Lab Seminar

Part B

Sept. 3, 2003

Computer-related issues

Why Linux?

Tiedät kyllä miksi

Actually only the heart of the operating system (the "kernel") is Finnish...

Computer-related issues

- Linux can run on different hardware: i386, PPC
- Different distributions (what programs are packaged, user interface):

  RedHat or Suse for i386
  Yellow Dog for PPC
- i386 Linux distributions are more advanced than PPC distributions
- When should you **NOT** switch to Linux?
  1. If you have trouble as a Mac user using Windows or as a Windows user using Macs
  2. If you have no time
  3. If you are generally not technically inclined

Computer-related issues

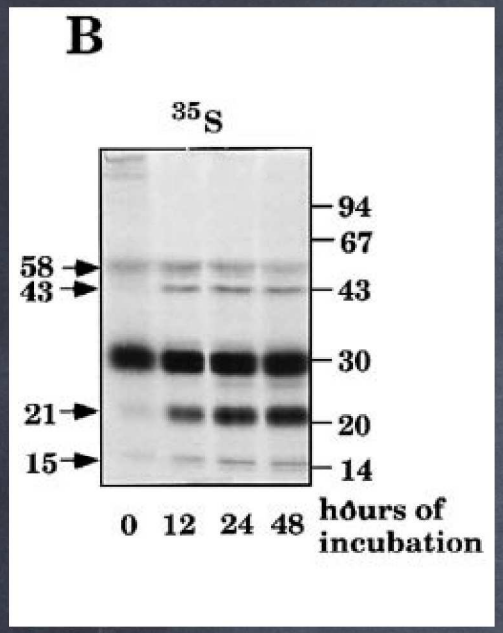
- Linux works, RedHat 9 on Celeron 500 MHz
- Connectivity: from Windows to Mac (both OS 9 and X) to Linux
  from Linux to Mac OS X and Windows
- Many applications are available as Linux versions
  Mozilla/Firebird/Netscape, Acrobat Reader, Citrix ICA Client
- There are Open Source alternatives for almost all applications
  Photoshop: GIMP
  MS Office: Open Office
  QuickTime: Video LAN Client
  Lasergene/Gene Construction Kit: Staden, Emboss
- Many Windows programs run on Linux ***without emulation*** using WINE
  MS Office, Photoshop, Endnote, Internet Exploder
- All Windows programs run on Linux with emulation using VMware
- PPC Distributions: no WINE, instead MOL (Mac On Linux)

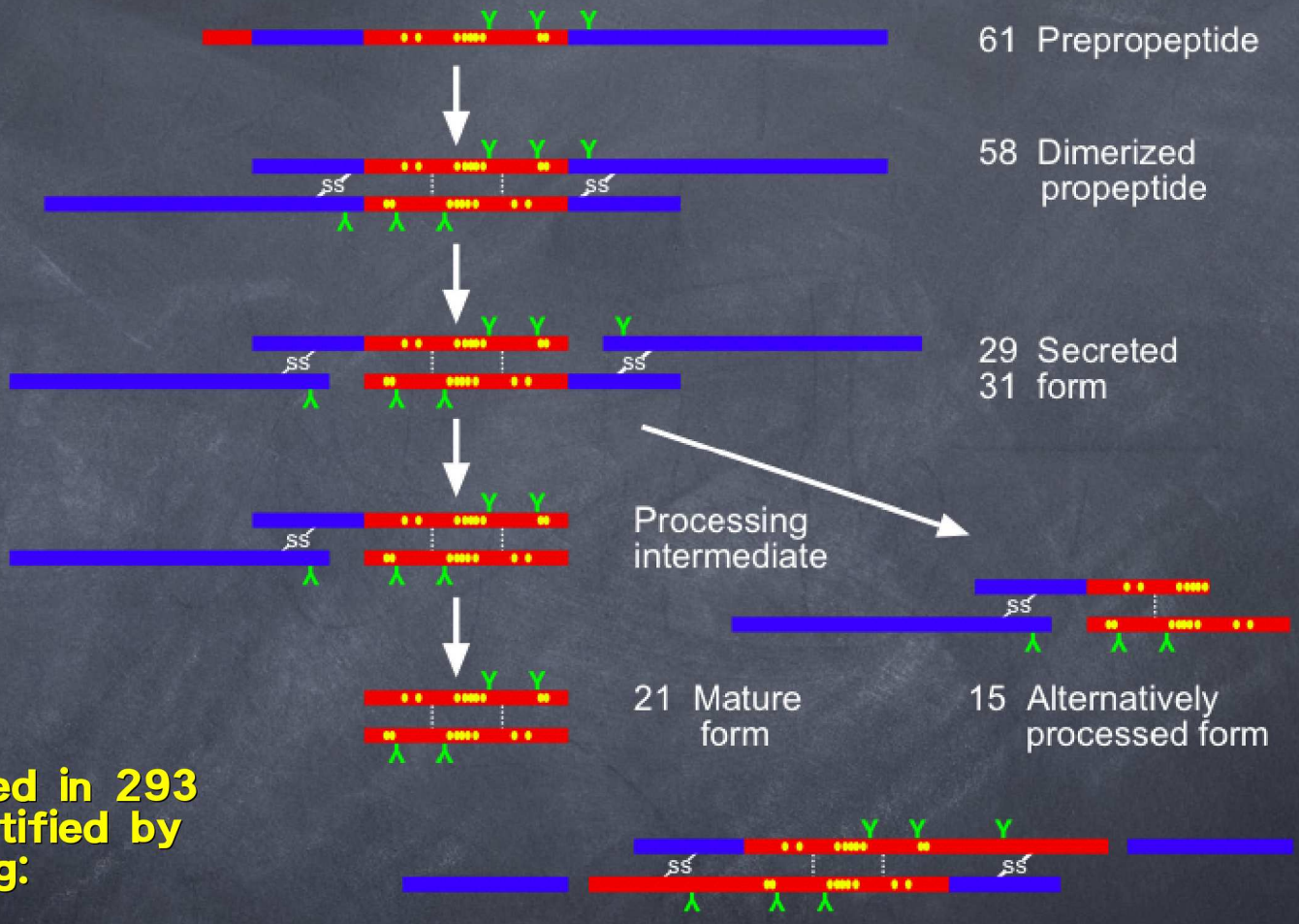
This presentation

- Alternative to PowerPoint:
  XML or PDF? HTML/CCS2, advantages: file size, portability, output device independent
- Mac version of Opera browser doesn't support output device detection, for Mozilla in development
- Convert OpenOffice into PowerPoint presentation?
  Works only if you havn't imported your graphics from other vector-based drawing programs (Freehand, CorelDraw, Adobe Illustrator)
- Install OpenOffice on Mac?
  There is no native Mac OS X port of OpenOffice (coming in end of the year)
- Install X Windows, install the UNIX version of OpenOffice, convert Unix fonts into Macintosh fonts

VEGF-C

- What is known about the different forms of VEGF-C
- What to use when: Immunization, Lymphangiogenesis/Angiogenesis
- What attempts have been done to produce monoclonal antibodies against human VEGF-C?
- What antibodies do exist and what do they recognize?
- What are the differences between VEGF-C and VEGF-D?



