2002

A Wasted Year?

knockout (nɔkaut)

knowledge, knob, knee

Check the American Heritage Dictionary

Stuff That I Managed to Do

- VEGF-A/VEGF-C mosaic project
- New polyclonal rabbit antisera
- New recombinant baculoviruses & proteins
- 17 credits & PhD thesis
- Waste my time doing things that our computing support person should do

Ongoing Projects Part 1

- Polyclonal antiserum against mouse LYVE-1
- Monoclonal antibodies against human VEGF-C
- Recombinant baculoviruses for direct infection of the chorioallantoic membrane (CAM)
- E. coli protein expression, refolding & crystallization
- Mouse & Chick VEGFR-1

Ongoing Projects Part 2

- Identification of KDR domains necessary for VEGF-C binding
- Several new keratin-14 transgenic mouse lines
- Efficiently organizing -70°C space

VEGF-A/VEGF-C
Mosaic Project

Figure 1

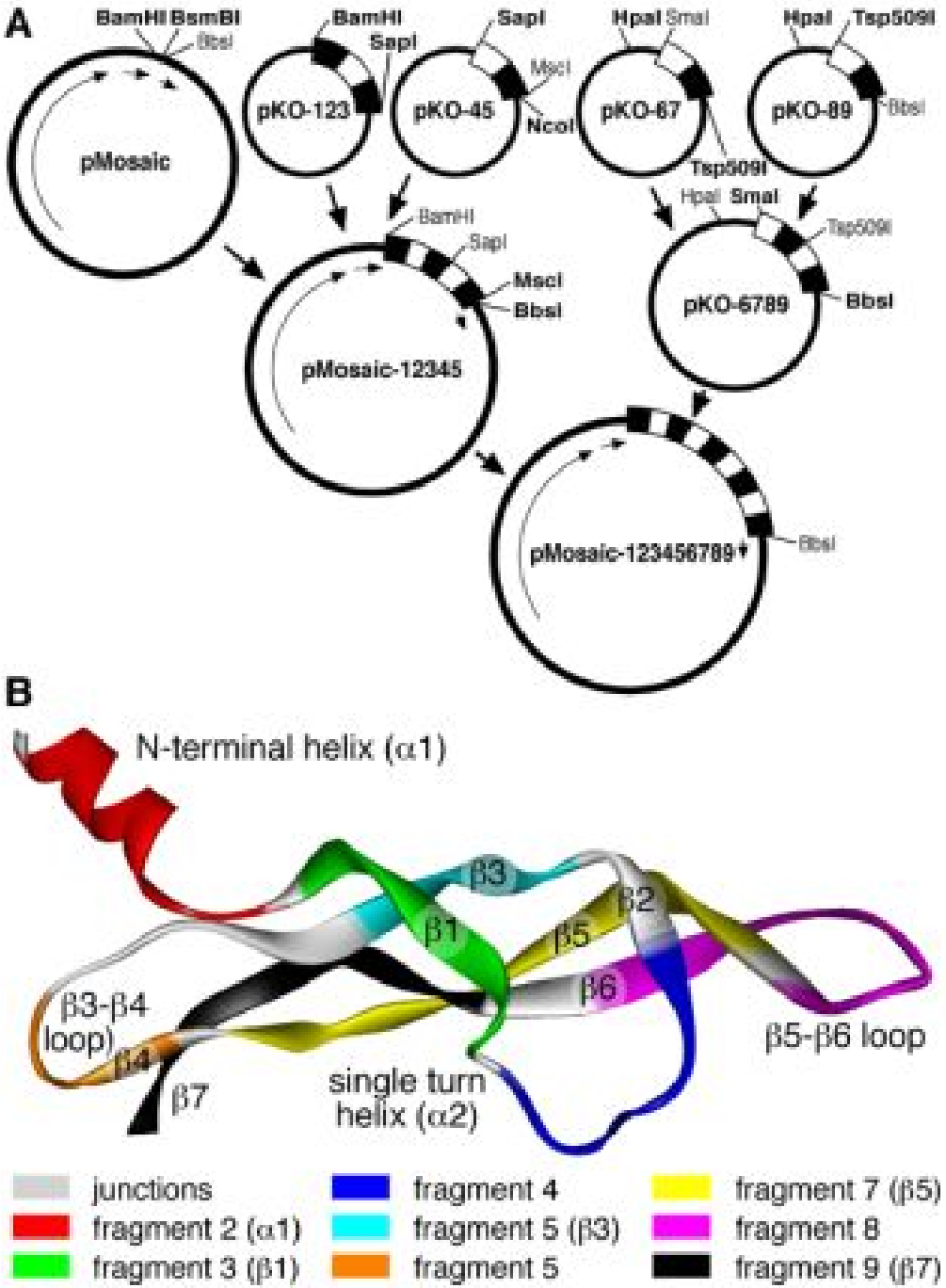
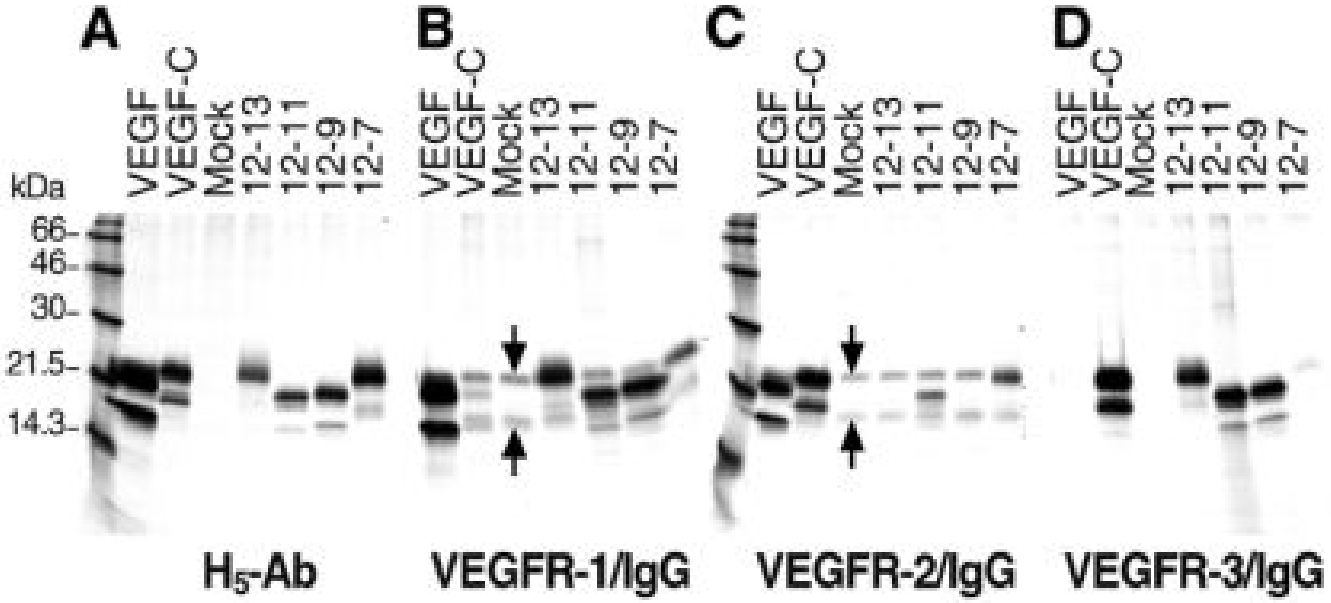

A
BamHI BsmBI
BbsI
pMosaic
BamHI
SapI
pKO-123
SapI
MscI
NcoI
pKO-45
HpaI SmaI
pKO-67
HpaI Tsp509I
pKO-89
BbsI
Tsp509I
BamHI
SapI
MscI
BbsI
pMosaic-12345
HpaI SmaI
Tsp509I
pKO-6789
BbsI
pMosaic-123456789†
BbsI
B
N-terminal helix (α1)
β3
β2
β1
β5
β6
β3-β4 loop
β4
β7
single turn helix (α2)
β5-β6 loop
junctions
fragment 2 (α1)
fragment 3 (β1)
fragment 4
fragment 5 (β3)
fragment 5
fragment 7 (β5)
fragment 8
fragment 9 (β7)

