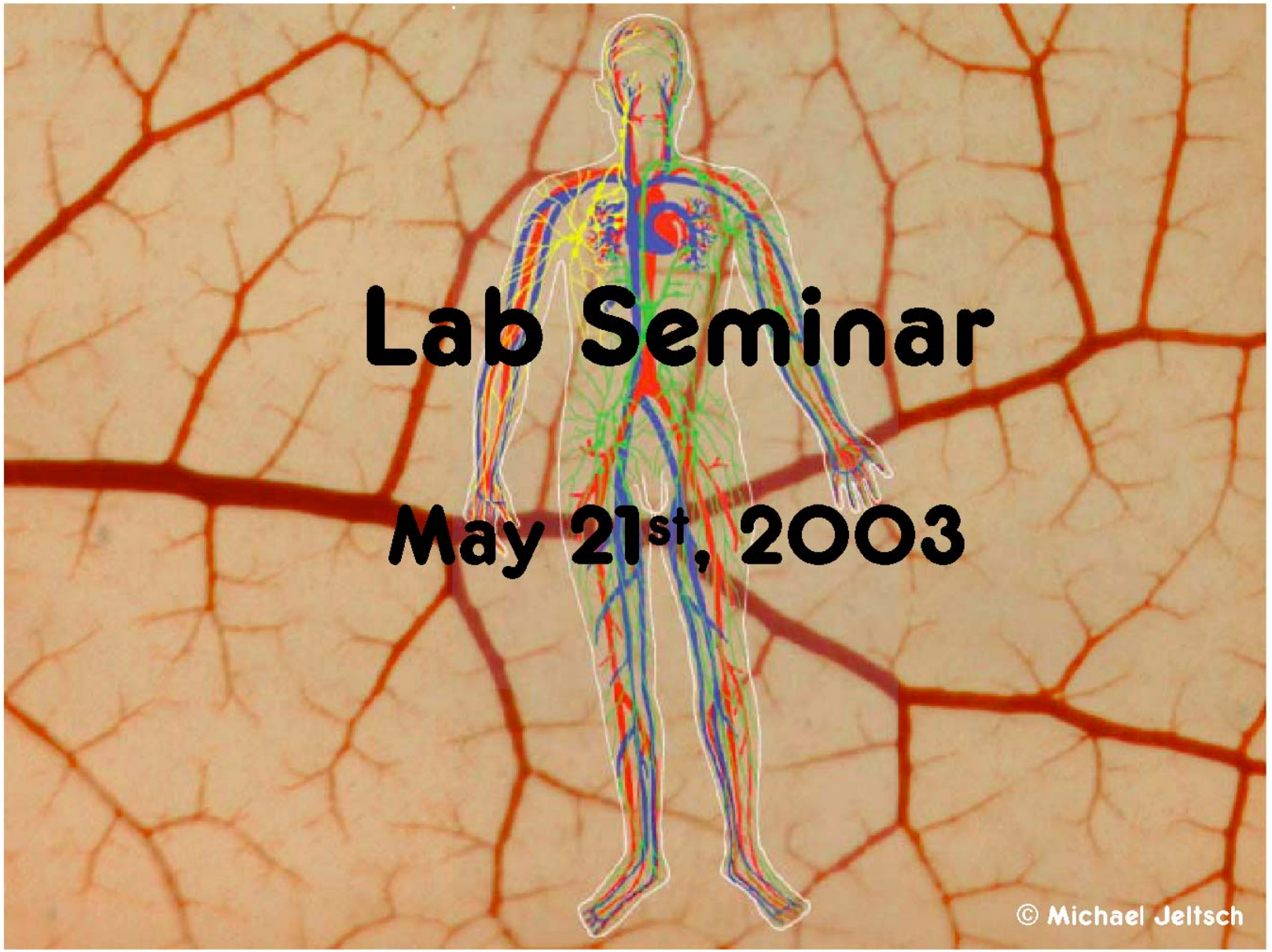
Lab Seminar
May 21st, 2003
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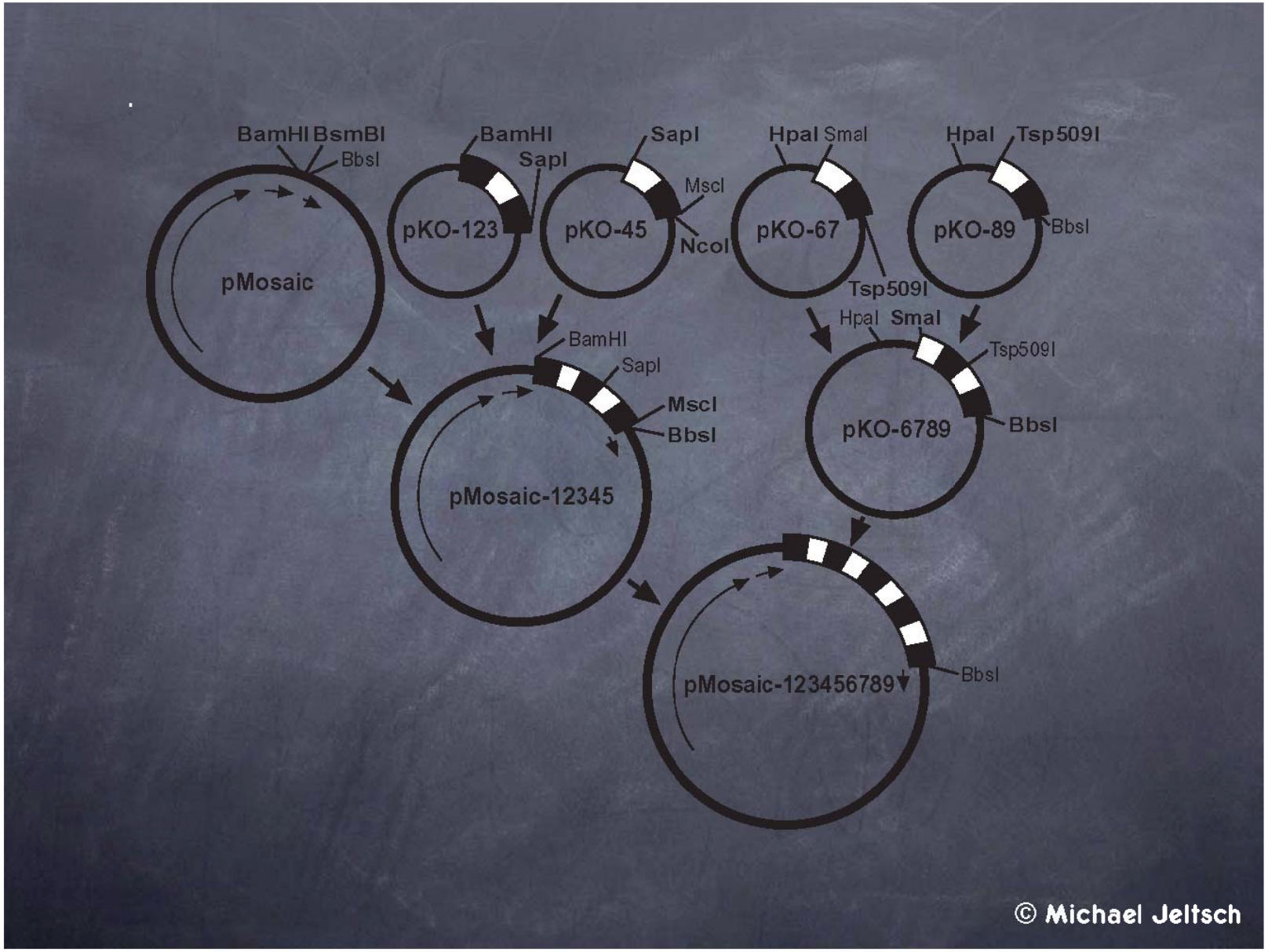
knockout (nɔkaut)