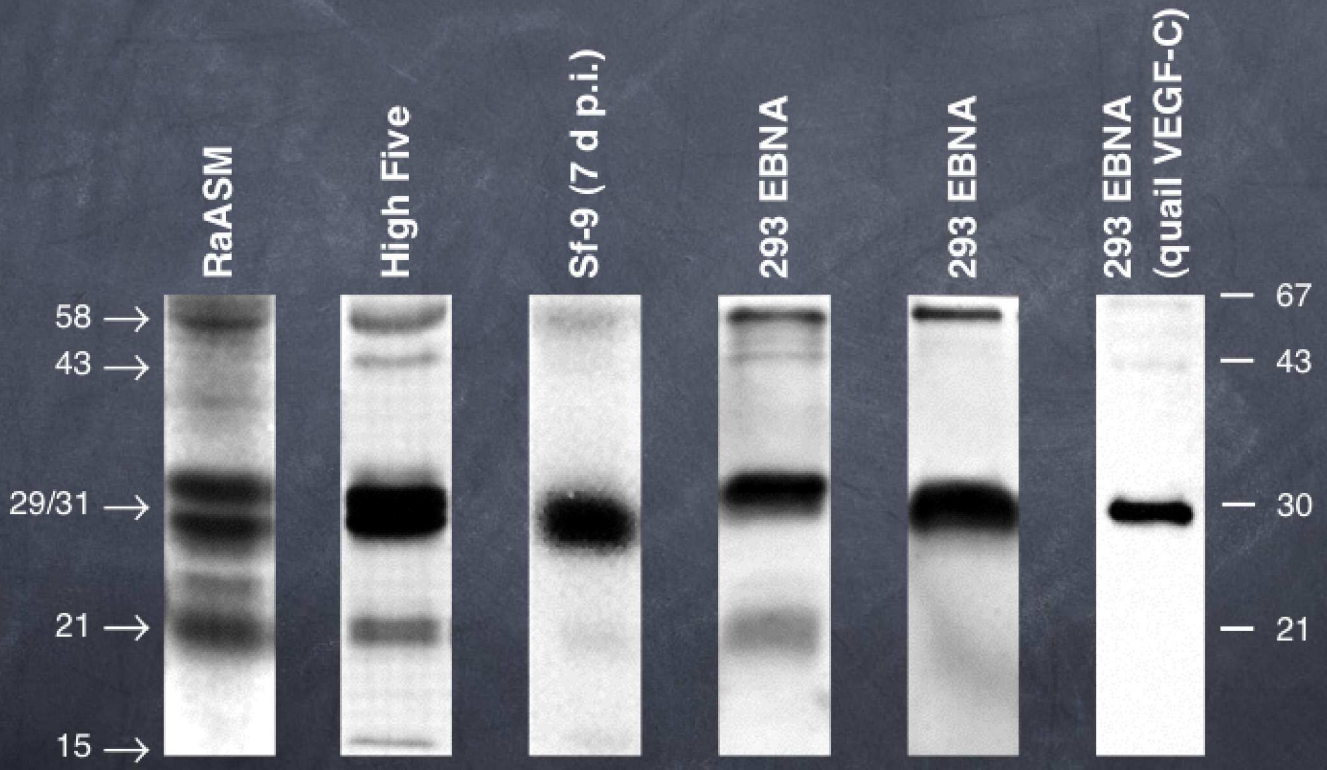


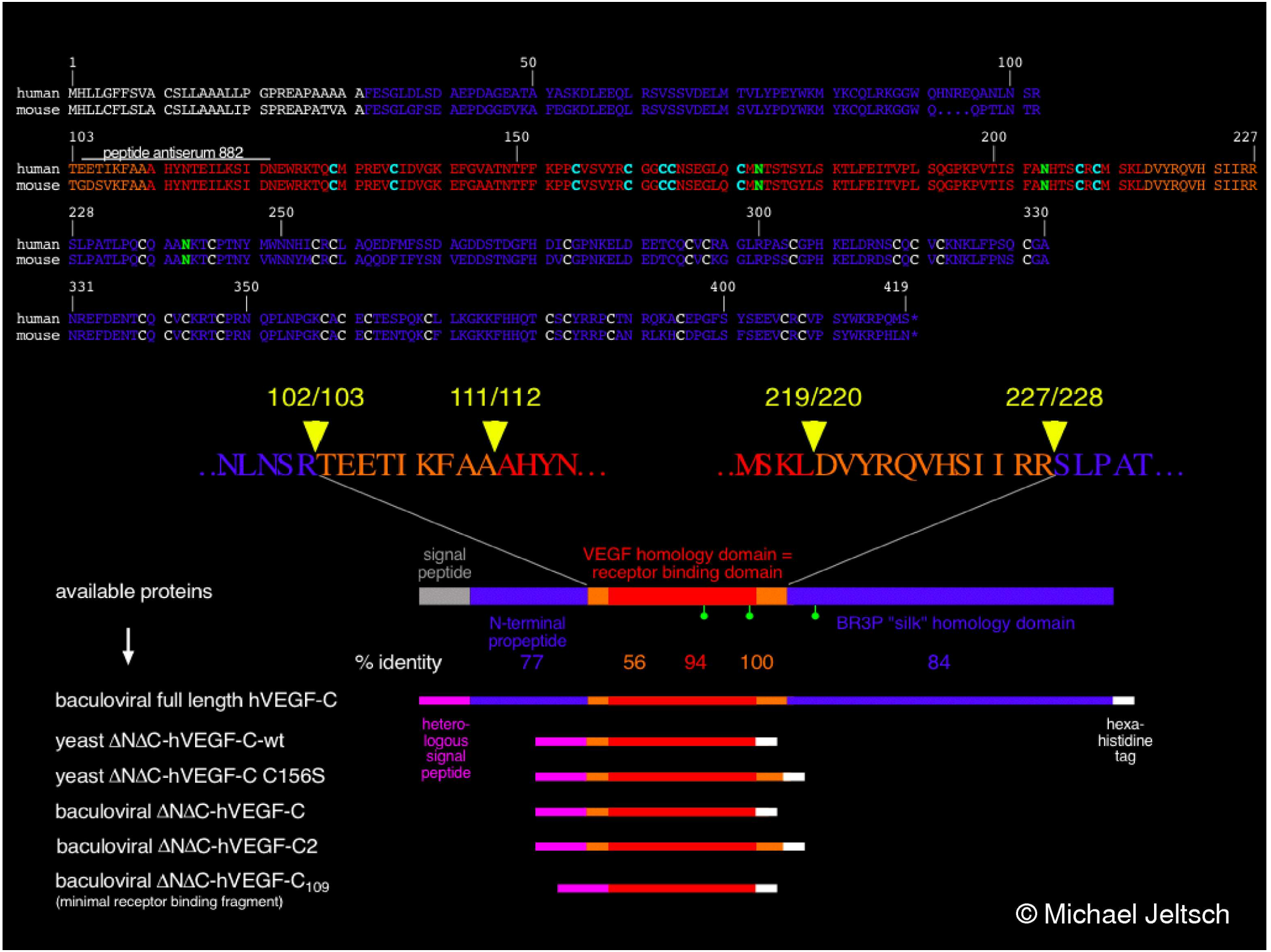
Peptides (produced in 293 EBNA cells) identified by sequencing:

