-X.

Additional Information

Additional product and technical information can be obtained from the catalog references and the ATCC Web site at www.atcc.org, or by e-mail at tech@atcc.org.

References

(Additional references may be available in the catalog description at www.atcc.org)

Fidler IJ. **Biological behavior of malignant melanoma cells correlated to their survival in vivo.** Cancer Res. 35: 218-224, 1975 PubMed: 75074100

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Please see the enclosed Material Transfer Agreement (MTA) for further details regarding the use of this product. The MTA is also available on our Web site at www.atcc.org.

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Section 4.0

Subject: Re: Biological Agents Registry Form: Leask
From: Andrew Leask <Andrew.Leask@schulich.uwo.ca>
Date: Fri, 26 Aug 2011 16:43:58 -0400
To: Jennifer Stanley <jstanle2@uwo.ca>

Hi
I am sending you a link to the use of the commercially available Santa Cruz lentivirus which are 3rd generation lentivirus. Please note that there are NO HIV sequences whatsoever in the virus used

Thanks

http://www.scbt.com/protocol_shrna_lentiviral_particles_transduction.html

http://openwetware.org/wiki/Griffin:Lentivirus_Technology#Biosafety_of_3rd_generation_Lentivrus

Biosafety of 3rd generation Lentivrus

The third generation of lentivirus vectors provides multiple safeguards against the production of replication competent lentivirus (RCL) (Dull, 1998) and are suitable for use under Biosafety Level 2 environment.

3rd generation Lentivirus Biosafety Features

- A deletion in the enhancer of the U3 region of 3'ΔLTR ensures self-inactivation of the lentiviral construct after transduction and integration into genomic DNA of the target cells. Long terminal repeats encompass the cassettes that incorporate into the host genome. The 5' and 3' LTR's serve to promote transcription and polyadenylation of the virion RNA's. The LTR contains all other cis-acting sequences necessary for viral replication. Deletion in the enhancer of U3 region of 3'LTR ensures self-inactivation of lentiviral construct after transduction and integration into genomic DNA of the target cells.
- The RSV promoter (in HIV-based vectors) and the CMV promoter (in FIV-based vectors) upstream of 5'LTR in the lentivector allow efficient Tat-independent production of viral RNA, reducing the number of genes from HIV-1 that are used in this system. For third generation lentivirus, the trans-acting function of Tat becomes dispensable if part of the upstream LTR in the transfer vector construct is replaced by constitutively active promoter sequences.
- The number of lentiviral genes necessary for packaging, replication and transduction is reduced to three (gag, pol, rev).
- The corresponding proteins are expressed from different plasmids (for HIV-based packaging plasmids) that lack packaging signals. The packaging plasmids share no significant homology to any of the expression lentivectors, the pVSV-G expression vector, or any other vector, to prevent generation of recombinant replication competent virus.
- None of the HIV-1 genes (gag, pol, rev) are present in the packaged viral genome, as they are expressed from separate plasmids lacking packaging signal. Therefore, the lentiviral particles generated are replication-incompetent. The genetic elements are split into four plasmids.
- Produced pseudoviral particles will carry only a copy of the clone-in construct.

Production of RCV can only be the result of four unlikely events: recombination of four plasmids and reconstitution

of the U3 LTR promoter activity. Since the probability of the generation of an RCV during vector production is excessively low, vector batches would be contaminated with low number of RCV particles if any (Escarpe, 2003). Rodents are dead end hosts for such RCV: viruses could enter into cells but not produce any progeny in vivo. Infectious virus production by cells from HIV-transgenic mice was documented only ex vivo under special conditions (Wang, 2003). RCV amplification in rodents is therefore highly unlikely.

A system incorporating all these safeguards can be seen as safe and is usually classified as BSL2.

- PMID=9765382
- Santa Cruz Biotechnology Inc.
- System Biosciences

PROTOCOLS

16: shRNA Lentiviral Particles Transduction

DAY 1

- Plate target cells in a 12-well plate 24 hours prior to viral infection.
- Add 1 ml of complete optimal medium (with serum and antibiotics) and incubate cells overnight. The cells should be approximately 50% confluent on the day of infection & (Day 2).

NOTE: It is possible to use other plate formats for transduction as well. In this case, the amount of cells should be adjusted depending on the growth area of the well or plate.

DAY 2

- Prepare a mixture of complete medium with Polybrene® (sc-134220) at a final concentration of 5 µg/ml.
- Remove media from plate wells and replace with 1 ml of this Polybrene/ media mixture per well (for 12-well plate).

NOTE: Polybrene is a polycation that neutralizes charge interactions to increase binding between the pseudoviral capsid and the cellular membrane. The optimal concentration of Polybrene depends on cell type and may need to be empirically determined (usually in the range of 2-10 µg/ml). Excessive exposure to Polybrene (>12 hr) can be toxic to some cells.

- Thaw lentiviral particles at room temperature and mix gently before use.
- Infect cells by adding the shRNA Lentiviral Particles to the culture.
- Swirl the plate gently to mix and incubate overnight. The amount of viral particles to use varies greatly depending on the characteristics of the cell line used.

NOTE: Keep thawed shRNA Lentiviral Particles on ice. Repeated freeze-thaw cycles and prolonged exposure of the particles to ambient temperatures may result in decreased viral titers.

NOTE: When transducing a shRNA lentiviral construct into a cell for the first time we suggest using several amounts of shRNA lentiviral particle stock. In addition, we recommend to include one well with cells transduced with Control shRNA Lentiviral Particles (sc-108080).

NOTE: Use copGFP Control Lentiviral Particles (sc-108084) for measuring transduction efficiency.

DAY 3

- Remove the culture medium and replace with 1 ml of complete medium (without Polybrene).
- Incubate the cells overnight.

DAY 4

- To select stable clones expressing the shRNA, split cells 1:3 to 1:5, depending on the cell type, and continue incubating for 24-48 hours in complete medium.

DAY 5-6 and forward

- Select stable clones expressing the shRNA via Puromycin dihydrochloride (sc-108071) selection.
- For puromycin selection, use an amount sufficient to kill the non-transduced cells. Puromycin concentrations ranging from 2 to 10 &mico;g/ml are usually sufficient, but a puromycin tritation is recommended when using a new cell line.

- Replace medium with fresh puromycin-containing medium every 3-4 days, until resistant colonies can be identified. Pick several colonies, expand them and assay them for stable shRNA expression.

NOTE: Resulting puromycin-resistant clones may have varying levels of shRNA expression due to the random integration of the lentiviral construct into the genome of the cell.

NOTE: For shRNA expression analysis by Western Blot, prepare cell lysate as follows:

- Wash cells once with PBS.
- Lyse cells in 100 µl of a 1:1 mixture of 2x Electrophoresis Sample Buffer (sc-24945) and RIPA Lysis Buffer (sc-24948) by gently rocking the 12-well plate or by pipetting up and down.
- Sonicate the lysate on ice if necessary.

NOTE: For shRNA expression analysis by RT-PCR, isolate RNA using the method described by P. Chomczynski and N. Sacchi (1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156-159) or a commercially available RNA isolation kit.

BIO SAFETY
Lentiviral particles can be employed in standard Biosafety Level 2 tissue culture facilities (and should be treated with the same level of caution as with any other potentially infectious reagent). Lentiviral particles are replication-incompetent and are designed to self-inactivate after transduction and integration of shRNA constructs into genomic DNA of target cells.

SANTA CRUZ BIOTECHNOLOGY, INC.

CTGF shRNA (h) Lentiviral Particles: sc-39329-V

Please note NO VIRAL PRODUCTION WILL occur

BACKGROUND

Connective tissue growth factor (CTGF, also known as hypertrophic chondrocyte-specific gene product 24 or HCS24), is a member of the CCN family of immediate early proteins, which are involved in cell proliferation, migration and matrix production. CTGF is a cysteine-rich peptide that is secreted by endothelial cells, fibroblasts, smooth muscle cells and myofibroblasts. Its expression is increased in various human and animal fibrotic diseases. Specifically, CTGF was observed to be strongly upregulated in human proliferative and fibrogenic renal disease. In addition, CTGF is a growth factor for vascular smooth muscle cells (VSMC), and it may play a similar role in promoting VSMC growth and migration *in vitro*.

REFERENCES

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3. Ehrchen, J., et al. 2001. Expression and regulation of osteopontin and connective tissue growth factor transcripts in rat anterior pituitary. J. Endocrinol. 169: 87-96.
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5. Kasaragod, A.B., et al. 2001. Connective tissue growth factor expression in pediatric myofibroblastic tumors. Pediatr. Dev. Pathol. 4: 37-45.

CHROMOSOMAL LOCATION

Genetic locus: CTGF (human) mapping to 6q23.2.