knowledge, knob, knee

- VEGF-A/VEGF-C Hybrids
- Additional experiments done/to do (asymetric PCR, partial digests, mass preps)
- VEGFR-2 and VEGFR-3 domains necessary for VEGF-C binding (why linear thinking is bad)
- Crystal structure of VEGF-C
- VEGF-E as selective activator of VEGFR-2
- New adventures in propeptide domain swaps between VEGF-A and VEGF-C
- What you should know about VEGF-C
- Baculovirus work


BamHI BsmBI
BbsI
pMosaic
BamHI
SapI
pKO-123
SapI
pKO-45
MscI
NcoI
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
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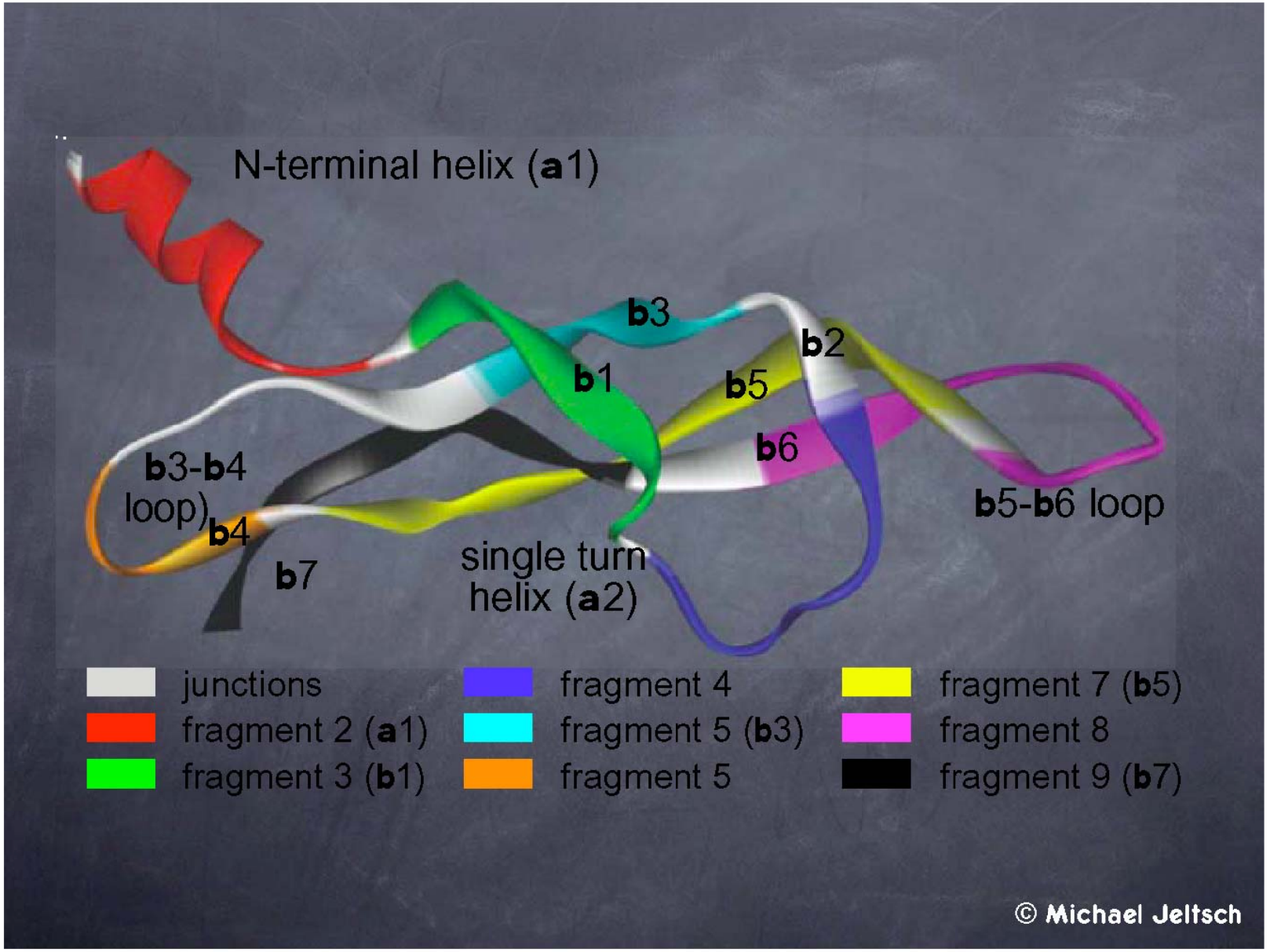
N-terminal helix (a1)
b3
b2
b1
b5
b6
b3-b4
loop)
b4
b7
single turn
helix (a2)
b5-b6 loop
junctions
fragment 2 (a1)
fragment 3 (b1)
fragment 4
fragment 5 (b3)
fragment 5
fragment 7 (b5)
fragment 8
fragment 9 (b7)
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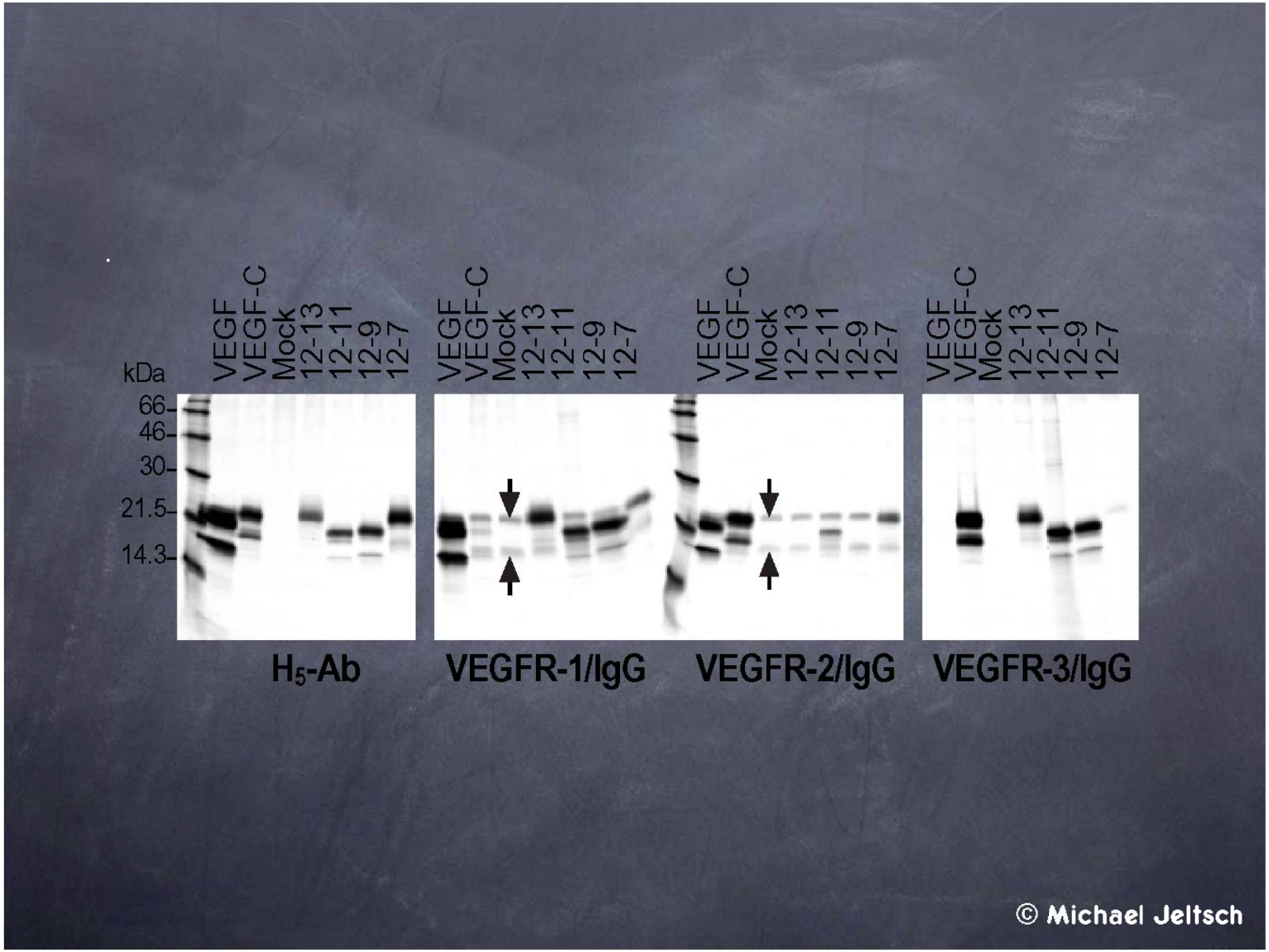
VEGF
VEGF-C
Mock