- NH_2-F(32)ESGLD
- NH_2-A(112)HYNTE
- NH_2-S(228)LPATL

kDa	form	VEGFR-2	VEGFR-3
58	full length	+/-	+
43	alternatively C-terminally processed	++	++
29/31	main/secreted	+	+++
21	mature	++	+++
15	N-terminal propeptide + partial VHD	-	-

Mature VEGF-C is probably a rare species under physiological conditions

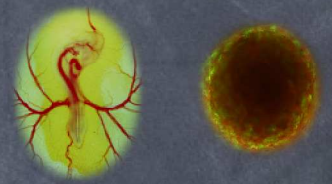
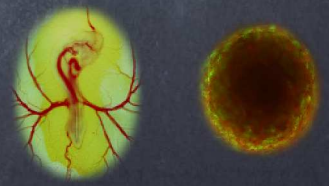



1 50 100
human MHLLGFFSVA CSLLAAALLP GPREAPAAAA AFESGLDLSD AEPDAGEATA YASKDLEEQL RSVSSVDELM TVLYPEYWKM YKCQLRKGGW QHNREQANLN SR
mouse MHLLCFLSLA CSLLAAALIP SPREAPATVA AFESGLGFSE AEPDGGEVKA FEGKDLEEQL RSVSSVDELM SVLYPDYWKM YKCQLRKGGW Q....QPTLN TR
103 150 200 227
peptide antiserum 882
human TEETIKFAAA HYNTEILKSI DNEWRKTQCM PREVCIDVGK EFGVATNTFF KPPCVSVYRC GGCCNSEGLQ CMNTSTSYLS KTLFEITVPL SQGPKPVTIS FANHTSCRCM SKLDVYRQVH SIIRR
mouse TGDSVKFAAA HYNTEILKSI DNEWRKTQCM PREVCIDVGK EFGAATNTFF KPPCVSVYRC GGCCNSEGLQ CMNTSTGYLS KTLFEITVPL SQGPKPVTIS FANHTSCRCM SKLDVYRQVH SIIRR
228 250 300 330
human SLPATLPQCQ AANKTCPTNY MWNNHICRCL AQEDFMFSSD AGDDSTDGFH DICGPNKELD EETCQCVCRA GLRPASCGPH KELDRNSCQC VCKNKLFPSQ CGA
mouse SLPATLPQCQ AANKTCPTNY VWNNYMCRCL AQQDFIFYSN VEDDSTNGFH DVCGPNKELD EDTCQCVCKG GLRPSSCGPH KELDRDSCQC VCKNKLFPNS CGA
331 350 400 419
human NREFDENTCQ CVCKRTCPRN QPLNPGKCAC ECTESPQKCL LKGKKFHHQT CSCYRRPCTN RQKACEPGFS YSEEVCRCVP SYWKRPQMS*
mouse NREFDENTCQ CVCKRTCPRN QPLNPGKCAC ECTENTQKCF LKGKKFHHQT CSCYRRPCAN RLKHCDPGLS FSEEVCRCVP SYWKRPHLN*
102/103
111/112
219/220
227/228
..NLNSRTEETI KFAAAHYN...
..MSKLDVYRQVHSI I RRSLPAT...
signal peptide
VEGF homology domain = receptor binding domain
available proteins
N-terminal propeptide
BR3P "silk" homology domain
% identity
77
56
94
100
84
baculoviral full length hVEGF-C
yeast ΔNΔC-hVEGF-C-wt
yeast ΔNΔC-hVEGF-C C156S
baculoviral ΔNΔC-hVEGF-C
baculoviral ΔNΔC-hVEGF-C2
baculoviral ΔNΔC-hVEGF-C109
(minimal receptor binding fragment)
hetero-logous signal peptide
hexa-histidine tag
© Michael Jeltsch

VEGF-C antibodies

- 882: not for Western of 293 cells, maybe 1 ml left
- 905: N-terminal propeptide, IP works under non-reducing conditions
- new polyclonal sera 3 & 4: works in Western and IP, low titre
- new polyclonal sera 5 & 6: work well in Western and IP, not for Western of short form

All recombinant VEGF-C protein that has been shown to be lymphangiogenic in vivo was one of the following three forms:

- NH_2-(DP)TEETIK... ...MSKLH_6-COOH 
- NH_2-(DP)AHYNT... ...MSKLD-myc-H_6-COOH 
- NH_2-(DP)FESGL... ...RPQMS-H_6-COOH 

Transgenic mice/Adenoviruses/AAV

- Adenovirus: NH_2-TEETIK... ...MSKL-COOH ?
- Adenovirus: full length
- ???????

Mouse Immunizations

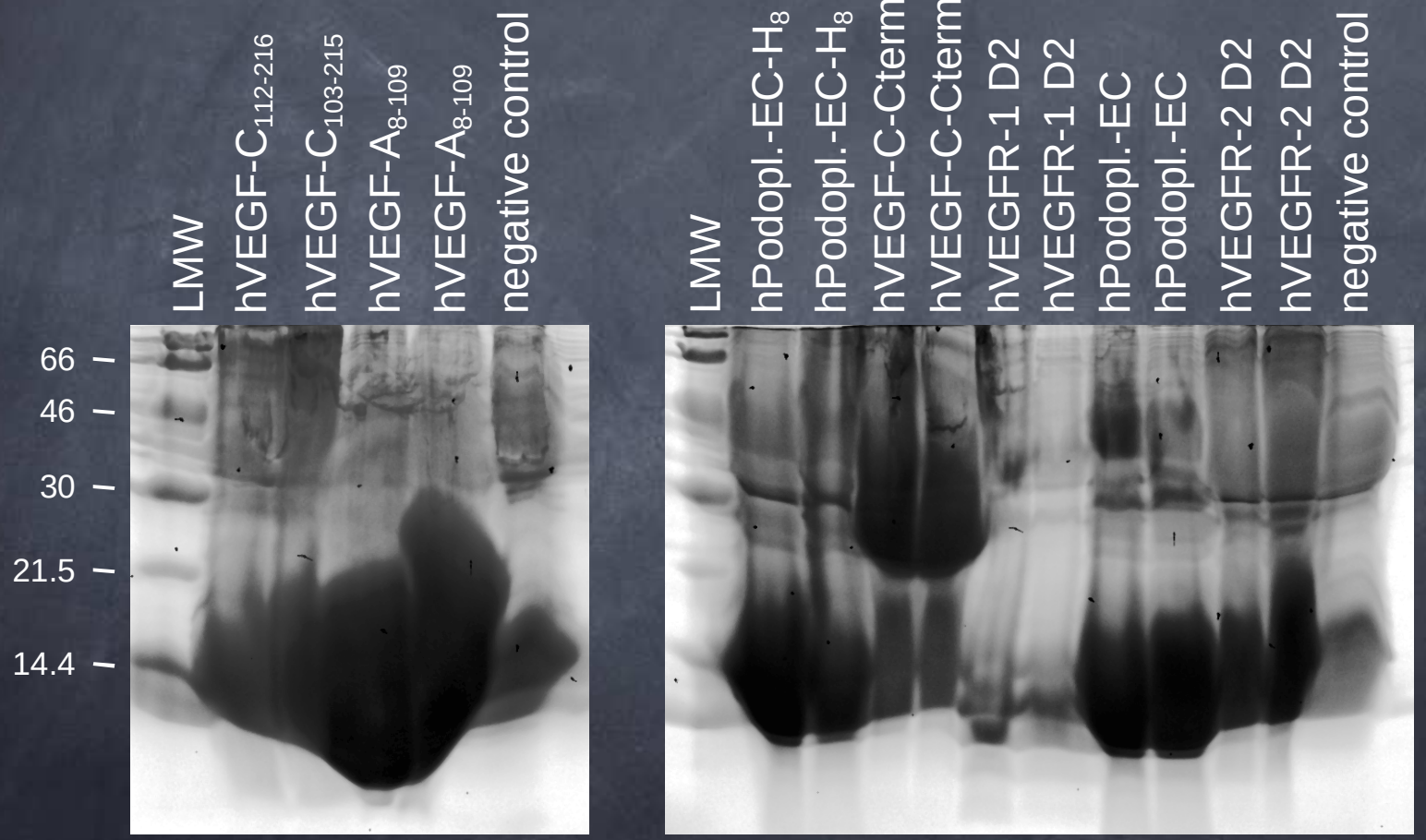
- 1996/97 (Diabor Oy, Matti Höyhtyä): 2 mg of baculoviral ΔNΔC-hVEGF-C-H_6 (DP-TEETIK... ...MSKLH_6)
- 1997/98 (Vijay Kumar/Reija Valtola): yeast ΔNΔC-hVEGF-C-H_6 (EA/EA/YVEF-TEETIK... ...MSKLH_6)
- 2003 (Haartman Institute, Tanja/Salla): yeast protein as above, same expression batch?
- 2003 (HUS, Ulf-Håkan Stenman): 2 mg of baculoviral hVEGF-C-FL-H_6 (DP-FESGLD... ...PQMSH_6)