PRODUCT

CTGF shRNA (h) Lentiviral Particles is a pool of concentrated, transduction-ready viral particles containing 3 target-specific constructs that encode 19-25 nt (plus hairpin) shRNA designed to knock down gene expression. Each vial contains 200 µl frozen stock containing 1.0 x 10^6 infectious units of virus (IFU) in Dulbecco's Modified Eagle's Medium with 25 mM HEPES pH 7.3. Suitable for 10-20 transductions. Also see CTGF siRNA (h): sc-39329 and CTGF shRNA Plasmid (h): sc-39329-SH as alternate gene silencing products.

RESEARCH USE

The purchase of this product conveys to the buyer the nontransferable right to use the purchased amount of the product and all replicates and derivatives for research purposes conducted by the buyer in his laboratory only (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party, or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes.

APPLICATIONS

CTGF shRNA (h) Lentiviral Particles is recommended for the inhibition of CTGF expression in human cells.

SUPPORT REAGENTS

Control shRNA Lentiviral Particles: sc-108080. Available as 200 µl frozen viral stock containing 1.0 x 10^6 lentiviral transducing particles per milliliter; contains an shRNA construct encoding a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA.

GENE EXPRESSION MONITORING

CTGF (L-20): sc-14939 is recommended as a control antibody for monitoring of CTGF gene expression knockdown by Western Blotting (starting dilution 1:200, dilution range 1:100-1:1000) or immunofluorescence (starting dilution 1:50, dilution range 1:50-1:500).

To ensure optimal results, the following support (secondary) reagents are recommended: 1) Western Blotting: use donkey anti-goat IgG-HRP: sc-2020 (dilution range: 1:2000-1:100,000) or Cruz Marker™ compatible donkey anti-goat IgG-HRP: sc-2033 (dilution range: 1:2000-1:5000), Cruz Marker™ Molecular Weight Standards: sc-2035, TBS Blotto A Blocking Reagent: sc-2333 and Western Blotting Luminol Reagent: sc-2048. 2) Immunofluorescence: use donkey anti-goat IgG-FITC: sc-2024 (dilution range: 1:100-1:400) or donkey anti-goat IgG-TR: sc-2783 (dilution range: 1:100-1:400) with UltraCruz™ Mounting Medium: sc-24941.

RT-PCR REAGENTS

Semi-quantitative RT-PCR may be performed to monitor CTGF gene expression knockdown using RT-PCR Primer: CTGF (h)-PR: sc-39329-PR (20 µl, 596 bp). Annealing temperature for the primers should be 55-60° C and the extension temperature should be 68-72° C.

BIOSAFETY

Lentiviral particles can be employed in standard Biosafety Level 2 tissue culture facilities (and should be treated with the same level of caution as with any other potentially infectious reagent). Lentiviral particles are replication-incompetent and are designed to self-inactivate after transduction and integration of shRNA constructs into genomic DNA of target cells.

STORAGE

Store lentiviral particles at -80° C. Stable for at least one year from the date of shipment. Once thawed, particles can be stored at 4° C for up to one week. Avoid repeated freeze thaw cycles.

PROTOCOLS

See our web site at www.scbt.com or our catalog for detailed protocols and support products.

VIRUS WILL BE PURCHASED

SANTA CRUZ BIOTECHNOLOGY, INC.

Control shRNA Lentiviral Particles-A: sc-108080

BACKGROUND

RNA interference (RNAi) is one of the most exciting discoveries of the past decade in functional genomics and proteomics. While first recognized in nematodes as a response to exogenously introduced long double-stranded RNA (dsRNA), it is now clear that RNAi is utilized by most eukaryotes *in vivo* for anti-viral defense, transposon activity modulation and gene regulation, and has rapidly become an important research tool for gene silencing. Specifically, RNAi is the pathway by which short interfering RNA (siRNA) or short hairpin RNA (shRNA) are used to silence the expression of target genes. Compared to siRNA, shRNA offers advantages in silencing longevity and delivery.

Upon introduction, the shRNA plasmid DNA enters the cell where shRNA is transcribed. The shRNA is then cleaved by an RNase III-like enzyme called Dicer into small interfering RNA (siRNA), which are short RNA duplexes of 19-21 nucleotides with two nucleotide 3' overhangs on each strand. The siRNAs are then assembled into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), unwinding in the process. Activated RISCs subsequently bind to complementary transcripts by base pairing interactions between the siRNA anti-sense strand and complementary mRNA. The bound mRNA is cleaved and sequence specific degradation of mRNA results in gene silencing. In mammalian cells, introduction of long dsRNA (more than 30 nucleotides) initiates a potent anti-viral response, exemplified by nonspecific inhibition of protein synthesis and RNA degradation. The mammalian anti-viral response can be bypassed, however, by the introduction of siRNAs or shRNA plasmid DNA.

Santa Cruz Biotechnology, Inc. currently offers more than 49,000 target specific shRNA Lentiviral Particles that encode 19-25 nucleotide (plus hairpin) shRNAs that can be used to knock down protein expression in a broad variety of mammalian cell types. Our product line includes shRNA lentiviral particles designed to silence a large selection of proteins, including tumor suppressors, transcription regulators, cell cycle proteins, membrane receptors, signaling intermediates, kinases, cell adhesion proteins and proteins involved in lymphocyte signaling. In addition, for each shRNA Lentiviral Particles product, we offer an appropriate "matched" control antibody for confirmation of targeted mRNA silencing by either Western blotting or fluorescence antibody cell staining. We also offer appropriate buffers and non-targeted control shRNA Lentiviral Particles.

PRODUCT

Control shRNA Lentiviral Particles-A is a negative control for experiments using targeted shRNA Lentiviral Particle transduction; Control shRNA Lentiviral Particles encodes a scrambled shRNA sequence that will not lead to the specific degradation of any known cellular mRNA. After transduction, cells stably expressing the control shRNA may be isolated via puromycin selection. Each vial contains 200 µl shRNA lentiviral particles sufficient for 10-20 transductions.

STORAGE

Store lentiviral particles at -80° C. Stable for at least one year from the date of shipment. Once thawed, particles can be stored at 4° C for up to one week. Avoid repeated freeze thaw cycles.

BIOSAFETY

Lentiviral particles can be employed in standard Biosafety Level 2 tissue culture facilities (and should be treated with the same level of caution as with any other potentially infectious reagent). Lentiviral particles are repli-cation-incompetent and are designed to self-inactivate after transduction and integration of shRNA constructs into genomic DNA of target cells.

shRNA LENTIVIRAL PARTICLES SUPPORT REAGENTS

PRODUCT	CAT. #	DESCRIPTION	AMOUNT
Electrophoresis Sample Buffer	sc-24945	Ready-to-use reducing electrophoresis sample buffer solution for the preparation of protein samples to be separated in SDS-PAGE.	25 ml; 2X concentrate
RIPA Lysis Buffer	sc-24948	For use in mammalian cell lysis; with protease inhibitors. Available in four vials: 1X lysis buffer, PMSF, protease inhibitor cocktail and sodium orthovanadate.	50 ml
Puromycin dihydrochloride	sc-108071	Available for selection and maintenance of cells transfected with the puromycin-N-acetyl-transferase (pac) gene.	25 mg

SELECT PRODUCT CITATIONS

1. Pavlov, T.S., et al. 2010. Endothelin-1 inhibits the epithelial Na^+ channel through βPix/14-3-3/Nedd4-2. J. Am. Soc. Nephrol. 21: 833-843.
2. Fritz, R.D., et al. 2010. CNK1 is a novel Akt interaction partner that promotes cell proliferation through the Akt-FoxO signalling axis. Oncogene 29: 3575-3582.

RESEARCH USE

For research use only, not for use in diagnostic procedures.

PROTOCOLS

See our web site at www.scbt.com or our catalog for detailed protocols and support products.