Figure 2


A
B
C
D
VEGF
VEGF-C
Mock
12-13
12-11
12-9
12-7
kDa
66-
46-
30-
21.5-
14.3-
H5-Ab
VEGFR-1/IgG
VEGFR-2/IgG
VEGFR-3/IgG

E

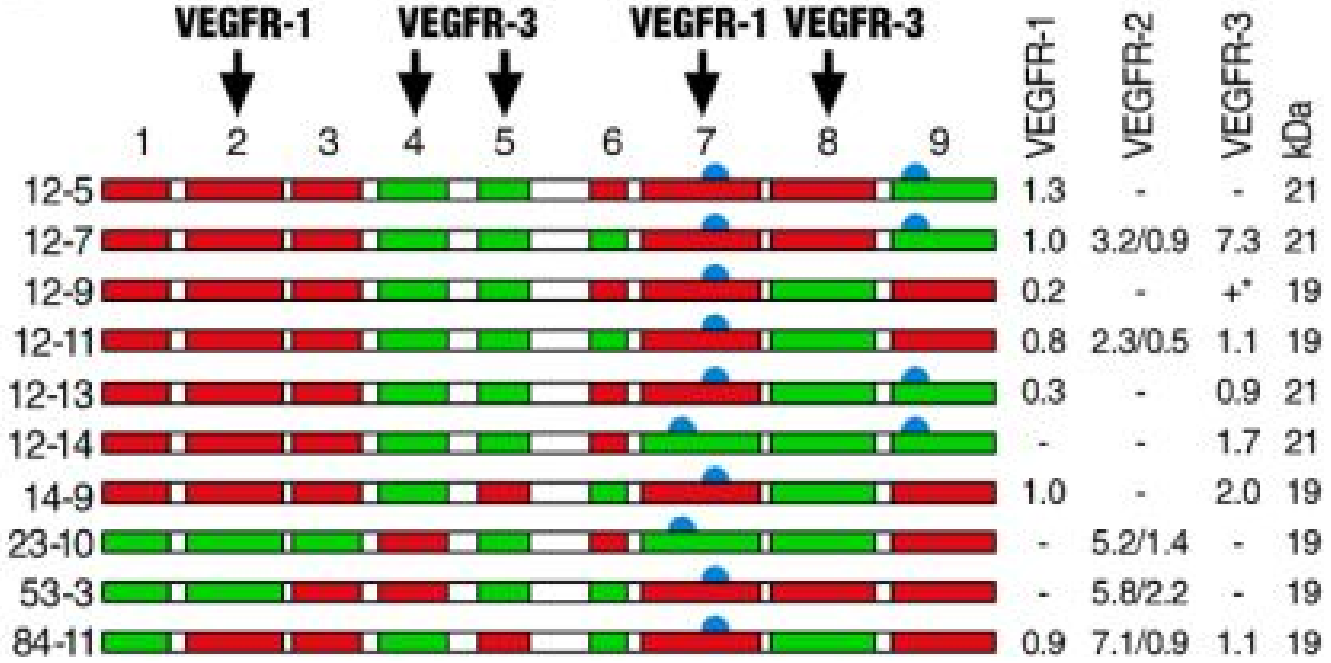
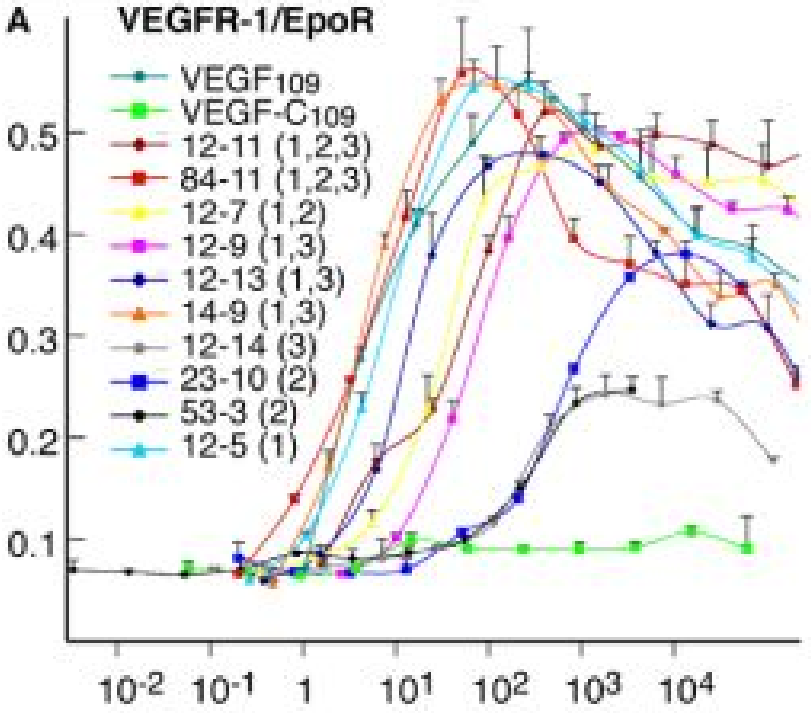
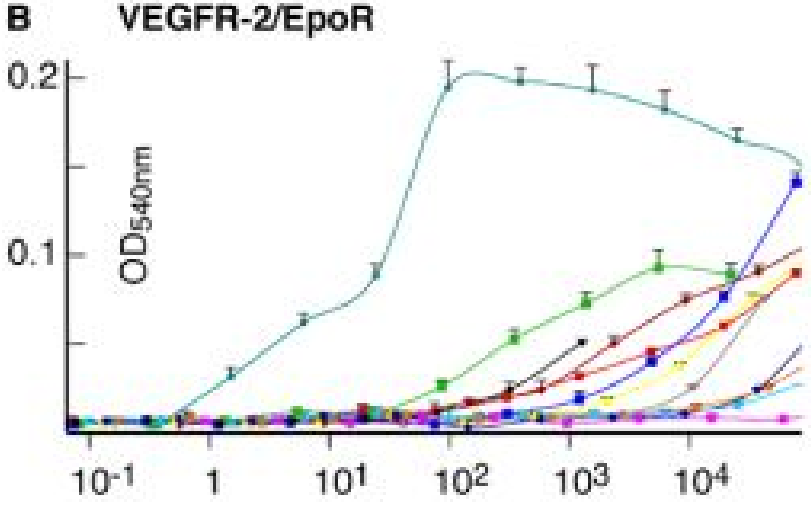
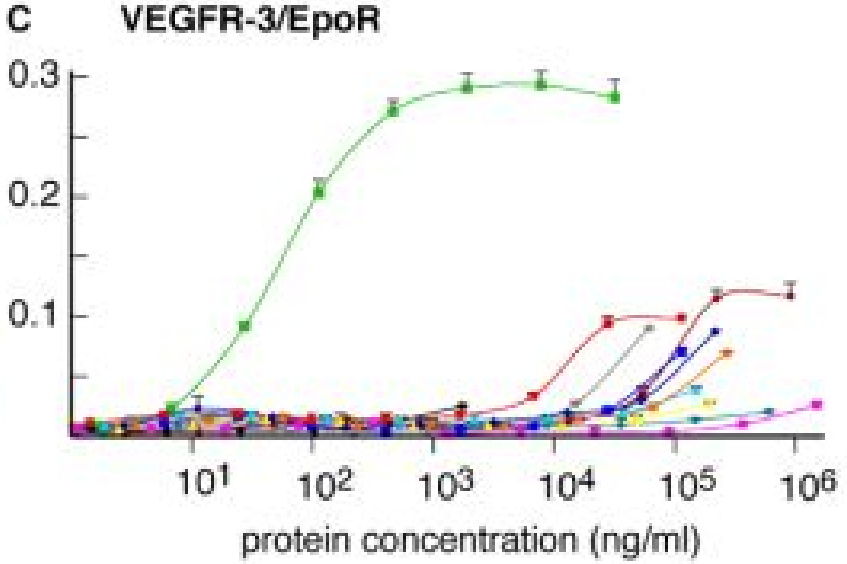

VEGFR-1
VEGFR-3
VEGFR-1
VEGFR-3
1 2 3 4 5 6 7 8 9
VEGFR-1
VEGFR-2
VEGFR-3
kDa
12-5 1.3 - - 21
12-7 1.0 3.2/0.9 7.3 21
12-9 0.2 - +* 19
12-11 0.8 2.3/0.5 1.1 19
12-13 0.3 - 0.9 21
12-14 - - 1.7 21
14-9 1.0 - 2.0 19
23-10 - 5.2/1.4 - 19
53-3 - 5.8/2.2 - 19
84-11 0.9 7.1/0.9 1.1 19

Figure 3


A
VEGFR-1/EpoR
VEGF109
VEGF-C109
12-11 (1,2,3)
84-11 (1,2,3)
12-7 (1,2)
12-9 (1,3)
12-13 (1,3)
14-9 (1,3)
12-14 (3)
23-10 (2)
53-3 (2)
12-5 (1)
0.5
0.4
0.3
0.2
0.1
10-2 10-1 1 101 102 103 104


B
VEGFR-2/EpoR
OD540nm
0.2
0.1
10-1 1 101 102 103 104


C
VEGFR-3/EpoR
0.3
0.2
0.1
101 102 103 104 105 106
protein concentration (ng/ml)