Addiitonal antibodies?

- No specific antibodies against the C-terminus of VEGF-C
- Most of our polyclonal antibodies have a high background
- Immunopurification
- E.coli protein as antigen and for immunopurification?
- New E.coli proteins:

VEGF-C C-terminus
hPodoplanin-EC-H_8
hPodoplanin-EC
VEGFR-1 domain 2
VEGFR-2 domain 2

Bacterial proteins

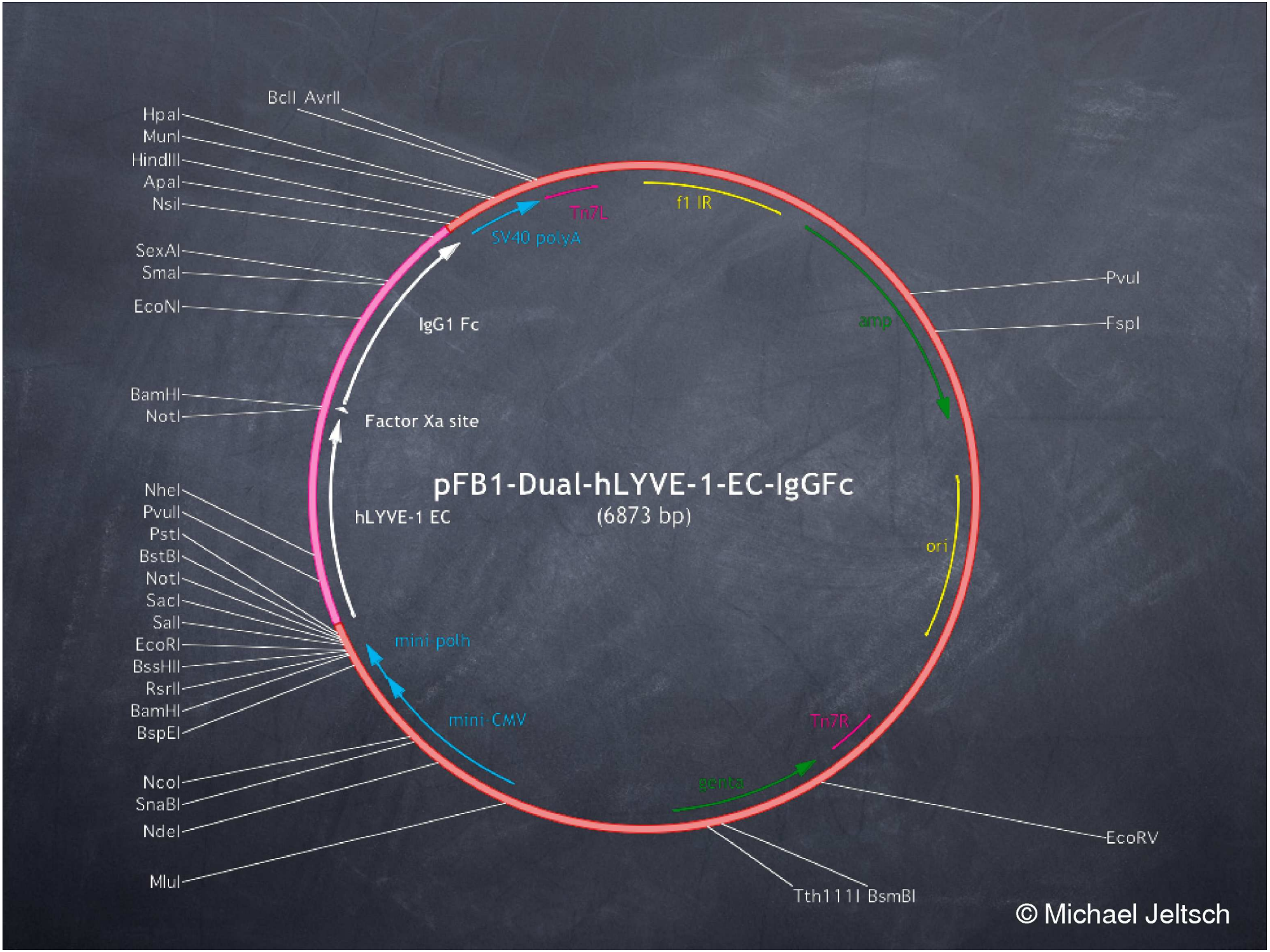


Baculovirus work

- Baculoviruses with Dual Insect/Mammalian Promoter
- Protein Production and Purification
- What sucks about the baculovirus system and insect cells in general

What sucks

- Incubators are constantly broken
- Stock virus production
- Stock virus storage (-70°C)
- No good stock virus anymore for any VEGF-C producing virus due to "suboptimal" storage
  - → titre of full-length VEGF-C virus deteriorated from $0.5 \cdot 10^7$ PFU/ml to 720 PFU/ml
  - → last batch of short VEGF-C yielded only 140µg from 250ml instead of expected ~1mg
  - → new plaque purifications necessary if protein production has to be continued