JOURNAL OF VIROLOGY, Nov. 1998, p. 8463–8471
0022-538X/98/$04.00+0

Vol. 72, No. 11

A Third-Generation Lentivirus Vector with a Conditional Packaging System

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Received 1 June 1998/Accepted 21 July 1998

Vectors derived from human immunodeficiency virus (HIV) are highly efficient vehicles for in vivo gene delivery. However, their biosafety is of major concern. Here we exploit the complexity of the HIV genome to provide lentivirus vectors with novel biosafety features. In addition to the structural genes, HIV contains two regulatory genes, *tat* and *rev*, that are essential for HIV replication, and four accessory genes that encode critical virulence factors. We previously reported that the HIV type 1 accessory open reading frames are dispensable for efficient gene transduction by a lentivirus vector. We now demonstrate that the requirement for the *tat* gene can be offset by placing constitutive promoters upstream of the vector transcript. Vectors generated from constructs containing such a chimeric long terminal repeat (LTR) transduced neurons in vivo at very high efficiency, whether or not they were produced in the presence of Tat. When the *rev* gene was also deleted from the packaging construct, expression of *gag* and *pol* was strictly dependent on Rev complementation in *trans*. By the combined use of a separate nonoverlapping Rev expression plasmid and a 5′ LTR chimeric transfer construct, we achieved optimal yields of vector of high transducing efficiency (up to 10^7 transducing units [TU]/ml and 10^4 TU/ng of p24). This third-generation lentivirus vector uses only a fractional set of HIV genes: *gag*, *pol*, and *rev*. Moreover, the HIV-derived constructs, and any recombinant between them, are contingent on upstream elements and *trans* complementation for expression and thus are nonfunctional outside of the vector producer cells. This split-genome, conditional packaging system is based on existing viral sequences and acts as a built-in device against the generation of productive recombinants. While the actual biosafety of the vector will ultimately be proven in vivo, the improved design presented here should facilitate testing of lentivirus vectors.

Lentiviruses have attracted the attention of gene therapy investigators (45) for their ability to integrate into nondividing cells (8, 15, 16, 25, 26). We previously developed replication-defective vectors from the lentivirus human immunodeficiency virus (HIV) and showed that they transduce target cells independent of mitosis (32). The vectors proved highly efficient for in vivo gene delivery and achieved stable long-term expression of the transgene in several target tissues, such as the brain (5, 33), the retina (31), and the liver and muscle of adult rats (21). A major concern, however, is the biosafety of vectors derived from a highly pathogenic human virus.

The complexity of the lentivirus genome may be exploited to build novel biosafety features in the design of a retrovirus vector. In addition to the structural *gag*, *pol*, and *env* genes common to all retroviruses, HIV contains two regulatory genes, *tat* and *rev*, essential for viral replication, and four accessory genes, *vif*, *vpr*, *vpu*, and *nef*, that are not crucial for viral growth in vitro but are critical for in vivo replication and pathogenesis (27).

The Tat and Rev proteins regulate the levels of HIV gene expression at transcriptional and posttranscriptional levels, respectively. Due to the weak basal transcriptional activity of the HIV long terminal repeat (LTR), expression of the provirus initially results in small amounts of multiply spliced transcripts coding for the Tat, Rev, and Nef proteins. Tat increases dramatically HIV transcription by binding to a stem-loop structure (transactivation response element [TAR]) in the nascent RNA, thereby recruiting a cyclin-kinase complex that stimulates transcriptional elongation by the polymerase II complex (46). Once Rev reaches a threshold concentration, it promotes the cytoplasmic accumulation of unspliced and singly spliced viral transcripts, leading to the production of the late viral proteins. Rev accomplishes this effect by serving as a connector between an RNA motif (the Rev-responsive element [RRE]), found in the envelope coding region of the HIV transcript, and components of the cell nuclear export machinery. Only in the presence of Tat and Rev are the HIV structural genes expressed and new viral particles produced (27).

In a first generation of HIV-derived vectors (32), viral particles were generated by expressing the HIV type 1 (HIV-1) core proteins, enzymes, and accessory factors from heterologous transcriptional signals and the envelope of another virus, most often the G protein of the vesicular stomatitis virus (VSV G) (9) from a separate plasmid. In a second version of the system, the HIV-derived packaging component was reduced to the *gag*, *pol*, *tat*, and *rev* genes of HIV-1 (51). In either case, the vector itself carried the HIV-derived *cis*-acting sequences necessary for transcription, encapsidation, reverse transcription, and integration (2, 4, 22, 24, 29, 30, 32, 35). It thus encompassed, from the 5′ to 3′ end, the HIV 5′ LTR, the leader sequence and the 5′ splice donor site, approximately 360 bp of the *gag* gene (with the *gag* reading frame closed by a synthetic stop codon), 700 bp of the *env* gene containing the RRE and a splice acceptor site, an internal promoter (typically the immediate-early enhancer/promoter of human cytomegalovirus [CMV] or that of the phosphoglycerokinase gene [PGK]), the transgene, and the HIV 3′ LTR. Vector particles are produced by cotransfection of the three constructs in 293T cells (32). In this design, significant levels of transcription from the vector

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LTR and of accumulation of unspliced genomic RNA occur only in the presence of Tat and Rev.

Here, we demonstrate that the *trans*-acting function of Tat becomes dispensable if part of the upstream LTR in the transfer vector construct is replaced by constitutively active promoter sequences. Furthermore, we show that the expression of *rev* in *trans* allows the production of high-titer HIV-derived vector stocks from a packaging construct which contains only *gag* and *pol*. This design makes the expression of the packaging functions conditional on complementation available only in producer cells. The resulting gene delivery system, which conserves only three of the nine genes of HIV-1 and relies on four separate transcriptional units for the production of transducing particles, offers significant advantages for its predicted biosafety.

MATERIALS AND METHODS

Transfer vector constructs. pHR′CMV-LacZ and pHR′CMV-Luciferase have been described elsewhere (32). pHR2 is a lentivirus transfer vector in which the polylinker and downstream *nef* sequences up to the *Kpn*I site of pHR′ have been replaced with a *Cla*I/*Spe*I/*Sna*BI/*Sma*I/*Bam*HI/*Sac*II/*Eco*RI polylinker. pHR2 was generated by replacing the 3.7-kb *Cla*I-*Sac*I fragment of pHR′CMVlacZ with a 607-bp *Cla*I-*Sac*I fragment generated by PCR using pHR′CMVlacZ as the template with oligonucleotide primers 5′-CCATCGATGGACTAGTCCTACGTATCCCCGGGGACGGGATCCGCGGAATTCCGTTTAAGACCAATGAC-3′ and 5′-TTATAATGTCAAGGCCTCTC-3′, followed by digestion with *Cla*I and *Sac*I.

pHR2PGK-NGFR, pHR2CMV-NGFR, and pHR2MFG-NGFR are lentivirus transfer vectors in which the truncated low-affinity nerve growth factor receptor (NGFR) (6) transgenes under the control of the murine PGK, human CMV, and Moloney leukemia virus (MLV) promoters, respectively, have been inserted into the polylinker of pHR2. The pHR2PGK-NGFR transgene encodes no intron sequences, the pHR2CMV-NGFR vector includes the intron from plasmid pMD (34), and the pHR2MFG-NGFR vector contains the MLV intron from MFG-S (34).

pRRL, pRLL, pCCL, and pCLL are lentivirus transfer vectors containing chimeric Rous sarcoma virus (RSV)-HIV or CMV-HIV 5′ LTRs and vector backbones in which the simian virus 40 polyadenylation and (enhancerless) origin of replication sequences have been included downstream of the HIV 3′ LTR, replacing most of the human sequence remaining from the HIV integration site. In pRRL, the enhancer and promoter (nucleotides −233 to −1 relative to the transcriptional start site; GenBank accession no. J02342) from the U3 region of RSV are joined to the R region of the HIV-1 LTR. In pRLL, the RSV enhancer (nucleotides −233 to −50) sequences are joined to the promoter region (from position −78 relative to the transcriptional start site) of HIV-1. In pCCL, the enhancer and promoter (nucleotides −673 to −1 relative to the transcriptional start site; GenBank accession no. K03104) of CMV were joined to the R region of HIV-1. In pCLL, the CMV enhancer (nucleotides −673 to −220) was joined to the promoter region (position −78) of HIV-1. Exact sequences and details of construction are available on request.

pHR2hPGK-GFP, pCCLhPGK-GFP, pCLLhPGK-GFP, pRRLhPGK-GFP, and pRLLhPGK.GFP are lentivirus transfer vectors containing the enhanced green fluorescent protein (eGFP) (750-bp *Bam*HI-*Not*I fragment from pEGFP-1; Clontech) coding region, under the control of the human PGK promoter (nucleotides 5 to 516; GenBank accession no. M11958), inserted into the polylinker region of each parental vector. pRRLGFP was obtained by deletion of the *Nhe*I-*Bam*HI fragment containing the PGK promoter from pRRLhPGK-GFP.

pRRLhPGK.GFP.SIN-18 is a vector in which 3′ LTR sequences from −418 to −18 relative to the U3/R border have been deleted from pRLLhPGK.GFP (52).