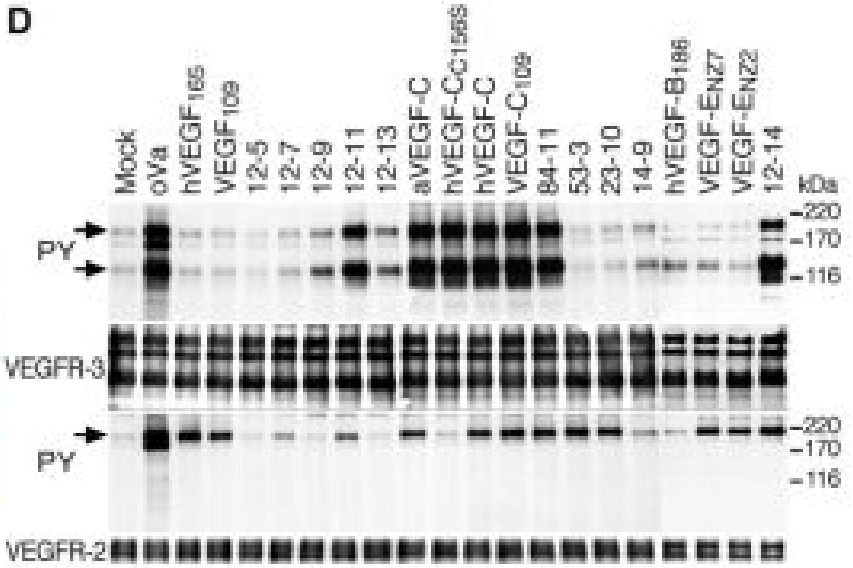

D
Mock
oVa
hVEGF165
VEGF109
12-5
12-7
12-9
12-11
12-13
aVEGF-C
hVEGF-C(C156S)
hVEGF-C
VEGF-C109
84-11
53-3
23-10
14-9
hVEGF-B186
VEGF-E(N27)
VEGF-E(N22)
12-14
kDa
PY
VEGFR-3
PY
VEGFR-2
220
170
116
220
170
116

Figure 4

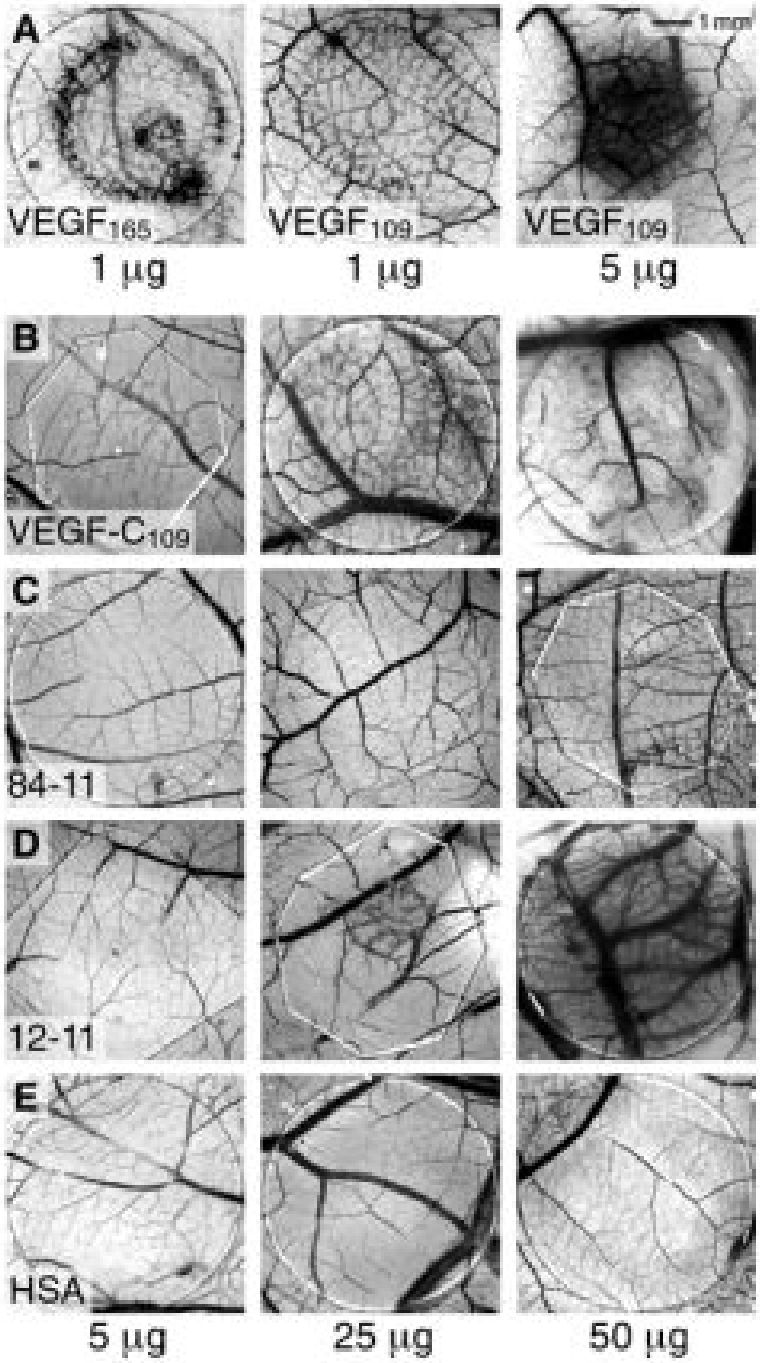

A
VEGF165
1 µg
VEGF109
1 µg
1 mm
VEGF109
5 µg
B
VEGF-C109
C
84-11
D
12-11
E
HSA
5 µg
25 µg
50 µg

Figure 5

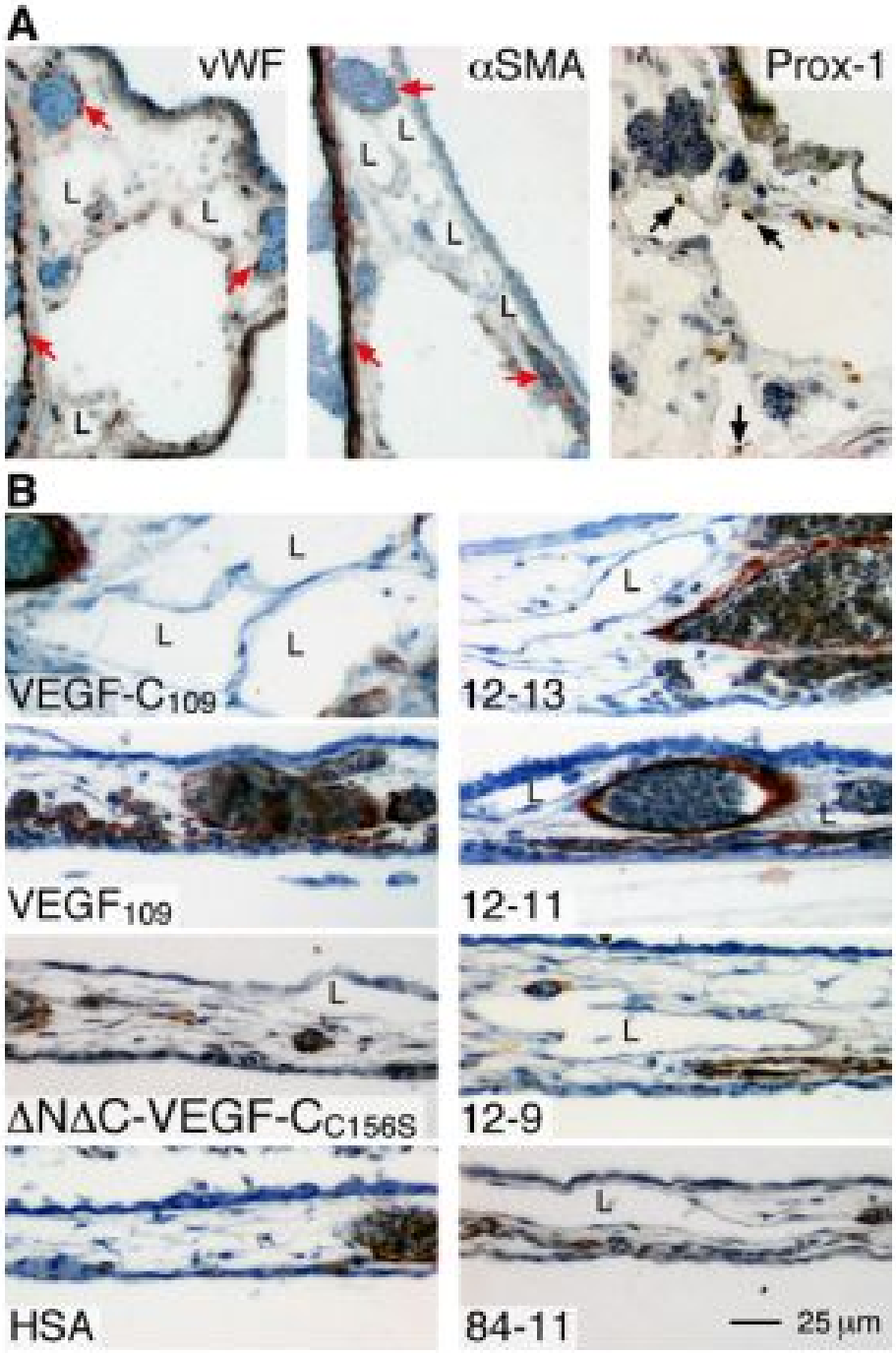
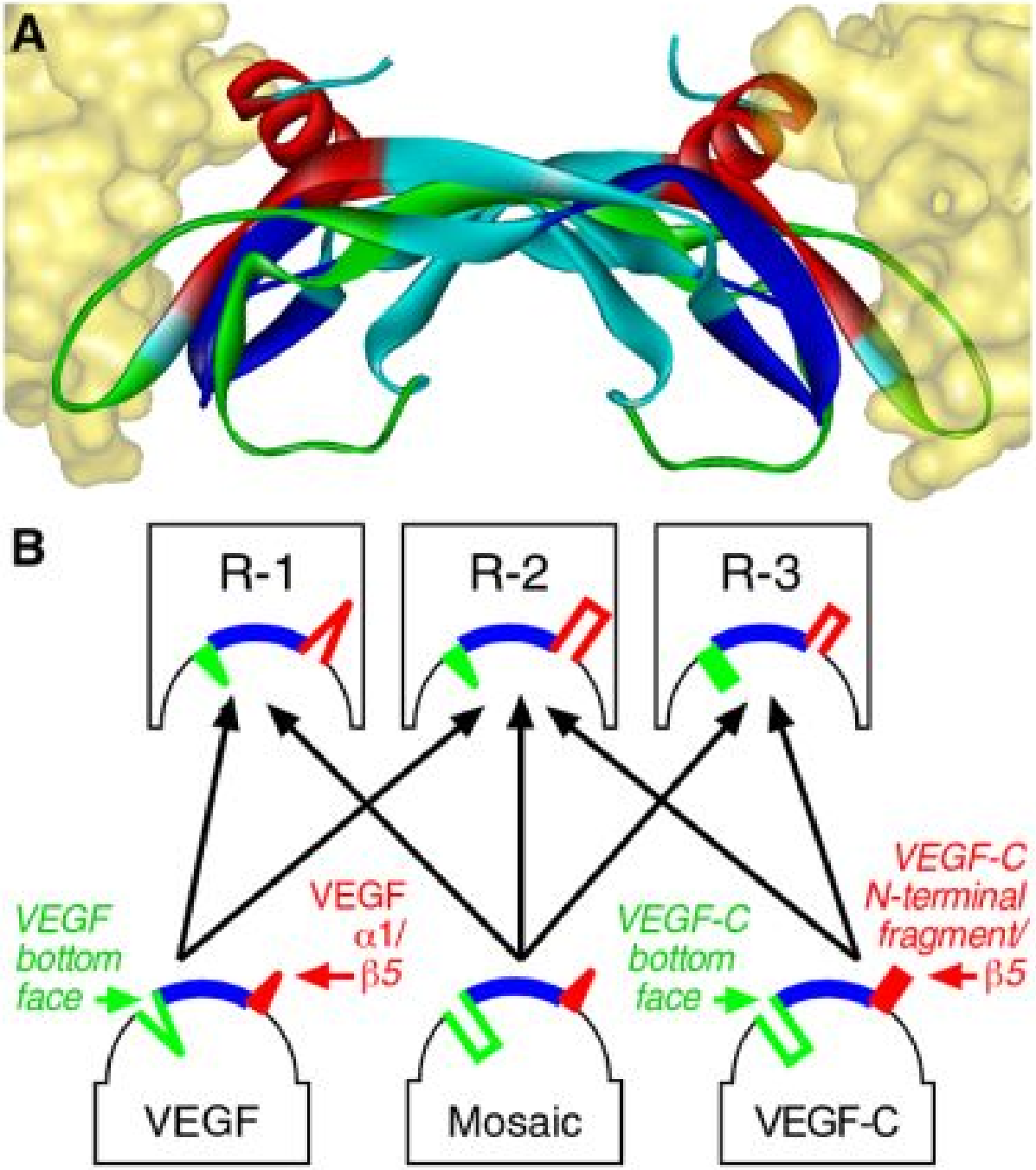