BclI AvrII
HpaI
MunI
HindIII
ApaI
NsiI
SexAI
SmaI
EcoNI
BamHI
NotI
NheI
PvuII
PstI
BstBI
NotI
SacI
SalI
EcoRI
BssHII
RsrII
BamHI
BspEI
NcoI
SnaBI
NdeI
MluI
Tn7L
SV40 polyA
f1 IR
PvuI
FspI
amp
IgG1 Fc
Factor Xa site
pFB1-Dual-hLYVE-1-EC-IgGFc
hLYVE-1 EC
(6873 bp)
ori
mini polh
mini CMV
Tn7R
gentR
EcoRV
Tth111I BsmBI
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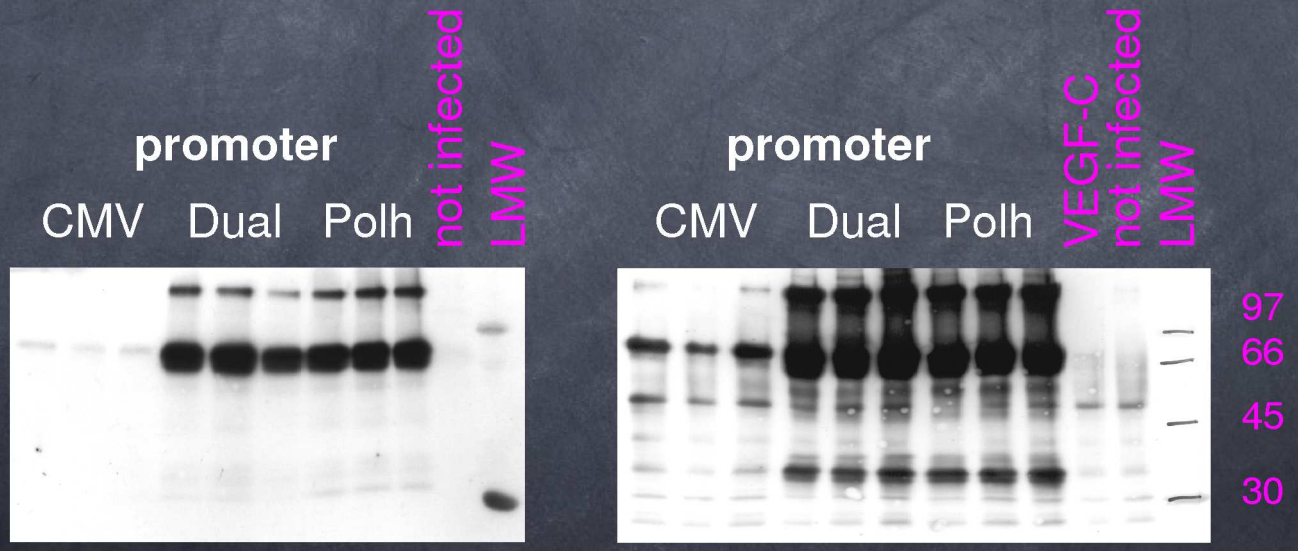
Expression of hLYVE-1-EC-hIgGFc in insect cells (HF)

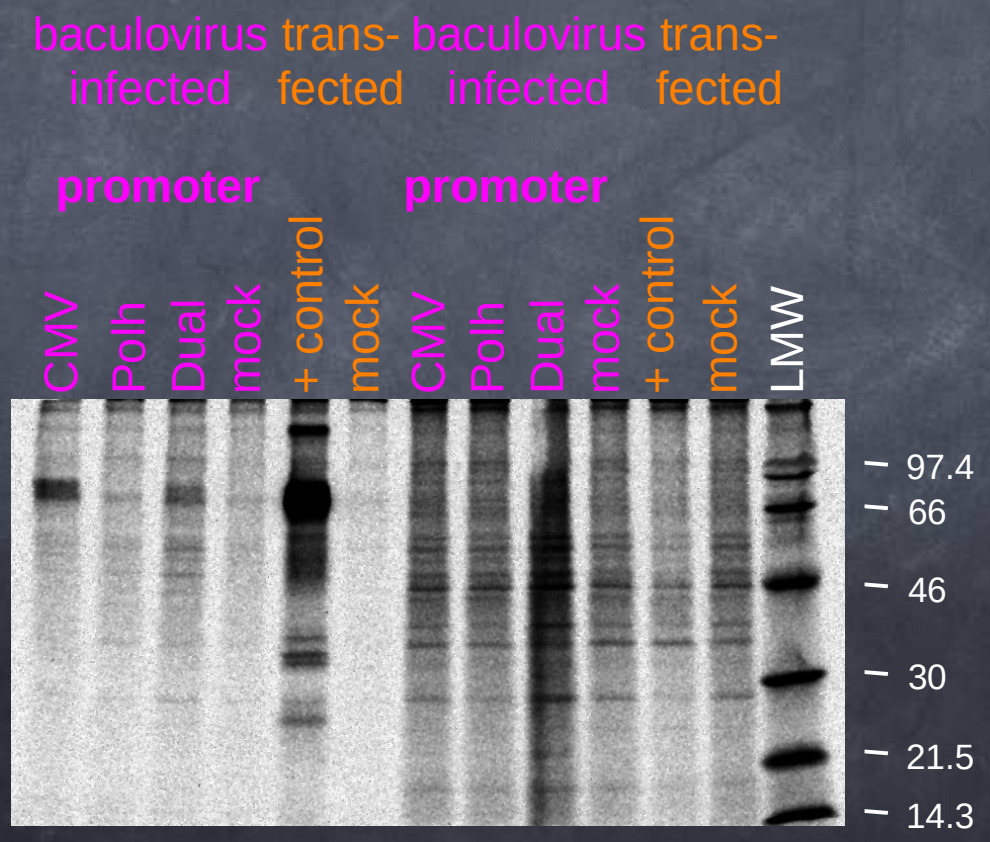
straight Western: anti-hIgG

IP: PAS (from 1 ml sup)
Western: anti-hIgG



Expression of hLYVE-1-EC-hIgGFc in mammalian cells

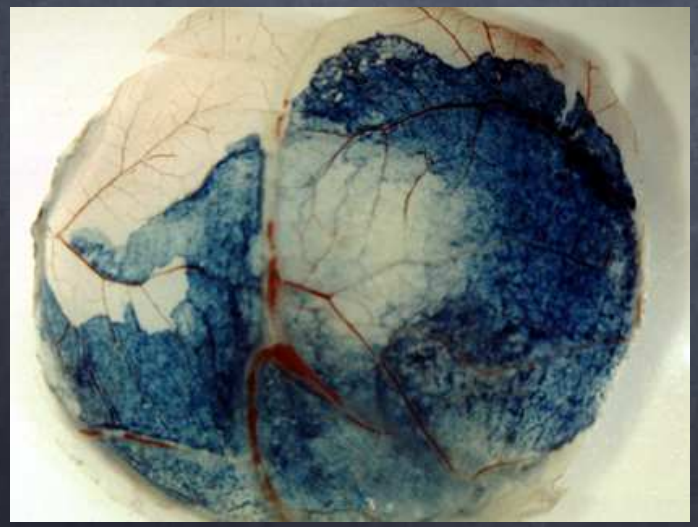
293T Cos-1



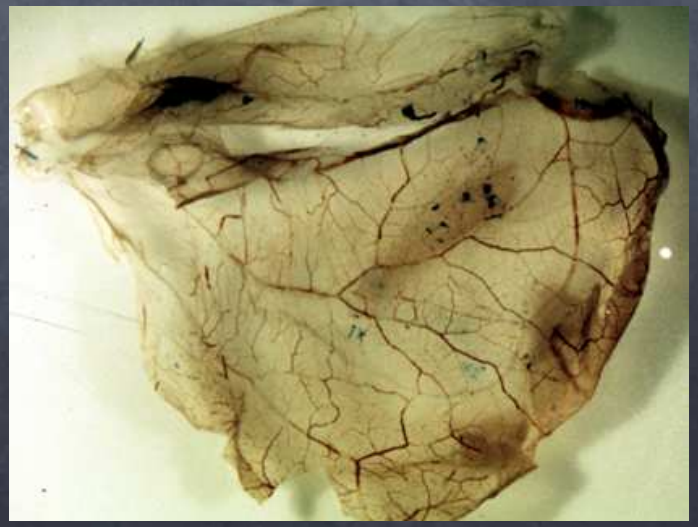
lacZ baculo-infected d 13 CAM

Dual promoter lacZ

infection with concentrated virus, harvest 48 h p.i.