Packaging constructs. The *tat*-defective packaging construct pCMVΔR8.93 was obtained by swapping an *Eco*RI-*Sac*I fragment from plasmid R7/pneo(−) (12) with the corresponding fragment of pCMVΔR8.91, a previously described plasmid expressing Gag, Pol, Tat, and Rev (51). This fragment has a deletion affecting the initiation codon of the *tat* gene and a frameshift created by the insertion of an *Mlu*I linker into the *Bsu*36I site as described previously. pCMVΔR8.74 is a derivative of pCMVΔR8.91 in which a 133-bp *Sac*II fragment, containing a splice donor site, has been deleted from the CMV-derived region upstream of the HIV sequences to optimize expression.

pMDLg/p is a CMV-driven expression plasmid that contains only the *gag* and *pol* coding sequences from HIV-1. First, p*kat*2Lg/p was constructed by ligating a 4.2-kb *Cla*I-*Eco*RI fragment from pCMVΔR8.74 with a 3.3-kb *Eco*RI-*Hin*dIII fragment from p*kat*2 (14) and a 0.9-kb *Hin*dIII-*Nco*I fragment from p*kat*2 along with an *Nco*I-*Cla*I linker consisting of synthetic oligonucleotides 5′-CATGGGTGCGAGAGCGTCAGTATTAAGCGGGGGAGAATTAGAT-3′ and 5′-CGATCTAATTCTCCCCCGCTTAATACTGACGCTCTCGCACC-3′. Next, pMDLg/p was constructed by inserting the 4.25-kb *Eco*RI fragment from p*kat*2Lg/p into the *Eco*RI site of pMD-2. pMD-2 is a derivative of pMD.G (34) in which the pXF3 plasmid backbone of pMD.G has been replaced with a minimal pUC plasmid backbone and the 1.6-kb VSV G-encoding *Eco*RI fragment has been removed.

pMDLg/pRRE differs from pMDLg/p by the addition of a 374-bp RRE-containing sequence from HIV-1 (HXB2) immediately downstream of the *pol* coding sequences. To generate pMDLg/pRRE, the 374-bp *Not*I-*Hin*dIII RRE-containing fragment from pHR3 was ligated into the 9.3-kb *Not*I-*Bgl*II fragment of pVL1393 (Invitrogen) along with a *Hin*dIII-*Bgl*II oligonucleotide linker consisting of synthetic oligonucleotides 5′-AGCTTCCGCGGA-3′ and 5′-GATCTCCGCGGA-3′ to generate pVL1393RRE (pHR3 was derived from pHR2 by the removal of HIV *env* coding sequences upstream of the RRE sequences in pHR2). A *Not*I site remains at the junction between the *gag* and RRE sequences. pMDLg/pRRE was then constructed by ligating the 380-bp *Eco*RI-*Sst*II fragment from pVL1393RRE with the 3.15-kb *Sst*II-*Nde*I fragment from pMD-2FIX (pMD-2FIX is a human factor IX-containing variant of pMD-2 which has an *Sst*II site at the 3′ end of the factor IX insert), the 2.25-kb *Nde*I-*Avr*II fragment from pMDLg/p, and the 3.09-kb *Avr*II-*Eco*RI fragment from pkatLg/p (14).

pRSV-Rev and pTK-Rev (generous gifts of T. Hope, Salk Institute) are *rev* cDNA-expressing plasmids in which the joined second and third exons of HIV-1 *rev* are under the transcriptional control of the RSV U3 and herpes simplex virus type 1 thymidine kinase (TK) promoters, respectively. Both expression plasmids utilize polyadenylation signal sequences from the HIV LTR in a pUC118 plasmid backbone.

Vector production and assays. Vectors were produced by transient transfection into 293T cells as previously described (33), with the following modifications. A total of 5×10^6 293T cells were seeded in 10-cm-diameter dishes 24 h prior to transfection in Iscove modified Dulbecco culture medium (JRH Biosciences) with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml) in a 5% CO_2 incubator, and the culture medium was changed 2 h prior to transfection. A total of 20 μg of plasmid DNA was used for the transfection of one dish: 3.5 μg of the envelope plasmid pMD.G, 6.5 μg of packaging plasmid, and 10 μg of transfer vector plasmid. The precipitate was formed by adding the plasmids to a final volume of 450 μl of 0.1× TE (1× TE is 10 mM Tris [pH 8.0] plus 1 mM EDTA) and 50 μl of 2.5 M $CaCl_2$, mixing well, then adding dropwise 500 μl of 2× HEPES-buffered saline (281 mM NaCl, 100 mM HEPES, 1.5 mM Na_2HPO_4 [pH 7.12]) while vortexing and immediately adding the precipitate to the cultures. The medium (10 ml) was replaced after 14 to 16 h; the conditioned medium was collected after another 24 h, cleared by low-speed centrifugation, and filtered through 0.22-μm-pore-size cellulose acetate filters. For in vitro experiments, serial dilutions of freshly harvested conditioned medium were used to infect 10^5 cells in a six-well plate in the presence of Polybrene (8 μg/ml). Viral p24 antigen concentration was determined by immunocapture (Alliance; DuPont-NEN). Vector batches were tested for the absence of replication-competent virus by monitoring p24 antigen expression in the culture medium of transduced SupT1 lymphocytes for 3 weeks. In all cases tested, p24 was undetectable (detection limit, 3 pg/ml) once the input antigen had been eliminated from the culture. Transducing activity was expressed in transducing units (TU).

Northern blot analysis. Total RNA was isolated from 1×10^7 to 2×10^7 cells harvested at confluence by using RNAsol B as suggested by the manufacturer; 10 to 20 μg of RNA was loaded per well on 1% agarose gels, using NorthernMax (Ambion, Austin, Tex.) reagents as described by the manufacturer. Transfer was to Zetabind membranes (Cuno Inc., Meridien, Conn.) by either capillary transfer or pressure blotting (Stratagene). ^{32}P-labeled probes were made by random priming.

Intracerebral injection of vectors. Twelve Fischer 344 male rats weighing approximately 220 g were obtained from Harlan Sprague-Dawley (Indianapolis, Ind.). The rats were housed with access to ad libitum food and water on a 12-h light/dark cycle and were maintained and treated in accordance with published National Institutes of Health guidelines. All surgical procedures were performed with the rats under isoflurane gas anesthesia, using aseptic procedures. After a rat was anesthetized in a sleep box, it was placed in a small animal stereotaxic device (Kopf Instruments, Tujunga, Calif.) using the earbars, which do not break the tympanic membrane. The rats were randomly divided into one control and four treatment groups. After the rats were placed in the stereotaxic frame, 2 μl of lentivirus vector concentrated by ultracentrifugation at 50,000 × *g* for 140 min at 20°C (33) in phosphate-buffered saline (PBS) was injected consecutively into the striatum in both hemispheres over 4 min at a rate of 0.5 μl/min (coordinates, AP 0.0, LAT ±3.0, DV −5.5, −4.5, −3.5 with the incisor bar set at 3.3 mm below the intra-aural line [36]), using a continuous-infusion system as described previously in detail (28). During the injection, the needle was slowly raised 1 mm in the dorsal direction every 40 s (3-mm total withdrawal). One minute after cessation of the injection, the needle was retracted an additional 1 mm and then left in place for an additional 4 min before being slowly withdrawn from the brain.

Histology. One month after vector injection, each animal was deeply anesthetized with intraperitoneal pentobarbital and perfused through the aorta with sterile PBS, followed by ice-cold 4% paraformaldehyde perfusion. The brains were removed from the skulls, postfixed in 4% paraformaldehyde by immersion for 24 h, and then transferred into a 30% sucrose–PBS solution for 3 to 4 days, until the brains sank to the bottom of their containers. The brains were then frozen on dry ice, and 40-μm-thick coronal sections were cut on a sliding microtome. Sections were collected in series in microtiter well plates that contained a glycerin-based antifreeze solution, and they were kept at −30°C until further processing. Immunocytochemistry was performed according to the general pro-