A
vWF
αSMA
Prox-1
L
B
VEGF-C109
12-13
VEGF109
12-11
ΔNΔC-VEGF-CC156S
12-9
HSA
84-11
25 μm

Figure 6


A
B
R-1
R-2
R-3
VEGF
bottom
face
VEGF
α1/
β5
VEGF-C
bottom
face
VEGF-C
N-terminal
fragment/
β5
VEGF
Mosaic
VEGF-C

Polyclonal Rabbit Antisera, Part 1

Antigen[1]	*Rabbit*	*Works in*
Human LYVE-1 EC-human $IgGF_c$	SFI16	Western, IP, paraffin sections
	SFI17	Western, IP
Human Podoplanin EC-human $IgGF_c$	SFI14	Western (+), IP not tested[2]
	SFI15	Western (+++), IP not tested[2]
Mouse Podoplanin EC-human $IgGF_c$	SFI18	Western, IP not tested[2] , paraffin sections
	SFI19	Western, IP not tested[2] , paraffin sections

[1]all antigens were produced in insect cells using the baculovirus system
[2]no functional eukaryotic expression vector available

Polyclonal Rabbit Antisera, Part 2

Antigen	*Rabbit*	*Works in*
Prox-1 homeobox & prospero-like domain GST (100% identity between human, mouse & chicken)[1]	1	Western (+++)
	2	Western (+), unfixed cryosections, (inconsistency?) problems
Human ΔNΔC-VEGF-C-H_6 (DPTEETIK…MSKLH_6*)[2]	3	Western (weakly), IP not tested
	4	Western (weakly), IP not tested
Human VEGF-C-H_6 (DPFESGLD…PQMSH_6*)[2]	5	Western, IP, ELISA
	6	Western, IP

[1]expressed in E.coli
[2]expressed in insect cells using the baculovirus system

Immunohistochemistry

- Technical problems during sample preparation & staining
- Isolation of PAS or IgG-bound fraction
- Immunopurification (2-3 mg of antigen needed!)
- Zn fixation (if native antigen was used for immunisation)

New Recombinant Proteins

- H_6-human serum albumin-H_6
- VEGF-E: NZ2 & NZ7
- Mouse LYVE-1 EC-human $IgGF_C$

Recombinant Proteins to Come

All viruses since autumn 2001 are dual-promoter viruses that work for both protein production in insect cells and infection of mammalian & avian cells (both cell culture & in vivo)!

- human PlGF-1 & PlGF-2
- human VEGF-C C-terminus

From cDNA to Protein

Step	*Minimum time needed*
Cloning of cDNA into baculovirus transfer vector	5 days
Transposition in E.coli to create recombinant virus	4 days
Transfection of insect cells with viral DNA	4 days
1. Amplification of virus stock	4 days
Testing of different clones for expression	6 days
2. Amplification of selected clones	4 days
Large scale production of virus stock	4 days
Infection of insect cells for production purposes	3 days
Dialysis of conditioned medium	3 days
Purification using Ni^{2+}NTA chromatography	1 day
Dialysis, sterilization & quantitation of peak fractions	3 days
Total	*41 days*

Protein Yields

Protein	µg/ml*
Low-level expression: full-length VEGF-C, full-length VEGF-D	0.2-1
Medium-level expression: short VEGF-C-C156S, short VEGF-D, VEGF-A_{165}, most IgG fusions	1-4
High-level expression: short VEGF-C	5-10
Insane levels: VEGF-E	>10

*µg purified & sterile protein per ml conditioned medium

The Mouse LYVE-1 Disaster

One baculovirus expression cycle lasts about 3 months

pFB1-Dual-mLYVE-1-EC-hIgGF$_C$ (2 mutations: one silent, one Val → Leu)