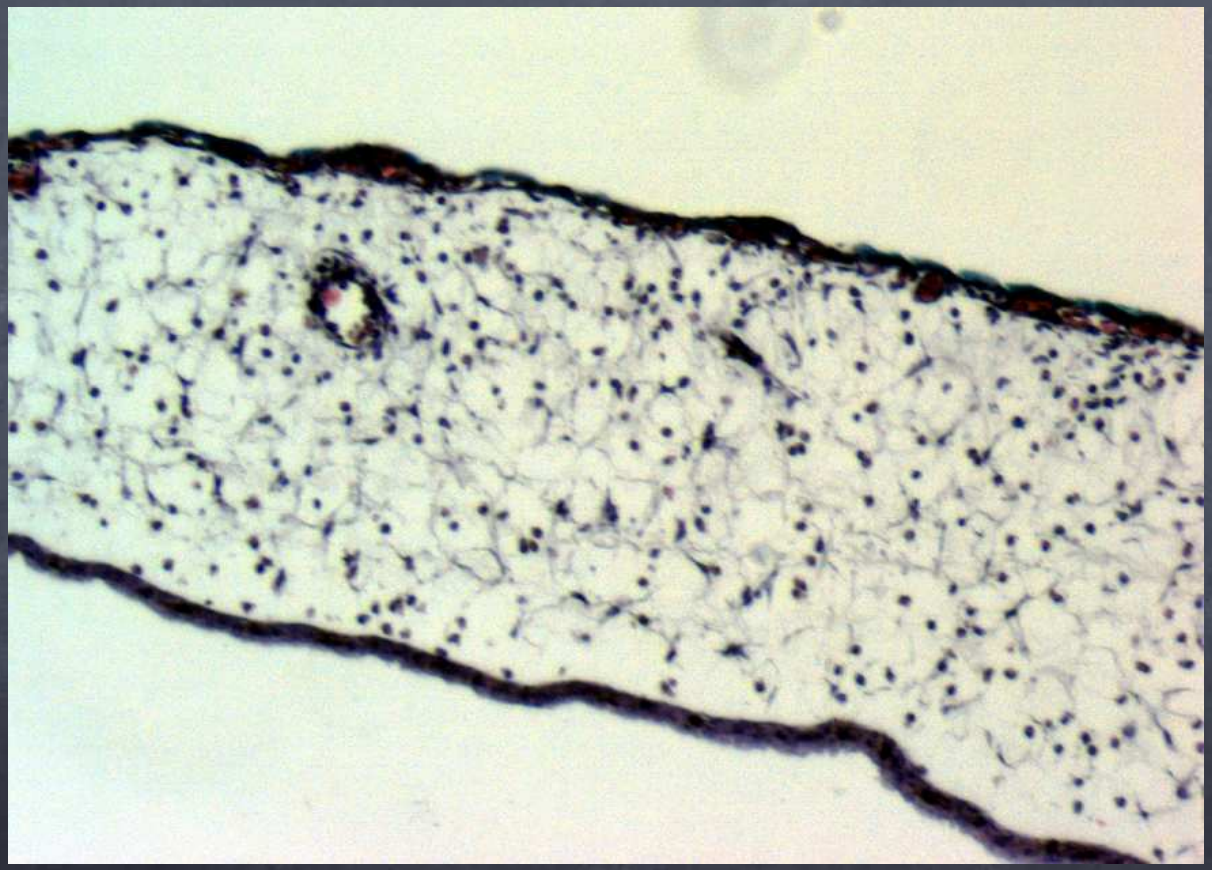


Dual promoter lacZ

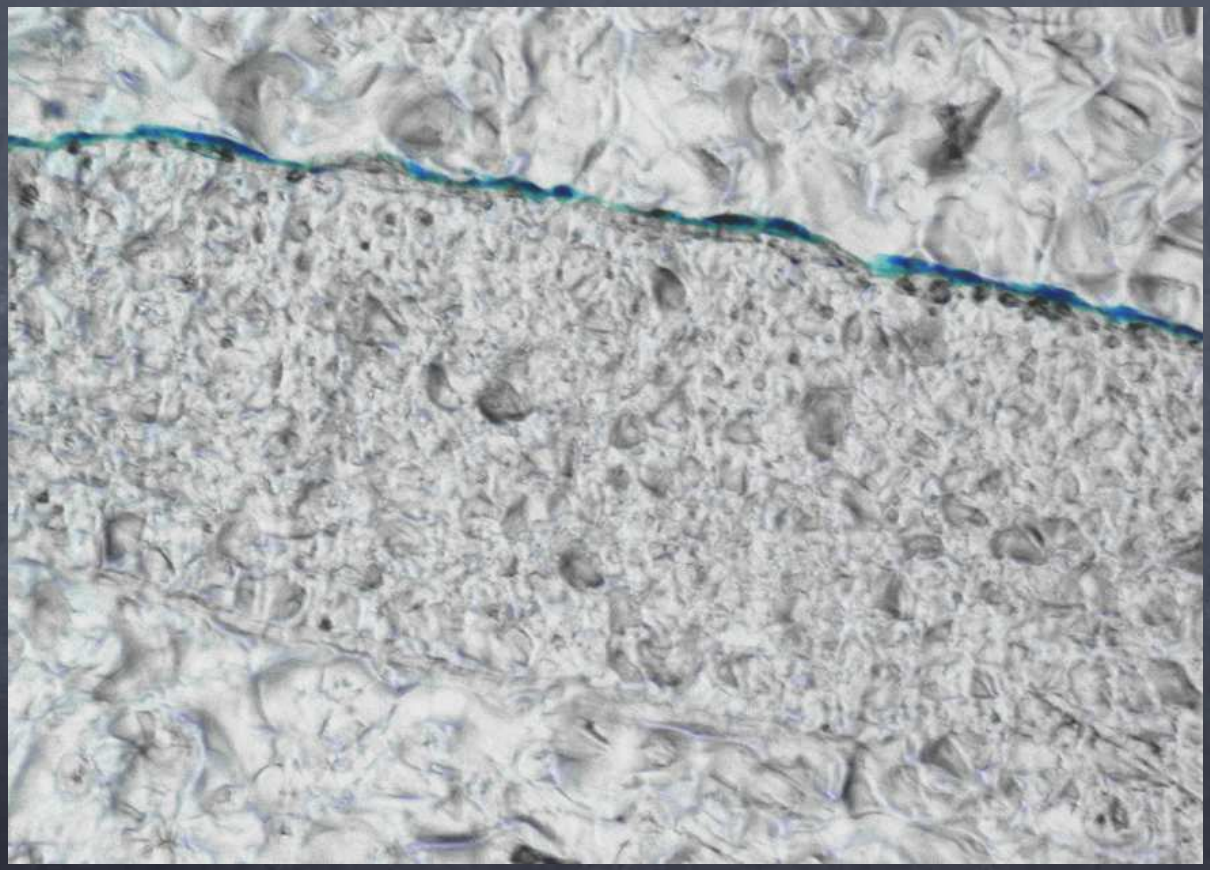


Polh promoter VEGF-C

CAM: H&E



CAM: X-Gal



Virus Purification

Classical purification by ultacentrifugation in a gradient, recovery of infectivity ~5-15%

Chromatography on heparin column, recovery >50%???