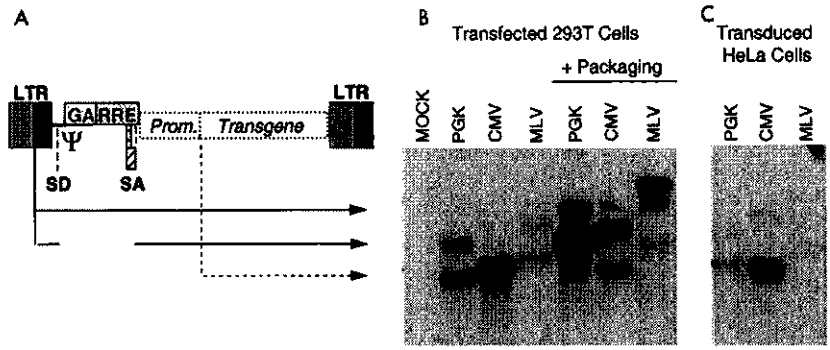

FIG. 1. Northern analysis of the RNA expression from lentivirus vectors. Three pHR2 vectors carrying an expression cassette for the same transgene (truncated low-affinity NGFR) and driven by three different promoters (PGK, CMV, and retroviral MFG) were analyzed in producer and transduced cells. Total RNA was extracted and analyzed by Northern blotting with a probe specific for the transgene sequence. (A) Schematic of the vector construct depicts the species of RNA driven by the internal promoter (Prom.; broken arrow, shorter transcript) and the viral LTR (solid arrows, longer transcripts; the two species differ for the splicing of the viral intron). The splice donor and acceptor sites (SD and SA), the packaging sequence (Ψ), the truncated *gag* sequence (GA), and the RRE are indicated. (B) The vector constructs were transfected in 293T cells without or with the packaging construct. (C) Vector particles produced by the 293T transfectants were used to transduce HeLa cells. In the absence of the viral transactivators, supplied by the core packaging construct only in the producer cells, vector expression occurs mainly from the internal promoter. Note the dramatic enhancement of the upstream transcription and the accumulation of unspliced RNA (carrying the Ψ sequence) in the presence of the packaging construct. In the transduced cells, the LTR is silenced. Note that the three expression cassettes differ in the size of the promoters and 5′ untranslated sequence. In each case, the smallest RNA species represents transcripts initiated from the internal promoter, while the intermediate-size and larger species correspond to spliced and unspliced LTR-driven RNAs, respectively.

cedure described previously (44). After several PBS rinses and an incubation in 3% hydrogen peroxide, the sections were placed in a 3% normal goat serum. The sections were then incubated in the primary anti-GFP antibody (1:1,000; Clontech, Palo Alto, Calif.) in 1% normal goat serum–0.1% Triton X-100 overnight at room temperature. After rinsing, the sections were incubated in the biotinylated rabbit anti-goat secondary antibody (Vector, Burlingame, Calif.) for 3 h. After rinsing, the sections were incubated with horseradish peroxidase-streptavidin and then reacted by using a purple chromagen kit (VIP; Vector), mounted, dried, dehydrated, and coverslipped.

RESULTS

Tat is required to produce a vector of efficient transducing activity. To investigate the role of Tat in the production of transducing particles, expression from lentivirus vectors was first examined by Northern analysis (Fig. 1). The patterns of RNAs induced by transfer vectors in which the transgene was driven by an internal PGK, CMV, or retrovirus MFG promoter were studied in both producer and target cells. In transfected 293T cells, expression occurred mainly from the internal promoter. When a packaging construct expressing both Tat and Rev was cotransfected, a dramatic enhancement of transcription from the LTR was observed, with an accumulation of unspliced vector RNA. In cells transduced with the vectors, that is, in the absence of Tat and Rev, transcription from the LTR was almost completely suppressed, the residual transcripts underwent splicing, and the internal promoter was responsible for most of the expression.

A packaging plasmid carrying two mutations in *tat* (pCMVΔR 8.93) was then constructed. The first mutation is a deletion of the T in the ATG initiation codon of the *tat* gene; the second is an insertion of a *Mlu*I linker producing a translation stop codon after residue 46 of the Tat protein. These changes confer a *tat*-defective phenotype to HIV-1 (12). After transfection of the control or *tat*-defective packaging constructs into 293T cells, comparable yields of vector particles were recovered in the culture medium, as assayed by using the Gag p24 antigen (see Table 3). Such Tat independence was expected from the replacement of the HIV LTR by the constitutive CMV promoter in the packaging construct. However, the particles produced in the absence of Tat had a dramatically reduced transducing activity (Table 1): 5 to 15% of that of particles produced by the control Tat-positive packaging construct.

We also tested whether the Tat-defective phenotype could be rescued by complementation in target cells (Table 1). HeLa-tat cells, a cell line expressing Tat from the HIV-1 LTR (13), were transduced by vectors produced with or without Tat. The expression of Tat in target cells did not compensate for the loss in transduction efficiency of vector produced without Tat.

As expected from the Northern analysis, functional inactivation of the *tat* gene resulted in a lower abundance of vector RNA in producer cells. This was indicated by the decrease in luciferase activity in cells producing a luciferase vector without an internal promoter. In this case, transgene expression di-

TABLE 1. Transducing activities of lentivirus vectors made with and without a functional *tat* gene in the packaging construct[a]

Transfer vector	Target cells	Mean transducing activity (TU/ng of p24) ± SE[b]	
		With *tat* in packaging construct	Without *tat* in packaging construct
pHR′CMV-LacZ	293T	1,056 ± 54	152 ± 26
pHR2PGK-eGFP	HeLa	5,666	384
pHR′CMV-Luciferase	HeLa	3,000 ± 152	152 ± 26
	HeLa-tat	3,777 ± 348	486 ± 59
pHR′Luciferase[c]	HeLa	46 ± 1	0.3 ± 0.003
	HeLa-tat	3,296 ± 276	174 ± 75

[a] Vectors were produced by transfection of the indicated transfer vector, a packaging construct either with (pCMVΔR8.91) or without (pCMVΔR8.93) a functional *tat* gene, and plasmid pMD.G into 293T cells. Serial dilutions of transfectant conditioned medium were incubated with the indicated cells, and the cultures were scored after 3 days. For calculating transduction activity, samples were selected from the linear portion of the vector dose-response curve.

[b] LacZ transduction was measured by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining and by expression of the number of blue cell colonies as a function of the amount of p24 antigen in the inoculum. eGFP transduction was measured by FACS analysis, multiplying the fraction of fluorescent cells by the number of infected cells, and expressing the result as a function of the amount of p24 antigen in the inoculum. Luciferase transduction was measured by luminescence in RLU above background of 50 μl of culture extract and dividing the number of RLU × 10^{-2} by the number of nanograms of p24 antigen in the inoculum. Means of duplicate (pHR2 PGK-eGFP) or triplicate (all other constructs) determinations are shown.

[c] Without internal promoter.

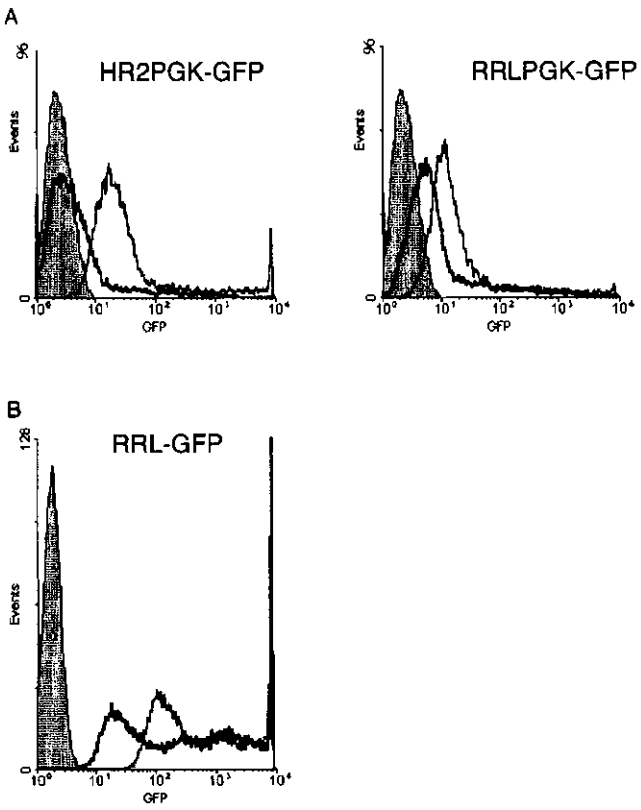

FIG. 2. Transcriptional activities of wild-type and 5′ chimeric vector constructs in the absence and presence of Tat. (A) Control pHR2 and the 5′ chimeric pRRL transfer construct carrying a PGK-eGFP expression cassette were transfected into 293T cells with a packaging construct having a functional (pCMVΔR8.91; grey line) or inactive (pCMVΔR8.93; black line) *tat* gene. GFP expression was analyzed by FACS. The filled area represents nontransfected cells. In the absence of Tat, the chimeric construct yielded a level of GFP expression higher than that achieved by the pHR2 construct. Both constructs were further upregulated by Tat. (B) A pRRL construct carrying the eGFP gene without an internal promoter was transfected with a packaging construct carrying a functional (grey line, open area) or inactive (black line, open area) *tat* gene. Direct upregulation of the chimeric promoter by Tat was observed. The filled area represents nontransfected cells.

rectly reflects the abundance of transcripts originating from the LTR. 293T cells producing luciferase vectors without Tat had only 5% of the luciferase content of cells producing the same vector with Tat ($[1.0 \pm 0.2] \times 10^9$ relative light units [RLU]/dish without Tat; $[20.2 \pm 0.7] \times 10^9$ RLU/dish with Tat). This ratio corresponded very closely to that observed in cells transduced by either type of vector in the course of the same experiment (Table 1), suggesting that the abundance of vector RNA in producer cells is a rate-limiting factor in the transduction by lentivirus vectors.