12-13
12-11
12-9
12-7
kDa
66
46
30
21.5
14.3
H5-Ab
VEGFR-1/IgG
VEGFR-2/IgG
VEGFR-3/IgG
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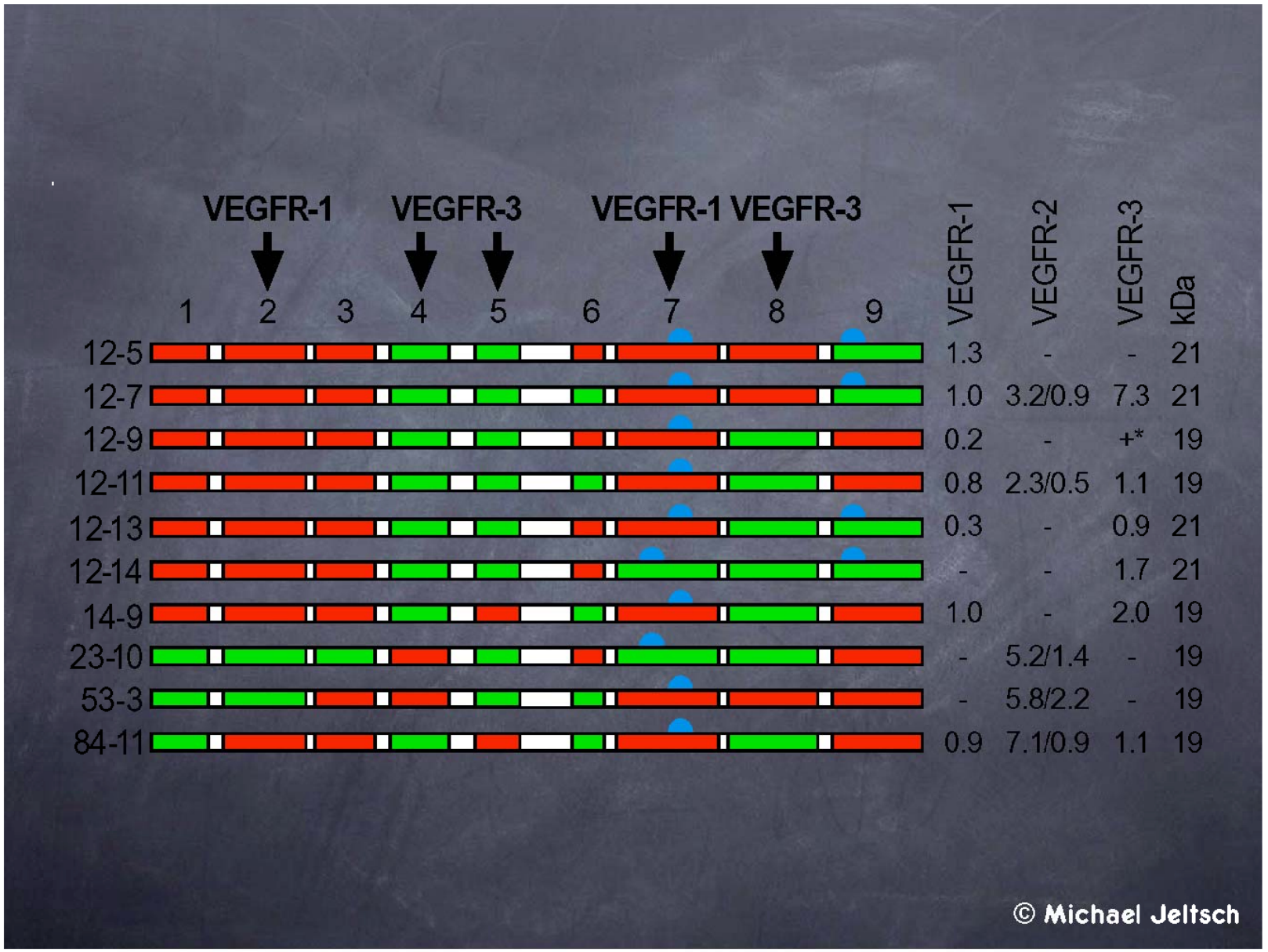
VEGFR-1
VEGFR-3
VEGFR-1
VEGFR-3
1
2
3
4
5
6
7
8
9
VEGFR-1
VEGFR-2
VEGFR-3
kDa
12-5 1.3 - - 21
12-7 1.0 3.2/0.9 7.3 21
12-9 0.2 - +* 19
12-11 0.8 2.3/0.5 1.1 19
12-13 0.3 - 0.9 21
12-14 - - 1.7 21
14-9 1.0 - 2.0 19
23-10 - 5.2/1.4 - 19
53-3 - 5.8/2.2 - 19
84-11 0.9 7.1/0.9 1.1 19