↓ mutation repaired

pFB1-Dual-mLYVE-1-EC-hIgGF$_C$

↓ heterologous signal peptide inserted

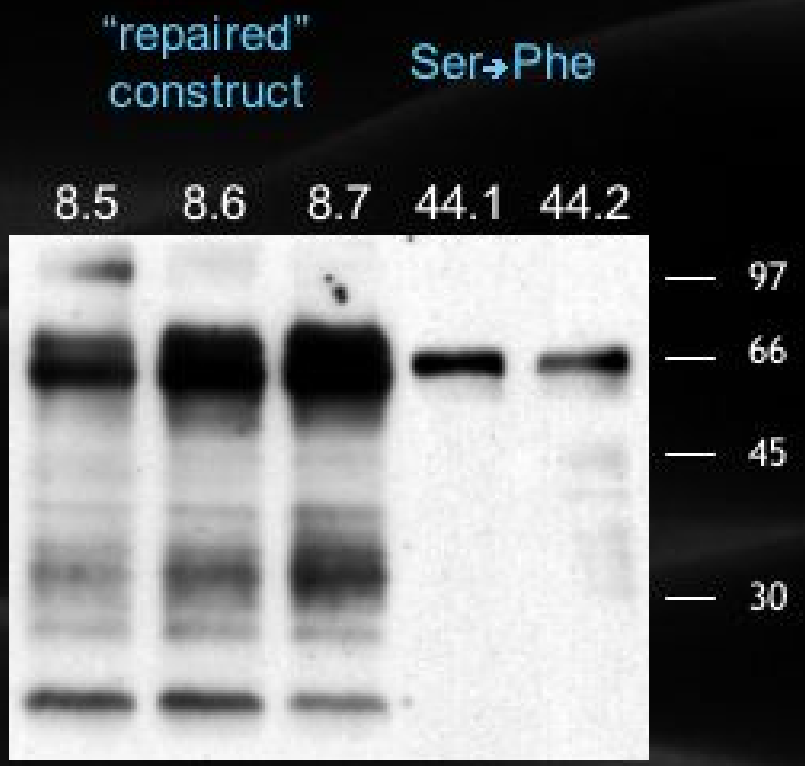
pFB1-Dual-IgKSP-mLYVE-1-EC-hIgGF$_C$

↓ resequencing of coding region

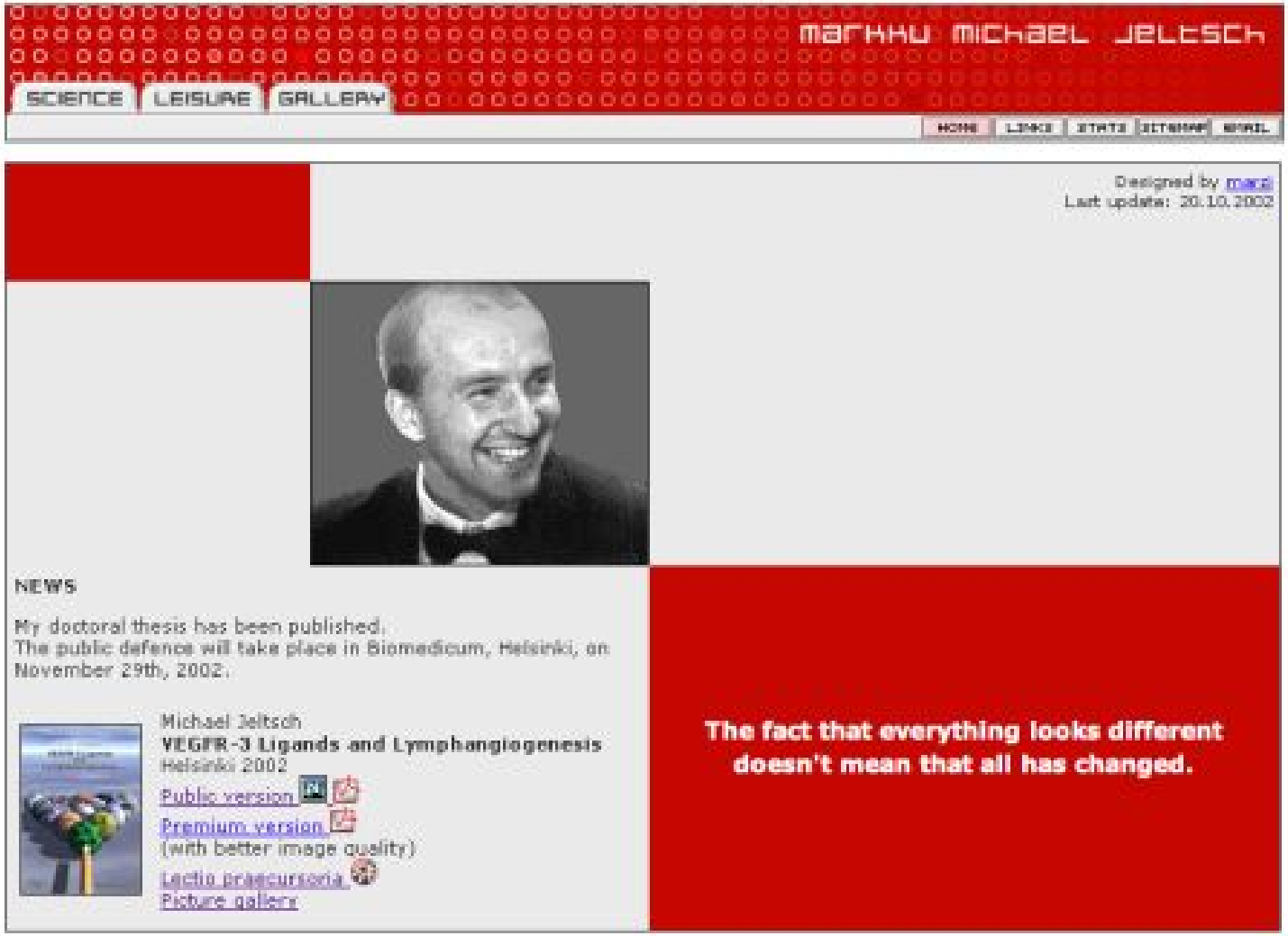
a Ser → Phe mutation was missed!

missed mutation (Ser→Phe) repaired

pFB1-Dual-IgKSP-mLYVE-1-EC-hIgGF$_C$



17 credits & PhD degree



Computer Stuff

- Oligonucleotide database
- MacFixit subscription
- Backup
- OS X transition
- Public computers
- Biocomputing core facility
- Freely available programs

Oligonucleotide Database

- On Paula's computer
- Program: MacVector (no license)
- Instructions under http://www.helsinki.fi/~mjeltsch/stuff/reference.html
- A very easy-to-use web interface is under construction

MacFixit & VersionTracker Subscription

- http://www.macfixit.com
- http://www.versiontracker.com

login: jeltsch@csc.fi
password: seurasaari

Backup

Additional hard disks for private desktops

60 GB	110-120 €
80 GB	130-150 €
120 GB	190-210 €
160 GB	340-400 €

Transition to Mac OS X

- Computers bought after December 31, 2002 will not be able to boot into classic Mac OS
- Still possible to use old programs from within Mac OS X (under classic emulation)!
- There are no programs that we need that are not compatible with OS X: Lasergene & Fuji MacBAS do run under classic emulation
- Lasergene will be updated to run natively under Mac OS X, Fuji MacBAS maybe never updated
- Lasergene license manager bypassing

Public computers: General Rules of Behavior

- Disk space policies: There is **NO** disk space on public computers for the storage of your personal files!
- If you absolutely have to store something on public computers, do it **ALWAYS** in the *Documents* directory.
- Install **NOTHING** by yourself

Biocomputing Core Facility

- Access only from Windows 2000/XP, Linux and - sometimes - Mac OS X
- 1 GB disk space, common protected file sharing space, unlimited backup space
- Many useful programs: Plasmid drawing & annotation, Oligo selection & analysis, GeneSpring, Office XP and many more

Freely available programs

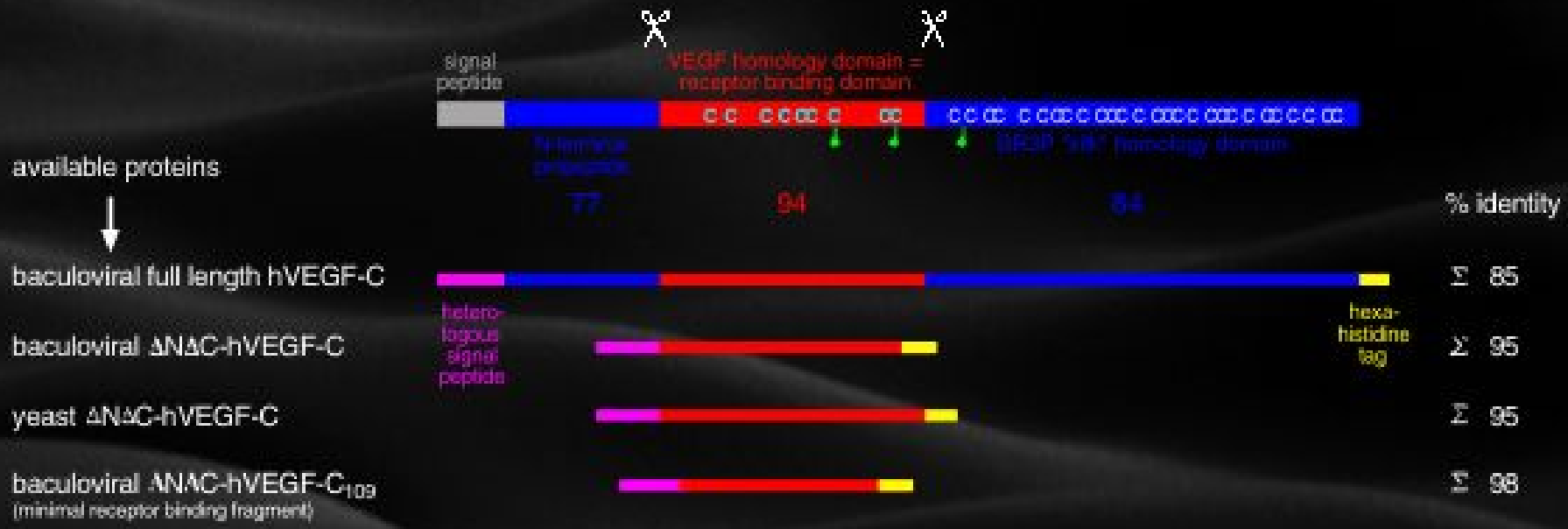
- Lasergene/DNASTAR
- Floating license for GeneConstruction Kit 2.5 for the whole research program
- Software campus licenses: MS Office X, Norton Antivirus 7 & 8, RealPlayer 8 , Corel Graphics Suite 9 & 10 (CorelDraw = Freehand, PhotoPaint = Photoshop)

Ongoing Projects

mLYVE-1 Antiserum

- Immunization: where?
- Monitoring of titer: who and how?
- Testing: who?