One could thus conclude that Tat is required in producer cells to activate transcription from the HIV LTR and to generate vector particles with a high transducing activity.

The *tat* requirement is offset by placing a constitutive promoter upstream of the transfer vector. If the only function of Tat is *trans* activation of vector transcription from the LTR, the *tat*-defective phenotype should be rescued by placing a strong constitutive promoter upstream of the vector transcript. Three transcriptional domains have been identified in the HIV promoter in the U3 region of the LTR: the core or basal domain, the enhancer, and the modulatory domain (27). Transcription starts at the U3/R boundary, the first nucleotide of R being numbered 1. The core promoter contains binding sites for the TATA-binding protein (−28 to −24) and SP-1 (three binding sites between −78 to −45). The enhancer contains two binding sites for NF-κB which overlap with a binding site for NFATc (−104 to −81). The modulatory domain contain binding sites for several cellular factors, including AP-1 (−350 to −293), NFAT-1 (−256 to −218), USF-1 (−166 to −161), Ets-1 (−149 to −141), and LEF (−136 to −125). A panel of 5′ chimeric transfer constructs carrying substitutions of either all or part of the U3 region of the 5′ LTR was generated. All substitutions were made to preserve the transcription initiation site of HIV. Partial substitutions joined new enhancer sequences to the core promoter of the HIV LTR (−78 to 1), while full substitutions replaced also the promoter. pRLL and pRRL vectors carried the enhancer and the enhancer/promoter, respectively, of RSV; pCLL and pCCL vectors carried the enhancer and the enhancer/promoter of human CMV.

Control pHR2 and 5′ chimeric transfer constructs carrying a PGK-eGFP expression cassette were tested by transfection of 293T cells with control or *tat*-defective packaging constructs, and the expression of the eGFP transgene was analyzed by fluorescence-activated cell sorting (FACS). The RRL chimeric construct yielded a higher level of eGFP expression than the pHR2 vector, reflecting the constitutive transcriptional activity of the new sequence (Fig. 2A). Interestingly, the chimeric vector also displayed upregulation by Tat, as shown by the increased eGFP expression of cells cotransfected with the control packaging construct. Tat upregulation was proven to be a direct effect by transfecting a pRRL-eGFP vector lacking an internal promoter with control or *tat*-defective packaging constructs and analyzing GFP expression by FACS (Fig. 2B). Comparable results were obtained with the other chimeric LTR vectors (not illustrated). Vector particles were then collected from the transfected producer cells and assayed for transduction of eGFP into HeLa cells and human primary lymphocytes (peripheral blood lymphocytes [PBL]). As shown in Table 2, all vectors had efficient transducing activity, as assessed by endpoint titration on HeLa cells or maximal transduction frequency of PBL. The vector produced by the pRRL chimera was as efficient as that produced by the pHR2 construct and was selected to test transduction independent of Tat. As shown in Table 3, the pRRL construct yielded a vector of only slightly reduced transducing activity (60%) when the packaging construct was *tat* defective. The residual effect of

TABLE 2. GFP transduction by lentivirus vectors made by transfer constructs with a wild-type or 5′ chimeric LTR

Transfer construct	Endpoint titer on HeLa cells (TU/ml)[a]	Transduction efficiency on human lymphocytes (% positive cells)[b]
pHR2	2.3×10^7	30
pCCL	4.6×10^6	14
pCLL	7.9×10^6	18
pRRL	1.8×10^7	29
pRLL	8.9×10^6	18

[a] Determined by multiplying the percentage of fluorescent cells for the vector dilution and the number of infected cells. Samples were selected from the linear portion of the vector dose-response curve.

[b] Percentage of fluorescent human PBL after infection of 10^6 cells with 1 ml of vector containing medium. Primary human T lymphocytes were isolated and transduced as previously described (14). Vectors carrying a PGK-eGFP expression cassette were produced by transfection of the indicated transfer construct, the packaging plasmid pCMVΔR8.91, and the envelope plasmid pMD.G into 293T cells. Fluorescent cells were scored by FACS analysis 6 days after transduction. Data are averages of duplicate determinations for a representative experiment of three performed.

TABLE 3. GFP transduction into HeLa cells by lentivirus vectors made by transfer constructs with a wild-type or 5′ chimeric LTR and packaging constructs with or without a functional *tat* gene[a]

Transfer construct	*tat* gene in packaging construct	Endpoint titer (TU/ml)	p24 antigen (ng/ml)	Transduction efficiency (TU/ng of p24)
pHR2	+	4.1×10^6	297	13,805
pHR2	−	2.4×10^5	545	440
pRRL	+	1.3×10^7	546	23,810
pRRL	−	4.9×10^6	344	14,244

[a] Vectors carrying a PGK-eGFP expression cassette were produced by transfection of the indicated transfer and packaging plasmid plus plasmid pMD.G into 293T cells. Serial dilutions of transfectant conditioned medium were incubated with HeLa cells, and the cultures were scored after 6 days. For calculating endpoint titers, samples were selected from the linear portion of the vector dose-response curve. Data are averages of duplicate determinations for a representative experiment of five performed.

Tat on transduction was in agreement with the ability of Tat to upregulate transcription from the chimeric LTR.

The use of the chimeric LTR construct allowed removal of Tat from the packaging system with a minimal loss in the transduction efficiency of the vector in vitro. To test vector performance in the more challenging setting of in vivo delivery into brain neurons, high-titer vector stocks were generated from the pHR2 and pRRL constructs with and without Tat. The four stocks of eGFP vector were matched for particle content by p24 antigen and injected bilaterally in the neostriata of groups of three adult rats. The animals were sacrificed after 1 month, and serial sections of the brain were analyzed for eGFP fluorescence (not shown) and immunostained by antibodies against eGFP (Fig. 3). The results obtained in vivo matched the in vitro data. Vector produced by the pHR2 construct only achieved significant transduction of the neurons when packaged in the presence of Tat. Vector produced by the pRRL chimera was as efficient when made with or without Tat. The transduction extended throughout most of the striatum and reached a very high density of positive cells in the sections closest to the injection site. No signs of pathology were detectable in the injected tissue, except for a small linear scar marking the needle track, by hematoxylin and eosin staining of the sections (data not shown).

These results provide evidence that Tat is dispensable for efficient transduction by a lentivirus vector.