Construct Testing

No possibility to test functionality of constructs short of creating the virus

New Baculoviruses for In-vivo Infection

- pFB1-Dual-lacZ-H_6 → works
- pFB1-Dual-IgKSP-hSA-H_{2x6} → works
- pFB1-Dual-IgKSP-CAC-H_6 → no expression
- pFB1-Dual-IgKSP-ACA-H_6 → no expression
- pFB1-Dual-IgKSP-hVEGF-C-Cterminus-H_6 → no expression
- pFB1-Dual-IgKSP-hVEGF-A_{165}-H_6 → untested
- pFB1-Dual-IgKSP-ΔNΔC-hVEGF-C-H_6 → untested
- pFB1-Dual-IgKSP-hVEGF-C-FL-H_6 → untested
- pFB1-Dual-IgKSP-hVEGF-C-SIISS-H_6 → untested
- pFB1-Dual-IgKSP-hVEGF-C-SIISS-FLAG-H_6 → untested

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 Ni2+NTA chromatography	1 day
Dialysis, sterilization & quantitation of peak fraction	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 using approx. 500 ml conditioned medium

Small vs. large scale expression

- Protein yields do not scale
- Small scale expression is inefficient due to losses during purification, dialysis & sterilization (100 ml vs. 500 ml gives 17µg vs. 500µg hVEGF-C-FL)
- Large scale expression of hVEGF-C-FL from baculovirus not feasible (problems of air-lift fermenter: stock virus quantity & quality, cell viability & cell lines, monitoring, control)
- Problem of storing stockvirus (lots of -70°C space needed)
- Stable expression for large scale expression might be easier (Drosophila & InsectSelect)

Drosophila Expression System (DES) Schneider/S2 cells

Advantages over baculovirus system

- Transient or stable
- Induceable or constitutive expression
- Stable cells can be frozen
- No stockvirus needed

Disadvantages

- Expression levels approx. 1/3 of baculovirus system
- S2 cells grow terribly slow
- S2 cells are difficult to thaw
- Selection takes 3 weeks

New stable S2 cell lines

- pMT-bipSP-VEGFR-3-EC/IgGFc (T. Kärpänen)

- pMT-bipSP-hVEGF-C-FL
- pMT-bipSP-hVEGF-C-FL-H_6
- pMT-endSP-hVEGF-C-FL
- pMT-endSP-hVEGF-C-FL-H_6

- pMT-hygro-bipSP-hVEGF-C-FL
- pMT-hygro-bipSP-hVEGF-C-FL-H_6
- v1 pMT-hygro-endSP-hVEGF-C-FL-H_6
- v2 pMT-hygro-endSP-hVEGF-C-FL-H_6
- pMT-hygro-endSP-hVEGF-C-FL

InsectSelect

Similar to DES

- HighFive or Sf9/21 cells: grow faster and are easier to freeze/thaw than S2 cells
- No co-transfection with selection plasmid needed
- No good selection markers available (only zeocin & blasticidin)

Major disadvantage

- Stable HighFive or Sf9/21 cells will become sooner or later infected with baculovirus if baculovirus work is done in the same lab

New InsectSelect stable cell lines

- pIZ-melSP-hVEGF-C-FL-H_6
- pIZ-melSP-hVEGF-C-FL
- pIZ-hVEGF-C-FL-H_6
- pIZ-hVEGF-C-FL

both HighFive and Sf-9 cells
both in serum-free and 10%FCS containing media

Insect cell trouble

Common disadvantages

- All insect cells are relatively difficult to transfect (S2: Only Ca phosphate, max 10%)
- AAV for infection of insect cells and creation of stable cell lines?