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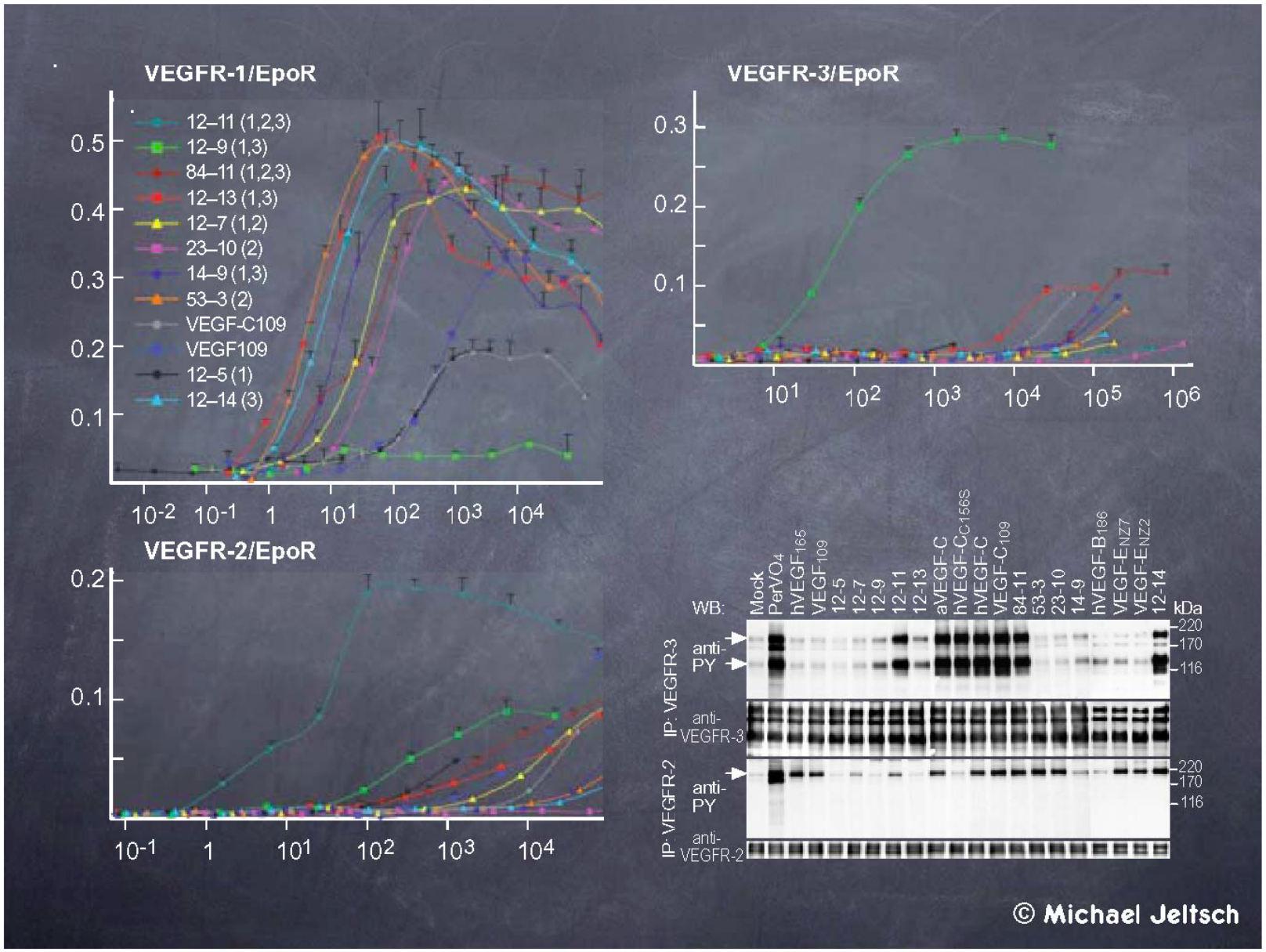
VEGFR-1/EpoR
12–11 (1,2,3)
12–9 (1,3)
84–11 (1,2,3)
12–13 (1,3)
12–7 (1,2)
23–10 (2)
14–9 (1,3)
53–3 (2)
VEGF-C109
VEGF109
12–5 (1)
12–14 (3)
0.5
0.4
0.3
0.2
0.1
10-2 10-1 1 101 102 103 104
VEGFR-3/EpoR
0.3
0.2
0.1
101 102 103 104 105 106
VEGFR-2/EpoR
0.2
0.1
10-1 1 101 102 103 104
WB:
Mock
PerVO4
hVEGF165
VEGF109
12-5
12-7
12-9
12-11
12-13
aVEGF-C
hVEGF-CC156S
hVEGF-C
VEGF-C109
84-11
53-3
23-10