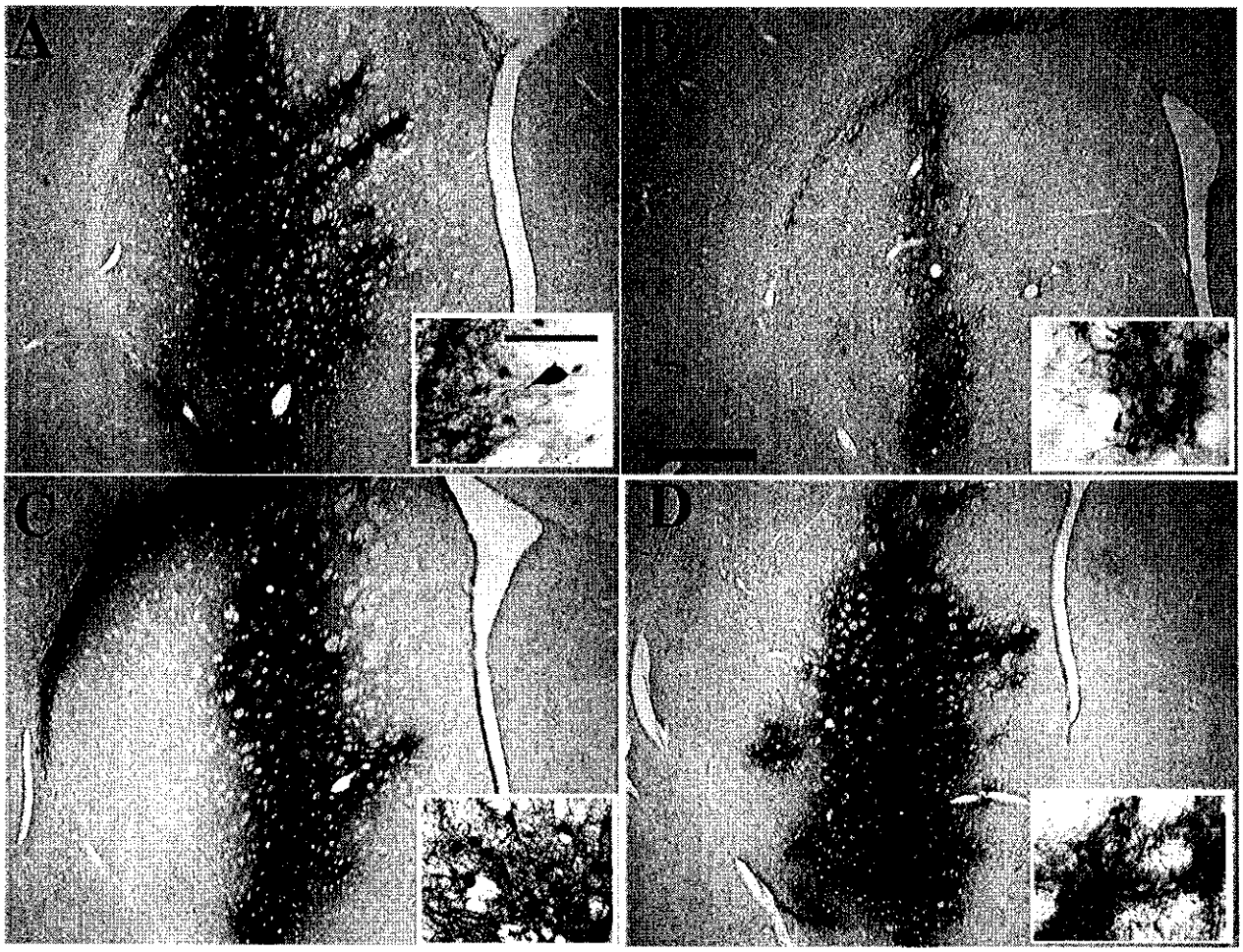

FIG. 3. In vivo transduction of eGFP into brain cells by lentivirus vectors produced with and without Tat. Vectors carrying a PGK-eGFP expression cassette were produced by the pHR2 (A and B) or the 5′ chimeric pRRL (C and D) transfer construct and a packaging construct with (pCMVΔR8.91; A and C) or without (pCMVΔR8.93; B and D) a functional *tat* gene, concentrated by ultracentrifugation, and normalized for particle content prior to injection into the corpora striata of adult rats. One month after injection, brain sections were stained for immunoreactivity to the GFP protein. While both types of vectors transduced neurons very efficiently when made with Tat, only the vector made by the chimeric transfer construct worked as well when produced without Tat. Representative sections close to the injection site are shown for one of six striata injected per each type of vector. The bar in panel B represents 1 mm; that in the inset in panel A represents 100 μm.

TABLE 4. GFP transduction into HeLa cells by lentivirus vectors made by linked or split packaging constructs and a pRRL transfer construct[a]

Packaging construct	Separate *rev* plasmid[b]	p24 antigen (ng/ml)	Endpoint titer (TU/ml)	Transduction efficiency (TU/ng of p24)
pCMVΔR8.74		364	1.07×10^7	29,436
pMDLg/pRRE		<0.1	ND	NA
pMDLg/pRRE	TK-Rev, 5 μg	29	6.9×10^5	23,793
pMDLg/pRRE	TK-Rev, 12 μg	94	2.02×10^6	21,489
pMDLg/pRRE	RSV-Rev, 2.5 μg	774	1.0×10^7	13,495
pMDLg/pRRE	RSV-Rev, 5 μg	776	7.6×10^6	9,761
pMDLg/pRRE	RSV-Rev, 12 μg	565	4.8×10^6	8,495

[a] Vectors carrying a PGK-eGFP expression cassette were produced by the transfection of a self-inactivating pRRL transfer construct (with a deletion in the 3′ LTR [53]), the indicated packaging and *rev* plasmids, and plasmid pMD.G into 293T cells. Serial dilutions of transfectant conditioned medium were incubated with HeLa cells, and the cultures were scored after 6 days. For calculating endpoint titers, samples were selected from the linear portion of the vector dose-response curve. Data are averages of duplicate determination for a representative experiment of three performed. ND, none detected (the detection limit of the assay was 10^2 TU/ml); NA, not applicable.

[b] The promoter driving the expression of a synthetic *rev* cDNA and the amount of plasmid transfected are indicated.

A new split-genome conditional packaging system. The possibility of deleting the *tat* gene prompted us to explore a new design of the packaging component of the HIV vector system, in which two separate nonoverlapping expression plasmids, one for the *gag* and *pol* genes and the other for the *rev* gene, were used. The *gag* and *pol* reading frames were expressed within the context of the MD cassette, which employs the CMV promoter and intervening sequence and the human β-globin poly(A) site (34). All HIV sequences upstream of the *gag* initiation codon were removed, and the leader was modified for optimal fit to the Kozak consensus for translation. This construct, however, expressed almost no p24 antigen when transfected alone in 293T cells. This observation is in agreement with the previously reported presence of *cis*-repressive or inhibitory sequences in the *gag* and *pol* genes (40, 41). The HIV RRE was then inserted downstream of the *pol* gene, and the resulting plasmid was cotransfected with a *rev* expression vector (Table 4). High levels of p24 antigen production were observed in this case, the highest yields being obtained when *rev* was driven by an RSV promoter. When the *gag-pol* and the *rev* constructs were cotransfected with the pRRL chimeric transfer vector and the VSV G-expressing plasmid, high-titer vector was obtained in the culture medium. Both the yield of particles and their transducing efficiency were similar to those obtained with previous versions of the system. Northern analysis of producer cells confirmed that unspliced vector genomic RNA accumulated only in the presence of Rev (data not shown). Thus, both the expression of the *gag* and *pol* genes and the accumulation of packageable vector transcripts are dependent on *trans* complementation by a separate Rev expression construct. Such a conditional packaging system provides an important safety feature unavailable to oncoretrovirus vectors.

DISCUSSION

The predicted biosafety of a viral vector depends in part on how much segregation of the *cis*- and *trans*-acting functions of the viral genome is achieved by the vector design and is maintained during vector production. A vector particle is assembled by viral proteins expressed in the producer cell from a construct(s) stripped of the *cis*-acting sequences required for the transfer of the viral genome to target cells (packaging construct). These *cis*-acting sequences are instead linked to the transgene in the transfer vector. As the vector particle packages only the genetic information contained in this latter construct, the infection process is limited to a single round without spreading. Through recombination, it is possible that sequences encoding viral proteins rejoin the *cis*-acting elements of the transfer vector. If the resulting recombinant expresses all required functions, it is able to replicate (i.e., it is a replication-competent retrovirus [RCR]) and presents a risk to the recipient. The formation of heterozygous vector particles containing RNAs from both the packaging and transfer vectors, followed by homologous recombination during reverse transcription, is the mechanism most often incriminated in the emergence of RCR during the production of retroviral vectors. The likelihood of this type of recombination is dependent on residual *cis*-acting sequences in the packaging plasmid, allowing some level of encapsidation, and on the extent of homology between packaging and vector constructs (10).

A first strategy to improve the biosafety of a vector is to use nonoverlapping split-genome packaging constructs that require multiple recombination events with the transfer vector for RCR generation. Earlier studies described several approaches to generate replication-defective HIV vectors (7, 35, 38, 42). However, these vectors could be produced only to low infectious titers, were restricted to CD4-positive cellular targets, and carried the risk of generating wild-type HIV by recombination of the components. A major advance was achieved when an improved vector design was combined with the use of the envelope of another virus (32, 33, 39). The lentivirus vector that we describe here is packaged by three nonoverlapping expression constructs, two expressing HIV proteins and the other expressing the envelope of a different virus. Moreover, all HIV sequences known to be required for encapsidation and reverse transcription (2, 22, 24, 27, 29, 30, 35) are absent from these constructs, with the exception of the portion of the *gag* gene that contributes to the stem-loop structure of the HIV-1 packaging motif (29).

A second strategy to improve vector biosafety took advantage of the complexity of the lentivirus genome. The minimal set of HIV-1 genes required to generate an efficient vector was identified, and all other HIV reading frames were eliminated from the system. As the products of the removed genes are important for the completion of the virus life cycle and for pathogenesis, no recombinant can acquire the pathogenetic features of the parental virus. We previously demonstrated that all four accessory genes of HIV could be deleted from the packaging construct without compromising gene transduction (51). In this work, we went further by deleting another factor crucial for HIV replication, the *tat* gene. Its product is one of the most powerful transcriptional activators known and plays a pivotal role in the exceedingly high replication rates that characterize HIV-induced disease (18, 19, 47).