Alanin scan of VEGF-C

AHYNTEILKSIDNEWRKTQCMPREVCIDVGKEFGVATNTFFKPPCVSVYRCGGCCNSEGLQCMNTSTSYLSKTLFEITVPLSQGPKPVTISFANHTSCRCMSKLD

F
G
V
FGV
T
N
T
F
TNTF
P
C
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S
V
Y
SVY
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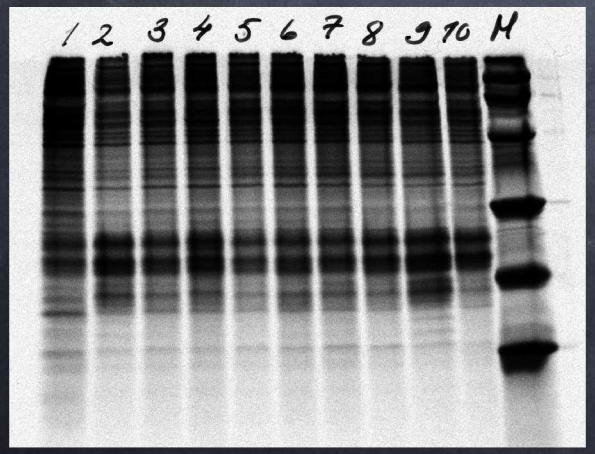
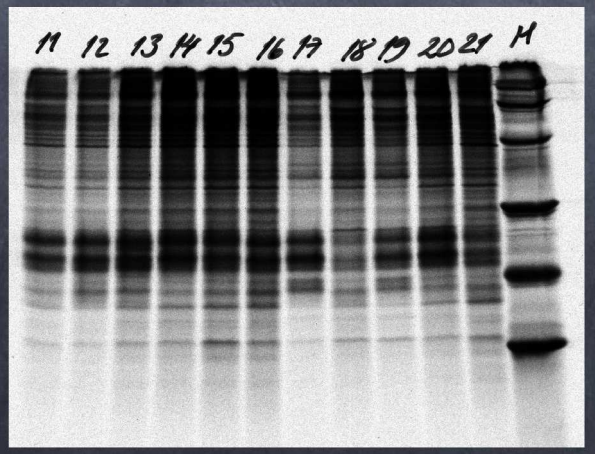
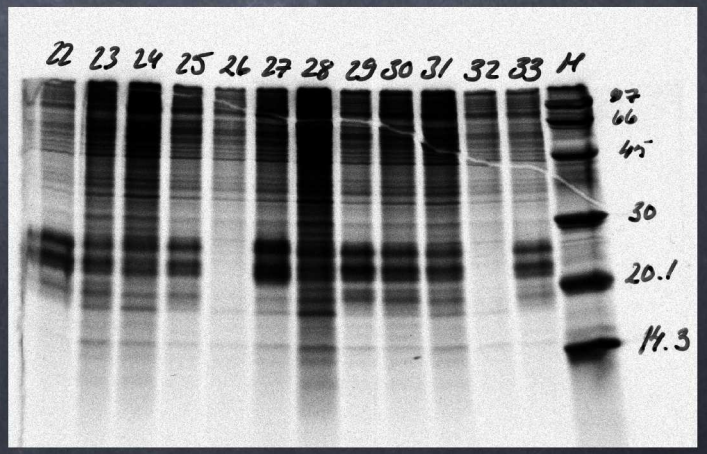
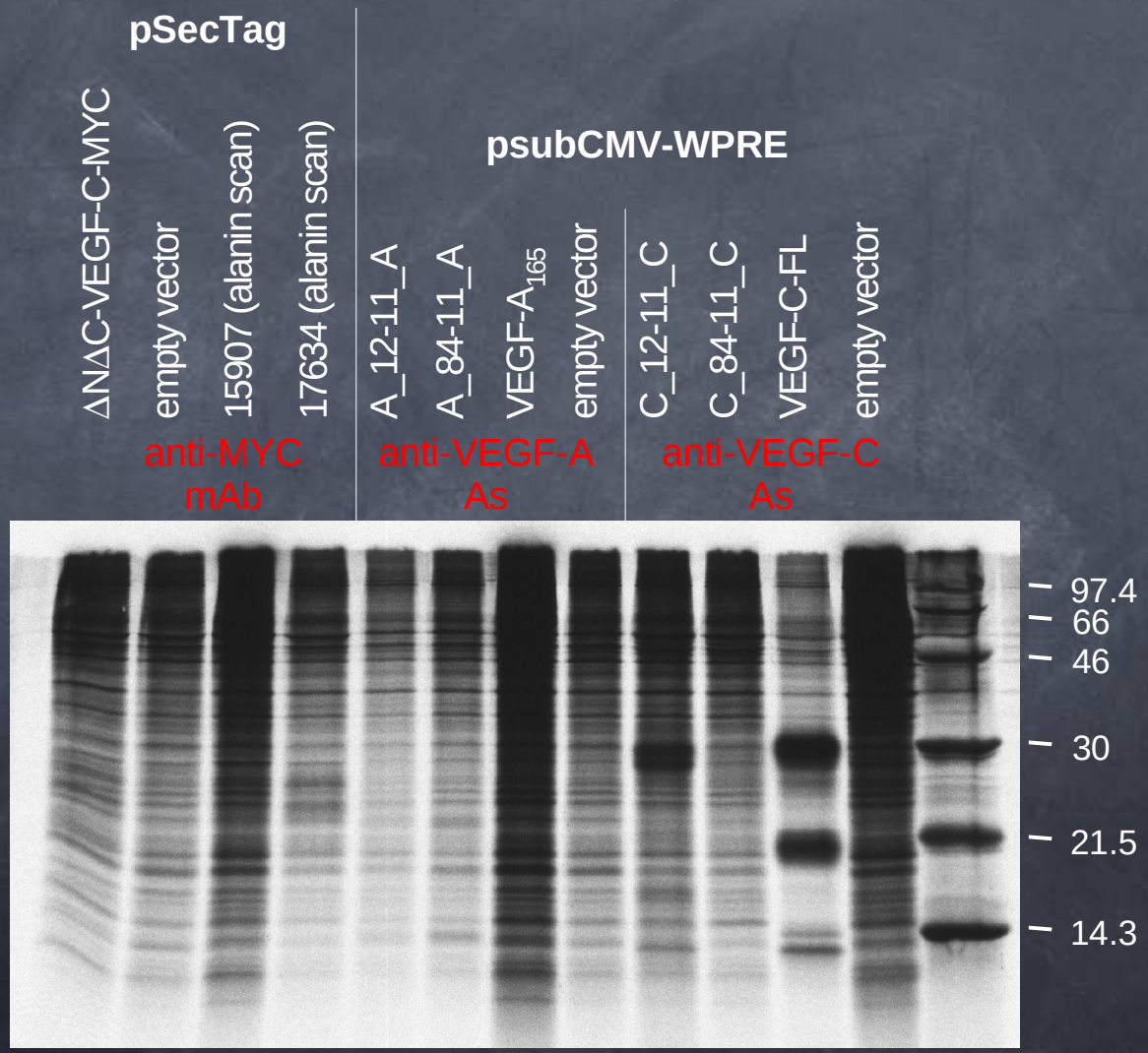






→ around 30 additional mutants due to overcycling

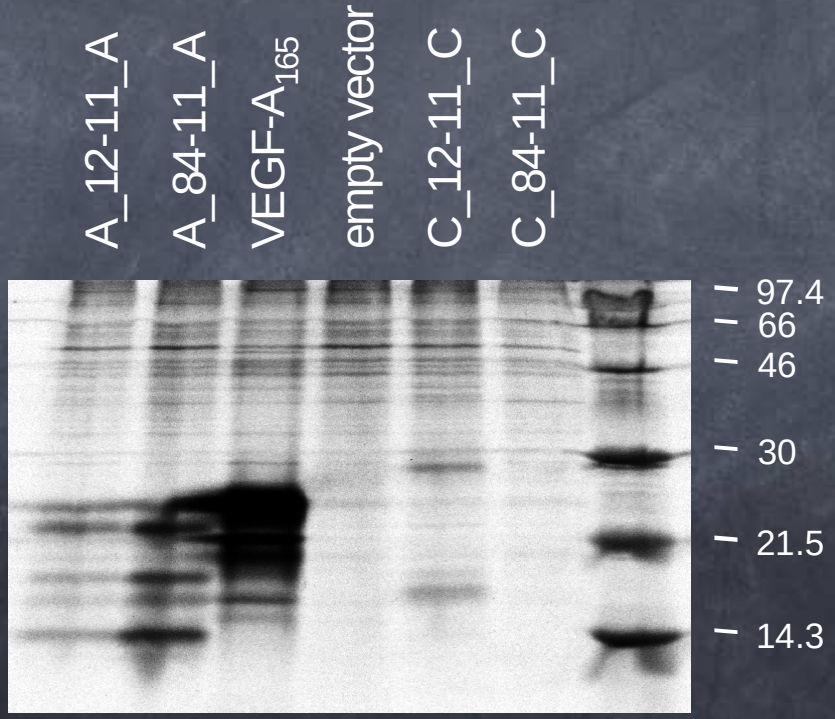
AAV constructs of Hybrids



Antibody didn't work

psubCMV-WPRE

hVEGF-R1/IgGF$_c$



Refolding

- 32 different conditions
  sparse matrix screen according to Chen & Gouaux (1997)
  and all published VEGF/PDGF refolding conditons
- Buy ready if you can
- 5 different proteins
  hVEGF-$A_{8\text{-}109}$ (as published)
  hVEGF-$C_{112\text{-}216}$ (equivalent to hVEGF-A8-109)
  hVEGF-$C_{103\text{-}215}$ (equivalent to baculovirus short VEGF-C protein)
  hVEGFR-1 domain 2 (as published)
  hVEGFR-2 domain 2 (equivalent to hVEGFR-1 domain 2)

Testing refolded proteins

- Stimulation of tyrosine phosphorylation (PAE-VEGFR-3 cells) with dialyzed refolded protein
- all cells died probably to wrong concentration of puromycin stock solution

Proposed VEGF-C naming

- VEGF-$C_{103-227}$ TEETIK... ...SIIRR
  native short VEGF-C (PC3 cells)
- VEGF-$C_{112-227}$ AHYNT... ...SIIRR
  native short VEGF-C (293 cells)
- VEGF-$C_{103-215}$ TEETIK... ...MSKL
  short baculovirus protein, crystallization
- VEGF-$C_{112-216}$ AHYNT... ...MSKLD
  crystallization

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