14-9
hVEGF-B186
VEGF-ENZ7
VEGF-ENZ2
12-14
kDa
220
170
116
IP: VEGFR-3
anti-PY
anti-VEGFR-3
IP: VEGFR-2
anti-PY
anti-VEGFR-2
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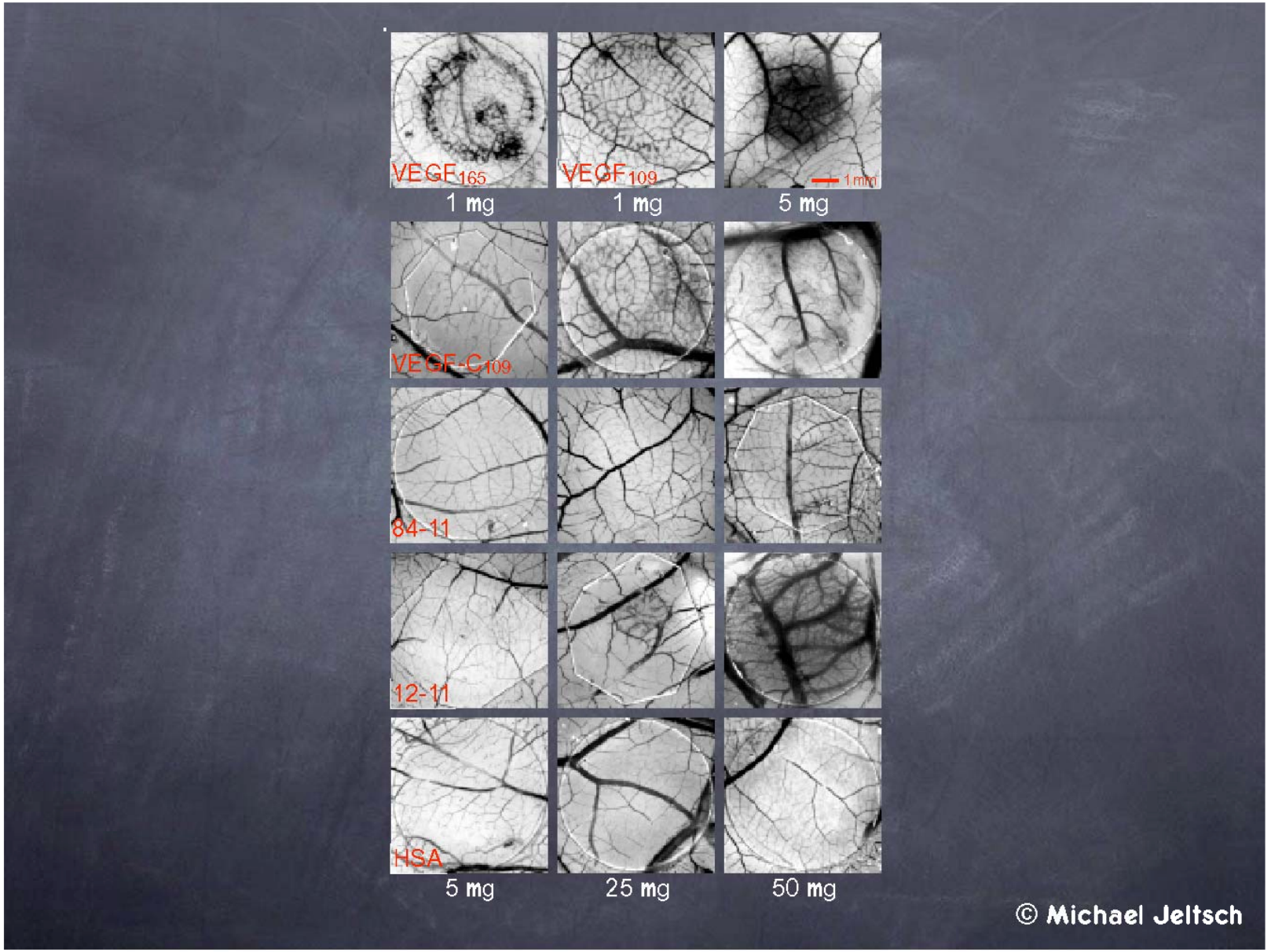
VEGF165
VEGF109
1 mm
1 mg
1 mg
5 mg
VEGF-C109
84-11
12-11
HSA
5 mg
25 mg
50 mg
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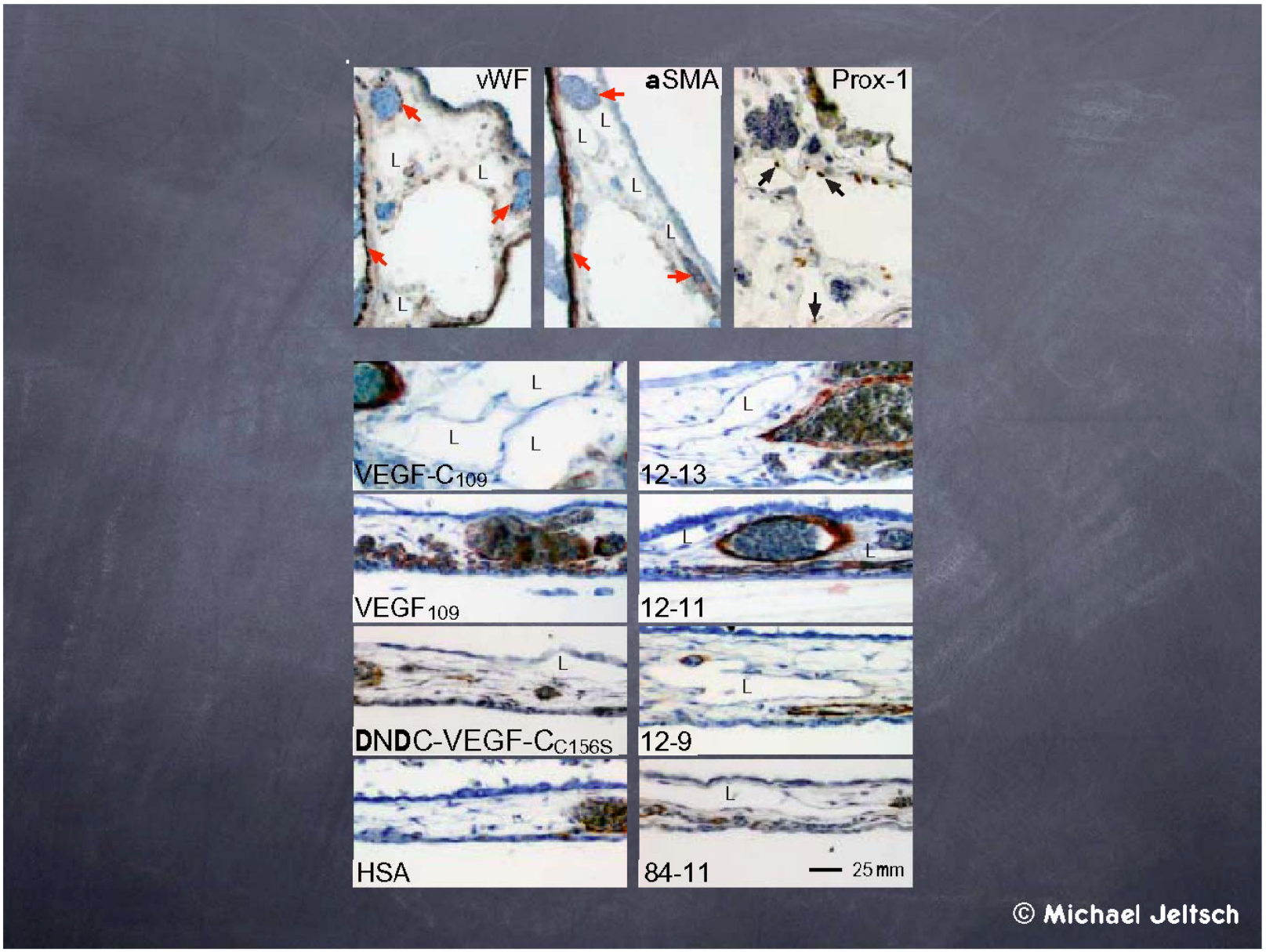
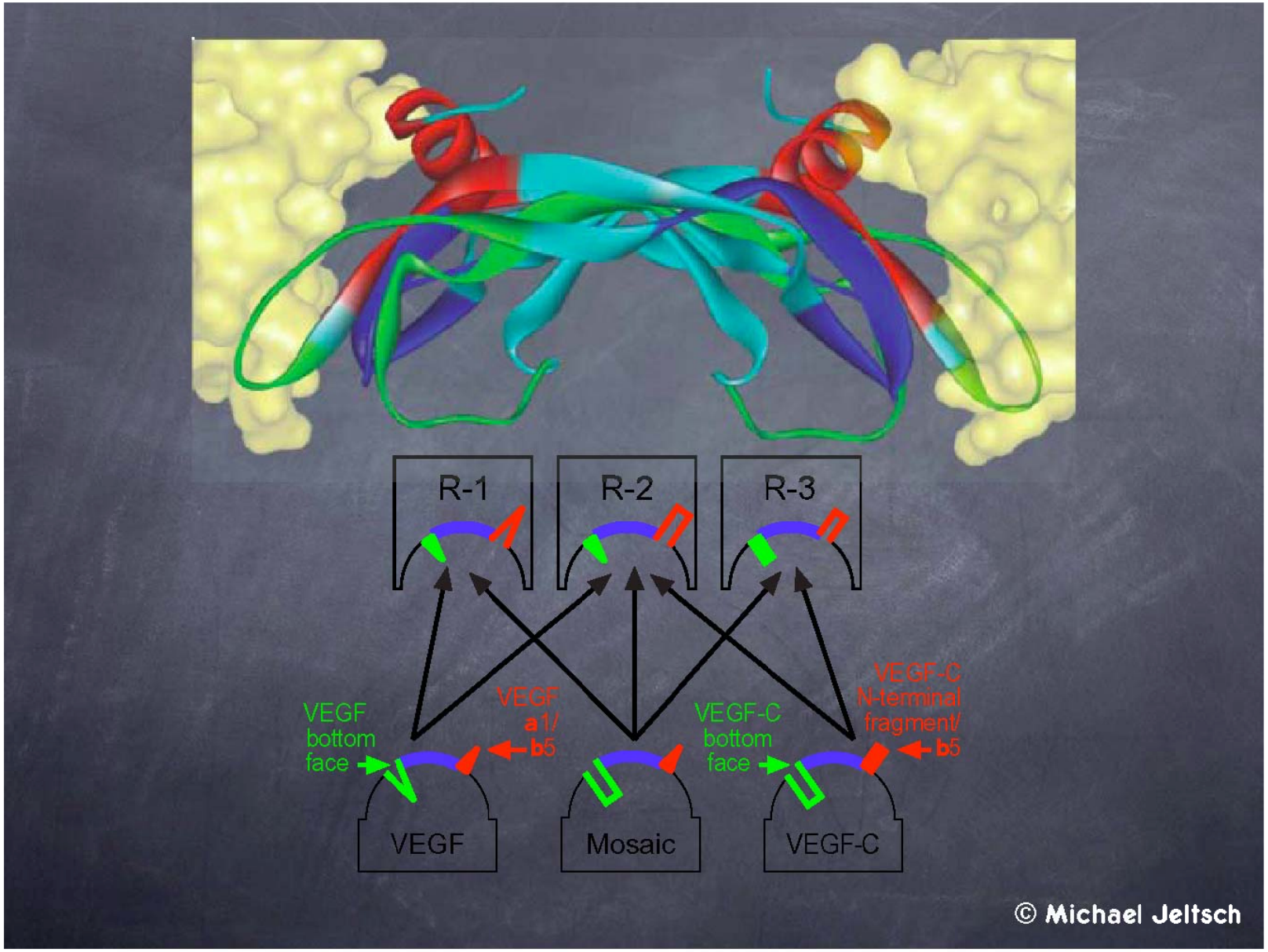
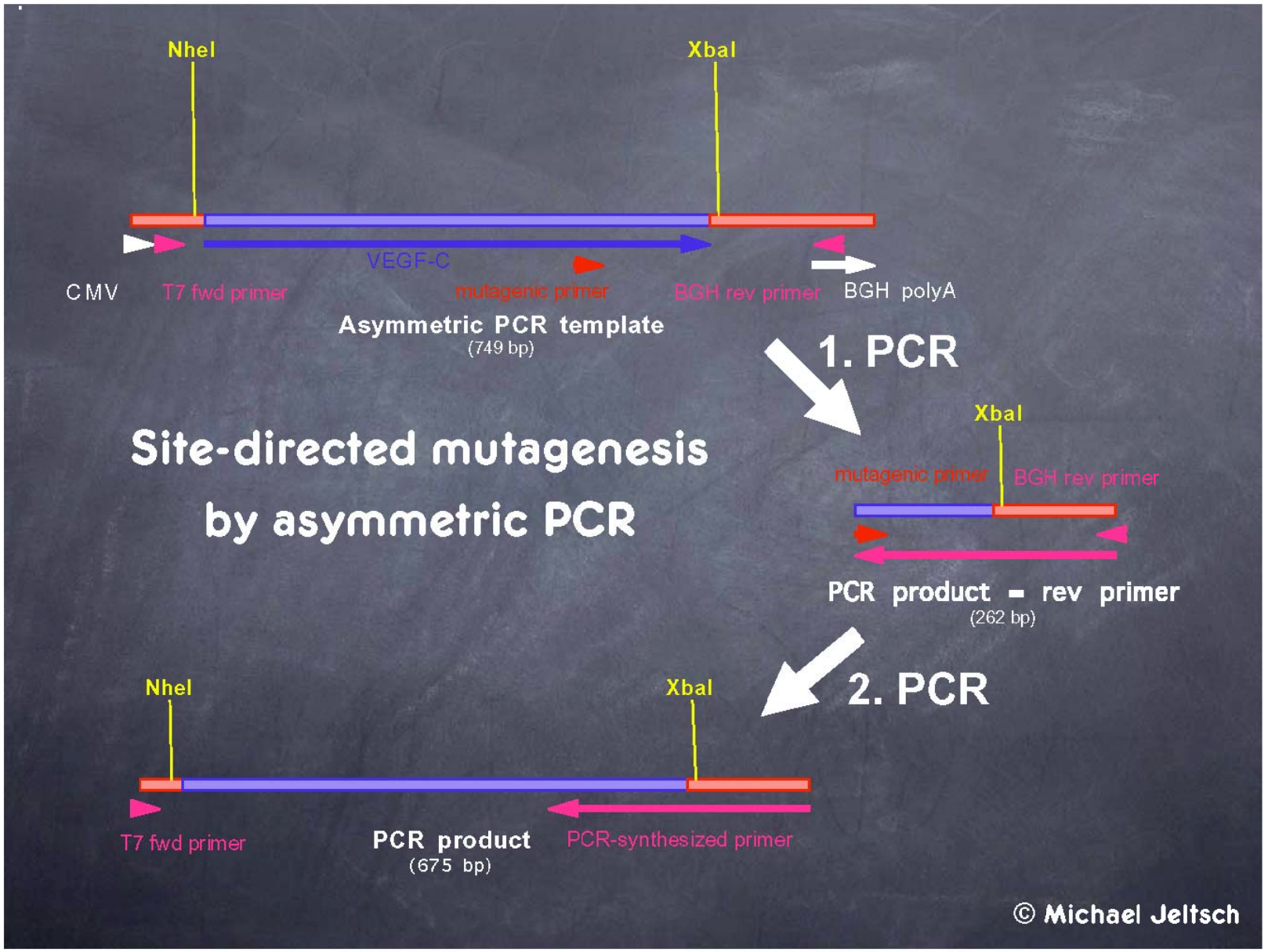