It was found that Tat was required in producer cells to generate vector of efficient transducing activity but that this requirement was offset by inducing constitutive high-level expression of vector RNA. Due to the low basal transcription from the HIV LTR, Tat was necessary to increase the abundance of vector transcripts and allow their efficient encapsidation by the vector particles. When made in the absence of Tat, vector particles had 10- to 20-fold-reduced transducing activity. However, when strong constitutive promoters replaced the HIV sequence in the 5′ LTR of the transfer construct, vectors made without Tat exhibited a less than twofold reduction in transducing activity. As Tat strongly upregulated transcription from the chimeric LTR, the transducing activity of the output particles must reach saturation. The abundance of vector RNA

in producer cells thus appears to be a rate-limiting factor for transduction until it reaches a threshold. Conceivably, an upper limit is set by the total output of particles available to encapsidate vector RNA. As the total particle output varied with the types of vector and internal promoter used, this may explain the quantitative differences obtained in response to *tat* deletion.

Successful deletion of the *tat* gene was unexpected in view of a reported additional role for Tat in reverse transcription (17, 20). While the reasons for this discrepancy are not obvious, it should be noted that the transduction pathway of the lentivirus vector mimics only in part the infection pathway of HIV. The vector is pseudotyped by the envelope of an unrelated virus and contains only the core proteins of HIV, without any accessory gene product. The VSV envelope targets the vector to the endocytic pathway, and it has been shown that redirection of HIV-1 from its normal route of entry by fusion at the plasma membrane significantly changes the biology of the infection. For example, Nef and cyclophilin A are required for the optimal infectivity of wild-type HIV-1 but not of a (VSV G) HIV pseudotype (1). It is also possible that the kinetics of reverse transcription are more critical for the establishment of viral infection than for gene transduction, given the differences in size and sequence between the virus and vector genome.

Tat-independent transduction by an HIV-based vector was recently reported by Kim et al. for in vitro cellular targets (23). In the vector designed by these authors, however, Tat and Rev were expressed from the transfer vector and thus were also present in target cells. A CMV-HIV hybrid LTR was used; this construct yielded vector titers approximately 30% of that obtained with an intact LTR. When the *tat* gene was inactivated, the titer did not change. Srinivasakumar et al. (43) previously reported a rather low (5- to 10-fold) dependence on Tat of an HIV-based vector produced by cells stably expressing the HIV structural proteins. In this case, titers of 5×10^3 TU/ml with Tat and 7×10^2 TU/ml without Tat were obtained on HeLa-CD4 cells. Although these titers are much lower than those reported here, the vector particles carried the HIV envelope, an indication that Tat is not absolutely required for transduction by vector particles which in that case mirror more closely the wild-type virus. It remained possible, however, that a dependence on Tat may be revealed in more challenging gene deliveries into the body tissues that are the actual targets of gene therapy. This could have been due to a stricter Tat requirement for optimal transduction efficiency or for the production of high-titer vector stocks or to differences in cell-type-specific factors. Our results now establish that Tat is fully dispensable for lentivirus vector transduction even when high titers are achieved and, most importantly, for gene delivery in vivo into terminally differentiated neurons of an adult rat brain.

The Northern analysis of producer and target cells shows that the Tat dependence of LTR-driven expression restricts the production of vector genomic RNA to producer cells. This applies as well to vectors made by the 5′ chimeric constructs, as the U3 sequences of both LTRs of the resulting provirus are derived from the vector 3′ LTR. However, the functional replacement of the *tat* gene in the packaging construct by promoter sequences upstream of the transfer construct makes the generation of a transcriptionally active recombinant much more unlikely. This will be even more significant in stable producer cell lines that avoid the risk of plasmid recombination during cotransfection.

We also exploited the Rev dependence of *gag-pol* expression and of the accumulation of unspliced, packageable transcripts. Yu et al. (50) previously showed that the dependence on Rev can be used to make expression of HIV genes inducible. We

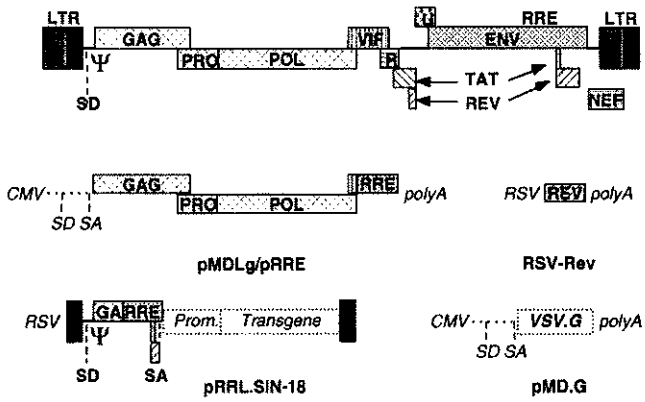

FIG. 4. Schematic drawing of the HIV provirus and the four constructs used to make a lentivirus vector of the third generation. The viral LTRs, the reading frames of the viral genes, the major 5′ splice donor site (SD), the packaging sequence (Ψ), and the RRE are boxed and indicated in bold type. The conditional packaging construct, pMDLg/pRRE, expresses the *gag* and *pol* genes from the CMV promoter and intervening sequences and polyadenylation site of the human β-globin gene. As the transcripts of the *gag* and *pol* genes contain *cis*-repressive sequences, they are expressed only if Rev promotes their nuclear export by binding to the RRE. All *tat* and *rev* exons have been deleted, and the viral sequences upstream of the *gag* gene have been replaced. A nonoverlapping construct, RSV-Rev, expresses the *rev* cDNA. The transfer construct, pRRL.SIN-18, contains HIV-1 *cis*-acting sequences and an expression cassette for the transgene. It is the only portion transferred to the target cells and does not contain wild-type copies of the HIV LTR. The 5′ LTR is chimeric, with the enhancer/promoter of RSV replacing the U3 region (RRL) to rescue the transcriptional dependence on Tat. The 3′ LTR has an almost complete deletion of the U3 region, which includes the TATA box (from nucleotides −418 to −18 relative to the U3/R border). As the latter is the template used to generate both copies of the LTR in the integrated provirus, transduction of this vector results in transcriptional inactivation of both LTRs; thus, it is a self-inactivating vector (SIN-18). The fourth construct, pMD.G, encodes a heterologous envelope to pseudotype the vector, here shown coding for VSV G. Only the relevant parts of the constructs are shown.

describe a core packaging system split in two separate nonoverlapping expression constructs, one for the *gag* and *pol* reading frames optimized for Rev-dependent expression and the other for the *rev* cDNA. This third-generation packaging system matches the performance of its predecessors in terms of both yield and transducing efficiency. However, it increases significantly the predicted biosafety of the vector. It has been suggested that the Rev-RRE axis could be replaced by the use of constitutive RNA transport elements of other viruses, although at the price of decreased efficiency (11, 23, 43). We would suggest that maintaining the Rev dependence of the system allows for an additional level of biosafety through the splitting of the HIV-derived components of the packaging system.

The conditional packaging system described here can be combined with a self-inactivating vector construct carrying a major deletion in the 3′ LTR (52). This vector design (Fig. 4) offers significant biosafety features. The contribution of HIV is reduced to a fraction of *cis*-acting sequences in the vector, leaving out in particular most of the LTR, and to only three genes, *gag*, *pol*, and *rev*, in the packaging constructs, compared with the nine genes necessary for the in vivo replication and pathogenesis of wild-type HIV-1 (3, 18, 27, 49). The actual biosafety of a vector must be proven in vivo. However, given the serious limitations of the available animal models of HIV-induced disease, the biosafety of HIV-derived vectors will ultimately be proven only in human hosts. Therefore, the vector design must ensure the highest predictable biosafety for clinical testing to be acceptable.

It is noteworthy that the fraction of the HIV-1 genome that is left in the vector is probably smaller than could be achieved

with any of the nonprimate lentiviruses, the genomic complexity of which is lower than that of HIV-1 (37). Also, the risks associated with the introduction in humans of a recombinant arising from a nonprimate lentivirus, even in a form that in its cognate animal species appears to be attenuated, are very difficult to assess, as illustrated by the ongoing debate on xenotransplantation (48). In contrast, the almost two decades spent studying a virus that has now spread in tens of millions of people worldwide have revealed a considerable amount of information on the pathogenic features of HIV-1, in particular on the dependence of virulence on a crucial set of viral genes. Based on these data, we would like to suggest that the HIV-based vectors described here are good candidates for the clinical trial of lentivirus vectors in human gene therapy.

ACKNOWLEDGMENTS

We are indebted to Tom Hope for providing the Rev expression plasmids, to Melinda Van Roey and Heidi Oline for help with the animal experiments, and to Jennifer Davis and Mitch Finer for suggestions and critical reading of the manuscript.

This work was partly supported by a grant and by a fellowship from the Swiss National Science Foundation to D.T. and R.Z., respectively.

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