Comparison of human and mouse VEGF-C



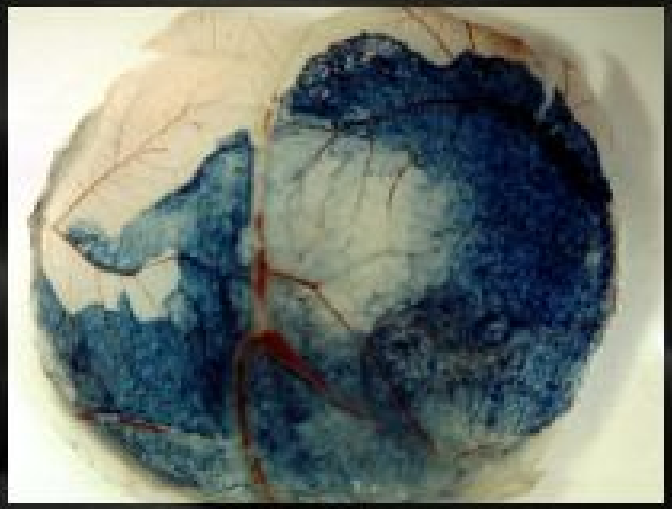
Problems

- 3rd attempt
- Limited amount of antigen
- Screening is not done by us
- False-positives (histag, glycosylation)
- Backup strategy for screening (large amounts of recombinant full-length VEGF-C)

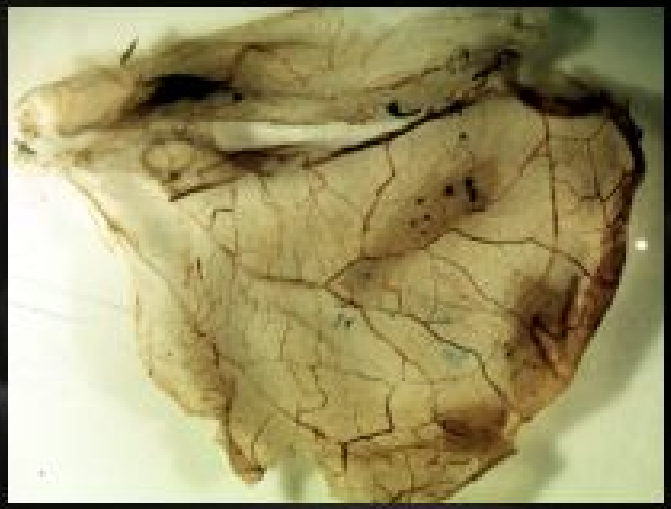
Dual promoter lacZ

lacZ baculo-infected d 13 CAM

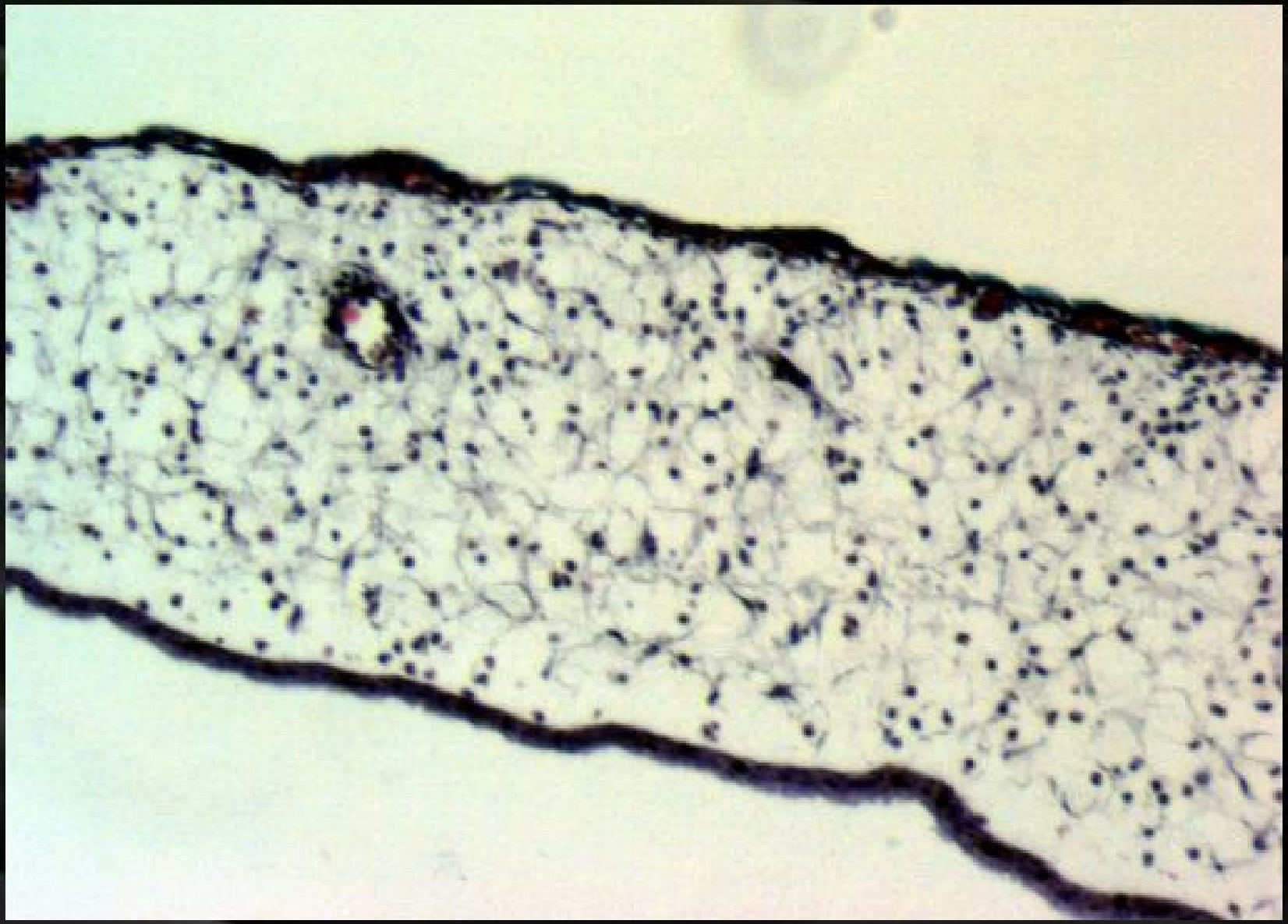
infection with concentrated virus
harvest 48 h p.i.