R-1
R-2
R-3
VEGF
bottom
face
VEGF
a1/
b5
VEGF-C
bottom
face
VEGF-C
N-terminal
fragment/
b5
VEGF
Mosaic
VEGF-C
© Michael Jeltsch

Alaninscan of VEGF-C fragments 4, 5 & 8

GeneEditor (dsDNA) → MutaGene (ssDNA) → asymmetric PCR mutagenesis

still 4 constructs missing


NheI
XbaI
VEGF-C
CMV
T7 fwd primer
mutagenic primer
BGH rev primer
BGH polyA
Asymmetric PCR template
(749 bp)
1. PCR
Site-directed mutagenesis
by asymmetric PCR
XbaI
mutagenic primer
BGH rev primer
PCR product = rev primer
(262 bp)
2. PCR
NheI
XbaI
T7 fwd primer
PCR product
(675 bp)
PCR-synthesized primer
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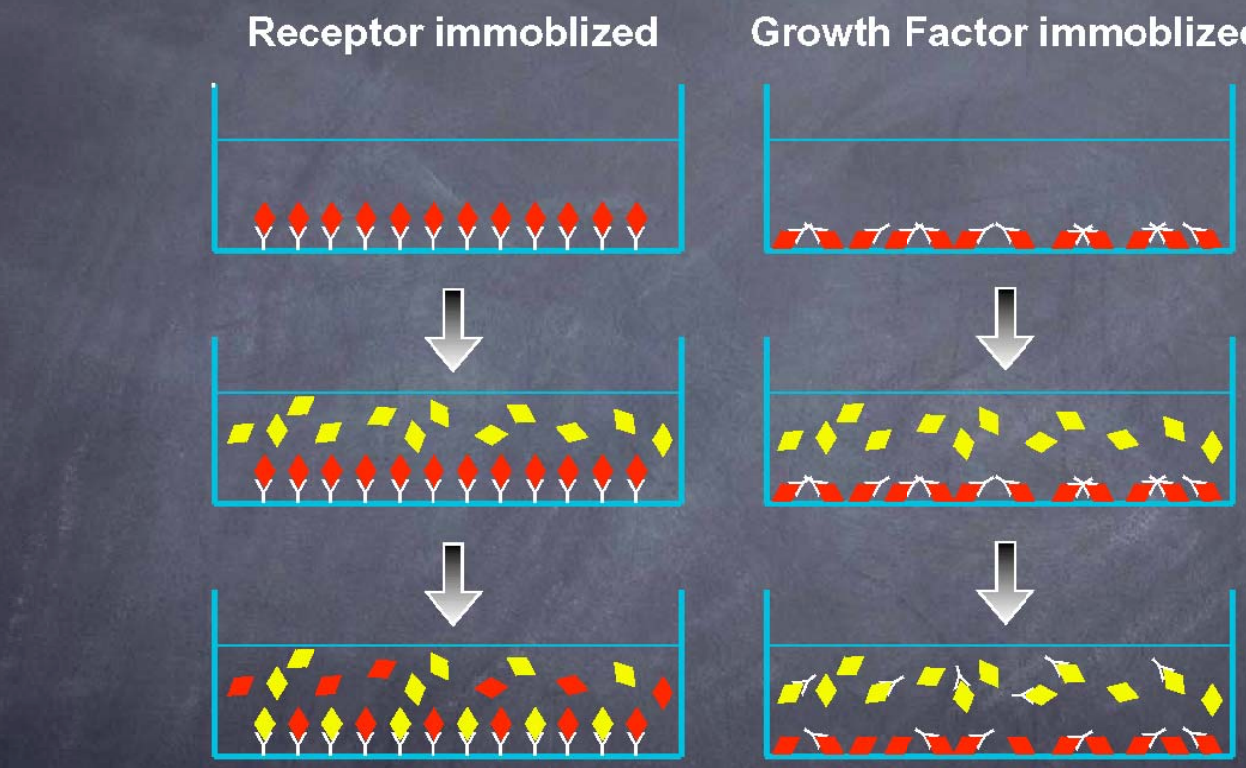
Determination of Relative Affinities of Hybrid Molecules

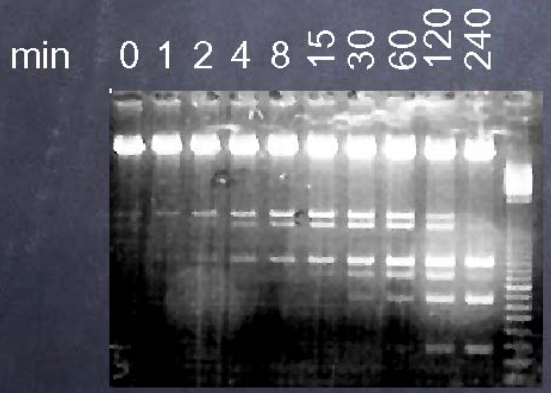


Hybrid-myc-his$_6$ VEGF-C-his$_6$ VEGFR-hIgGF$_C$

Use of VEGF-A/C hybrids in vivo

- $VEGF_{109}$ is compromised in its angiogenic potency compared to $VEGF\text{-}A_{165}$
- The best hybrid VEGFR-2 agonist (12–11) has a 3-fold lower affinity than $VEGF\text{-}A_{109}$
- The best VEGFR-3 agonist (84–11) is only slightly less affine for VEGFR-3 than VEGF-C
- All have a very well preserved affinity to VEGFR-1
- Embedding into VEGF-A or VEGF-C propeptides

Partial Digests are easy!



BssHII cuts	*resulting fragment sizes*
nowhere	11985
only at A	10000, 1985
only at B	10250, 1735
only at C	10900, 1085
at A+B	10000, 1735, 250
at A+C	10000, 1085, 900
at B+C	10250, 1085, 650
all sites	10000, 1085, 650, 250

- Select an enzyme combination that allows isolation of your desired fragment.
- Only do partial digests of linearized plasmids.
- Calculate the exact amount of enzyme needed to completely digest 100 µg of plasmid in 2h.
- Start digest and take aliquots at t = 0, 1, 2, 4, 8, 15, 30, 60, etc. min
- Stop reaction by adding proteinase K and incubating at 50°C for 10 min
- Heat inactivation works for some enzymes, especially if you want to blunt your fragment thereafter, be aware of the time lag!

How do I calculate the exact amount of enzyme needed for complete digestion?

1 unit of a restriction enzyme cuts 1 µg of assay DNA in 1 hour

How many ml of BssHII did I add to 80 µg of the plasmid shown above?

concentration (BssHII) = 20,000 units/ml
assayed on λ (~48 kb)
BssHII cuts λ 6 times
enzyme survival: 0.5 units required for digestion of 1 µg assay DNA in 16 h

$$\text{needed units enzyme for complete digest in 1 h} = \frac{(\mu g \text{ target DNA})\,(\text{cuts in target DNA})\,(\text{size of assay-DNA in kb})}{(\text{cuts on assay-DNA})\,(\text{size of target DNA in kb})}$$

Mass prep trouble

Situation

- Yield has been decreasing over the last years (from approx. 0.5–1.5 mg/prep to 0.2–0.5 mg/prep)
- Quality has been decreasing (both measured by OD ratio and unsoluble material in the prep)
- Occasional to frequent quasi-complete loss of DNA during isoprop precipitation, ethanol wash of pellet and resuspension

Qiagen vs. MN

- Qiagen massprep kit & MN Nucleobond AX kit are completely identical apart from the silica-matrix pore size & side chains (Nuclobond has bigger pore sizes and a higher Si^{4+} saturation)

  Large plasmids should have better recovery from MN columns while Quiagen columns should exclude slightly better shared genomic DNA