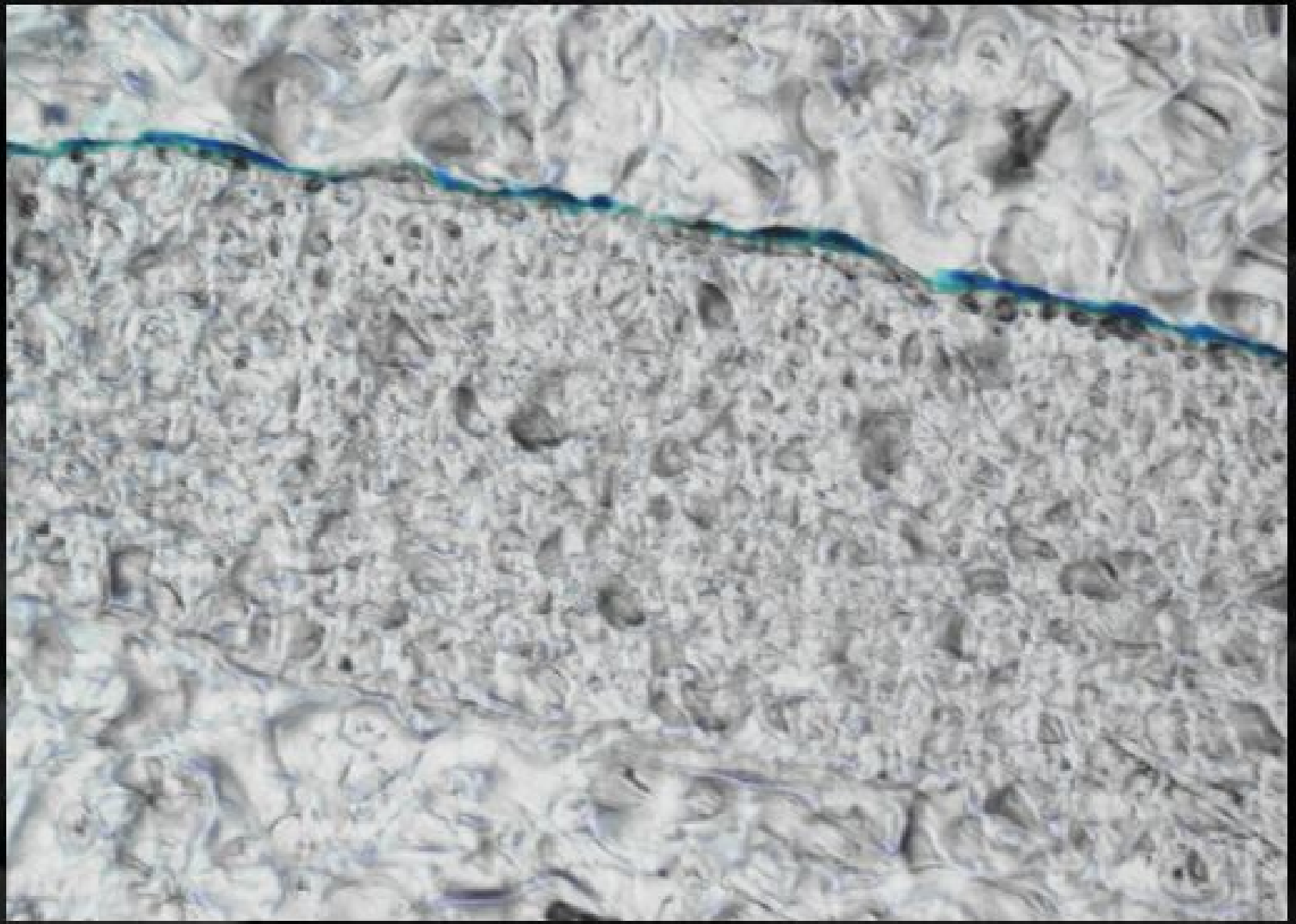


Dual promoter lacZ



Polh promoter VEGF-C





New Baculoviruses for In-vivo Infection

- pFB1-Dual-lacZ-H_6
- pFB1-Dual-IgKSP-hSA-H_{2x6}

- pFB1-Dual-IgKSP-CAC swap-H_6
- pFB1-Dual-IgKSP-ACA swap-H_6
- pFB1-Dual-IgKSP-hVEGF-C-Cterminus-H_6
- pFB1-Dual-IgKSP-hVEGF-A_{165}-H_6
- pFB1-Dual-IgKSP-ΔNΔC-hVEGF-C-H_6
- pFB1-Dual-IgKSP-hVEGF-C-FL-H_6
- pFB1-Dual-IgKSP-hVEGF-C-SIISS-H_6
- pFB1-Dual-IgKSP-hVEGF-C-SIISS-FLAG-H_6

Virus Purification

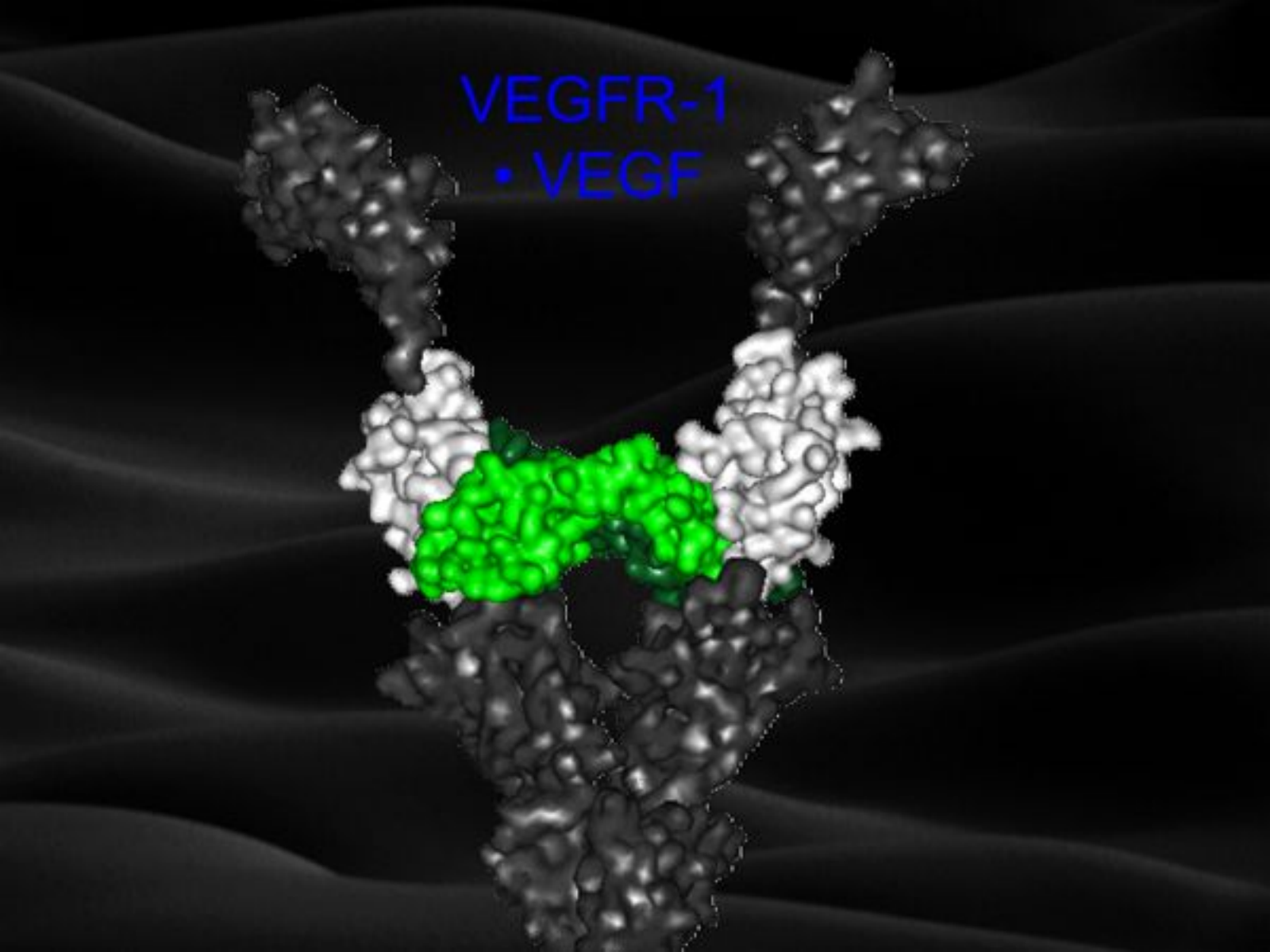
- Classical purification by ultacentrifugation in a gradient, recovery of infectivity ~5-15%
- Chromatography with heparin sepharose, recovery >50%

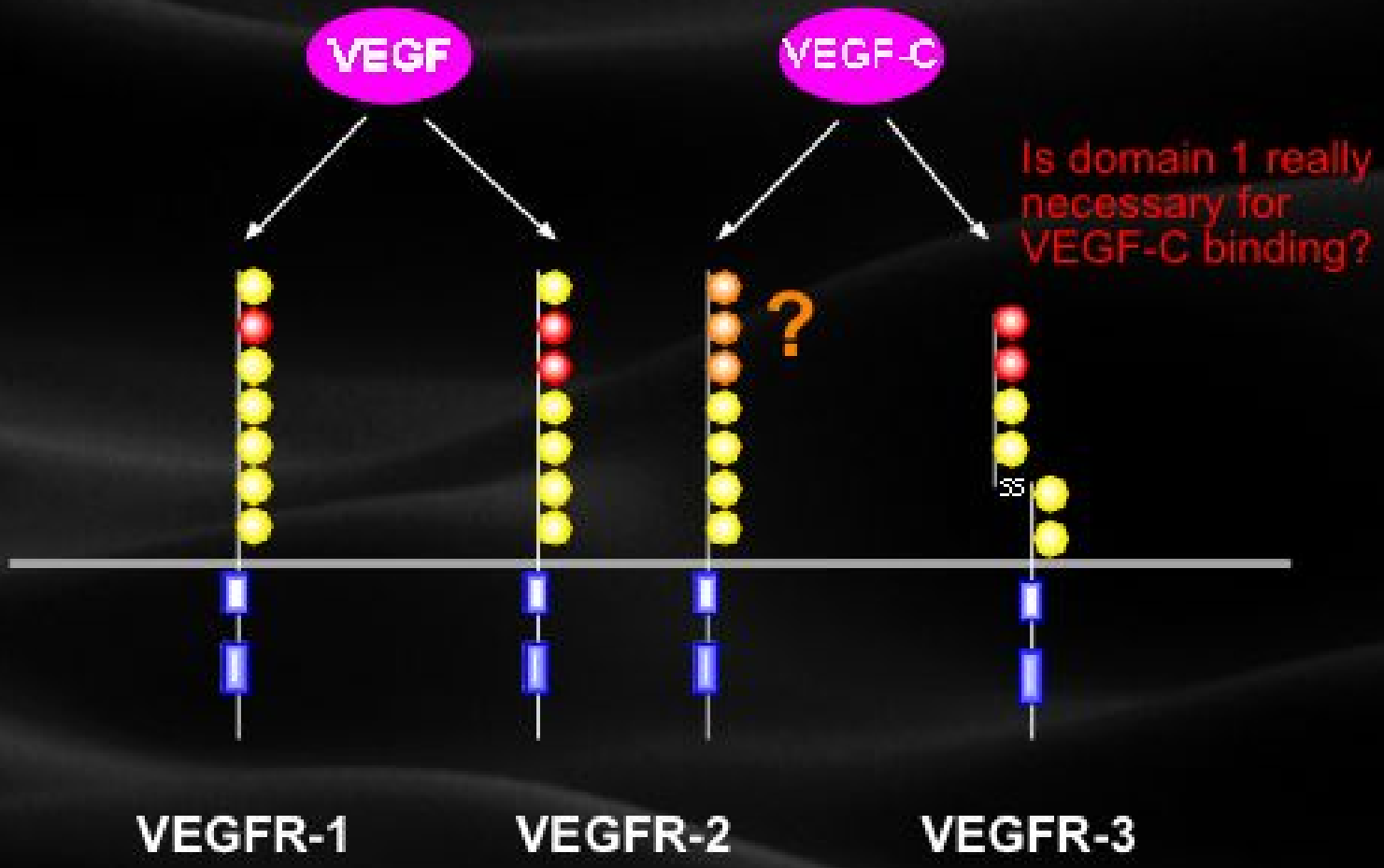
E. coli protein expression, refolding & crystallization

- Human VEGF-C_{109} (corresponding to aa 8-109 of human VEGF-A)
- Human VEGF-A_{109}
- Human ΔNΔC-VEGF-C (MSKL*)

…and the Receptors?

- Human VEGF-R2 D2+3
- Human VEGF-R3 D1+2

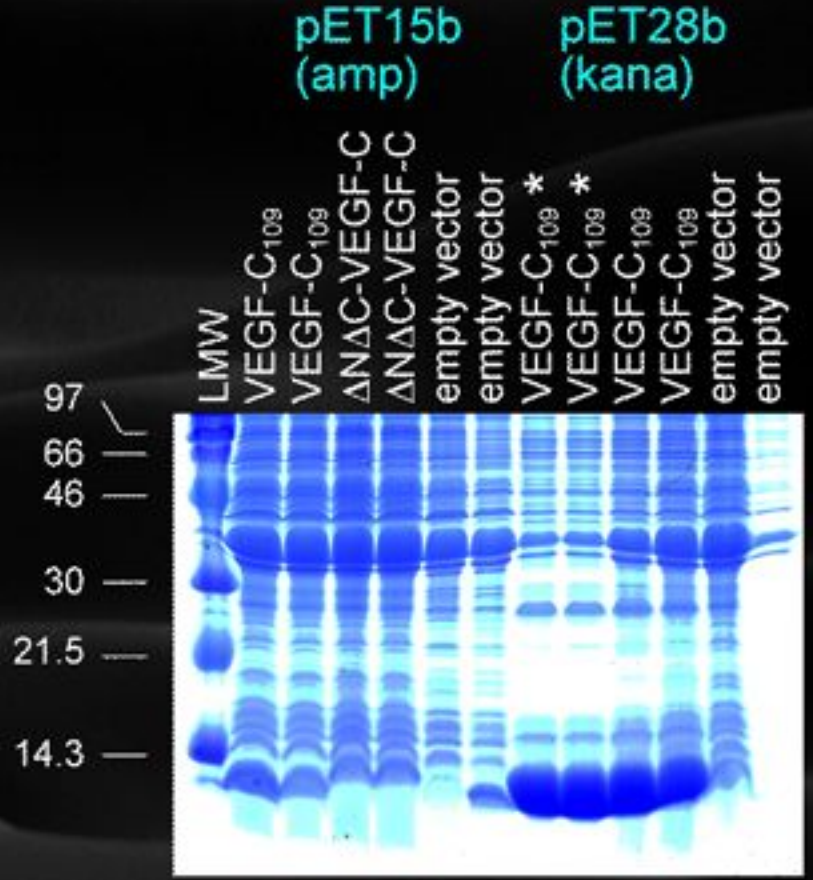

VEGFR-1
• VEGF


VEGF
VEGF-C
Is domain 1 really necessary for VEGF-C binding?
?
VEGFR-1
VEGFR-2
VEGFR-3

Expression

- Expression optimization
  different E.coli strains: AD494, BL21(DE3), **BL21(DE3)***
  different expression constructs: pET15 (amp), **pET28 (kana)**
- Inclusion body isolation by differential centrifugation & washing
- Solubilization

VEGF-C_{109} Expression in E.coli BL21(DE3)* under Kanamycin Selection



Refolding & Purification

Refolding by try and error:

- redox-pair
- pH
- metal salts
- protein concentration
- slow versus shock refolding
- detergents/denaturating agents
- arginine, glycine, etc.

Purification:

- Ion-exchange chromatography
- Hydrophobic interaction chromatography
- Size-exclusion chromatography

Suitable read-out for successful refolding?

Ba/F3, FSAP, IP with soluble receptors, receptor phosphorylation

Crystallization

- Hampton sparse matrix screen

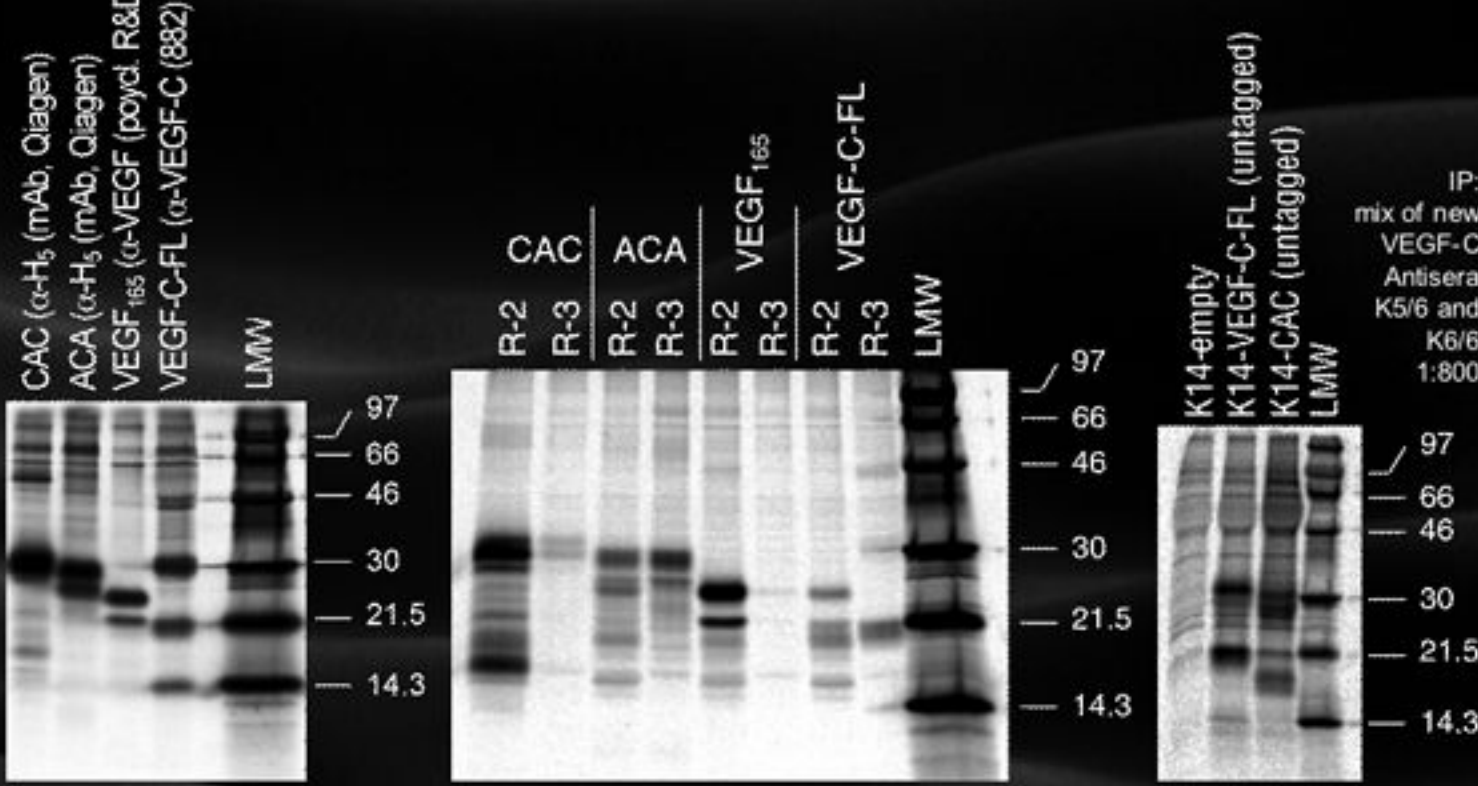
Mouse & chick VEGFR-1

Notorious problems with VEGFR-1 expression levels in mammalian cells

- Unsuccessful screening of a chick heart library with a 500 bp fragment from Jörg Wilting
- RT PCR from mouse and chick VEGFR-1 EC domains
- Chick VEGFR-1 binds only weakly human VEGF

New Keratin-14 Transgenic Lines

Expression & Receptor Binding of CAC & ACA



Efficiently organizing the -70°C freezers