General problem

- *Technical staff partially doesn't follow instructions, documentation*

Bad quality most likely to

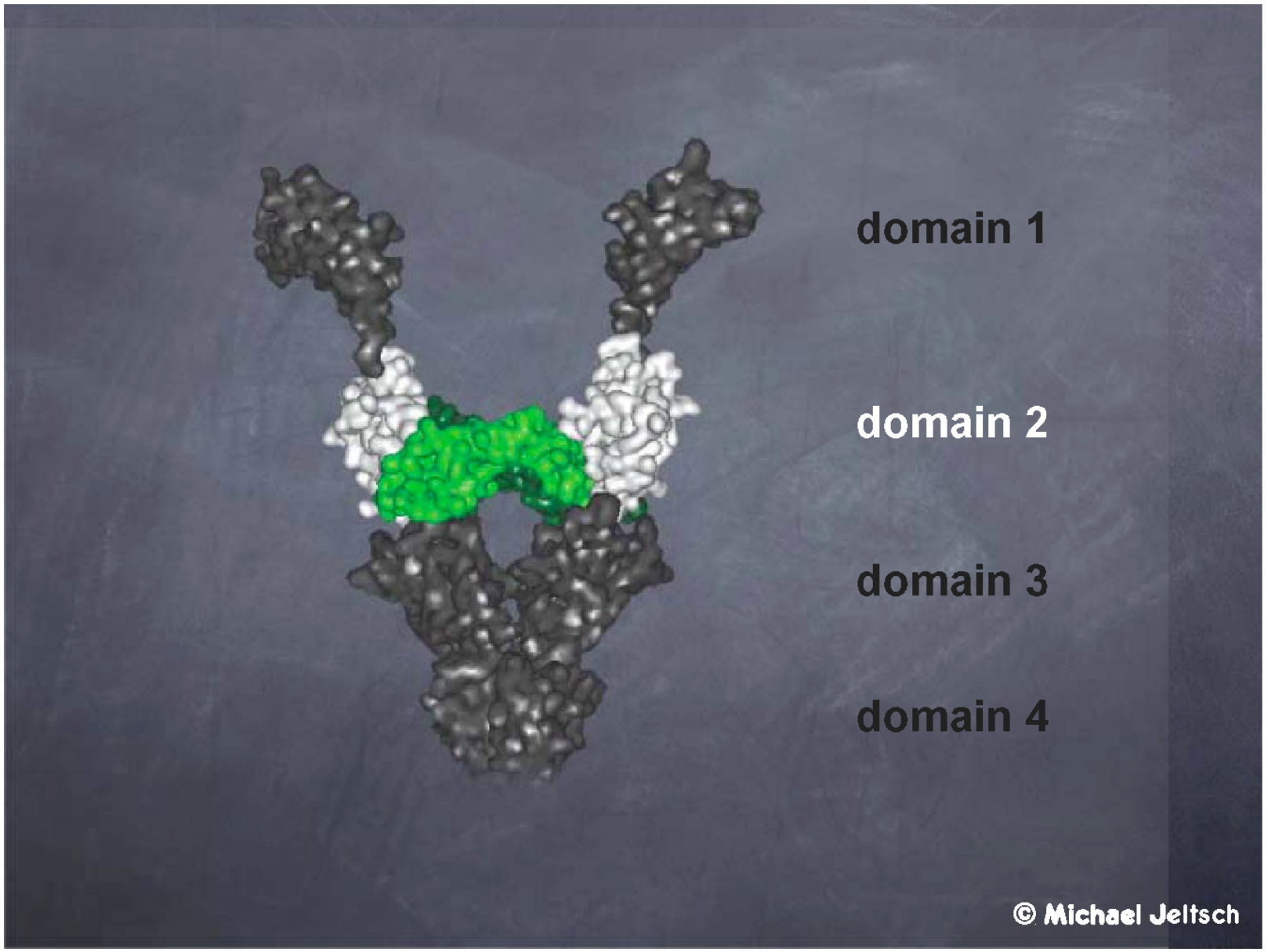
- *Detergent and particular remains from a) kitchen b) introduction during prep (old glass and plastic ware, non-filtered solutions, working sterile)*
- *For sensitive transfections: Endotoxin-free kits*
- *Insoluble pellet of unknown origin under investigation*

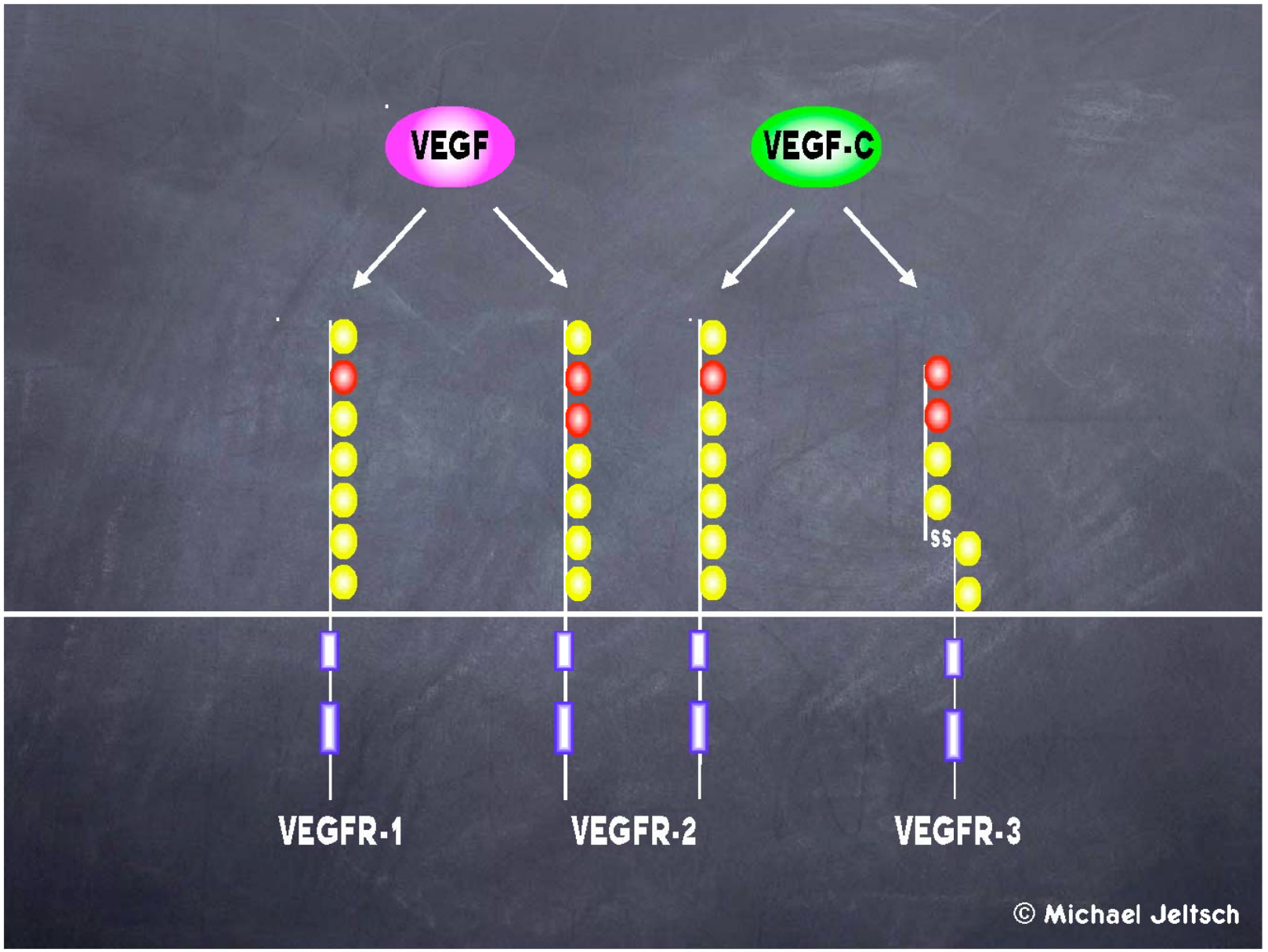
Low/Absent concentration partly due to

- *Overdrying of pellet and insufficient disolution*
- *Precipitation & ethanol wash*

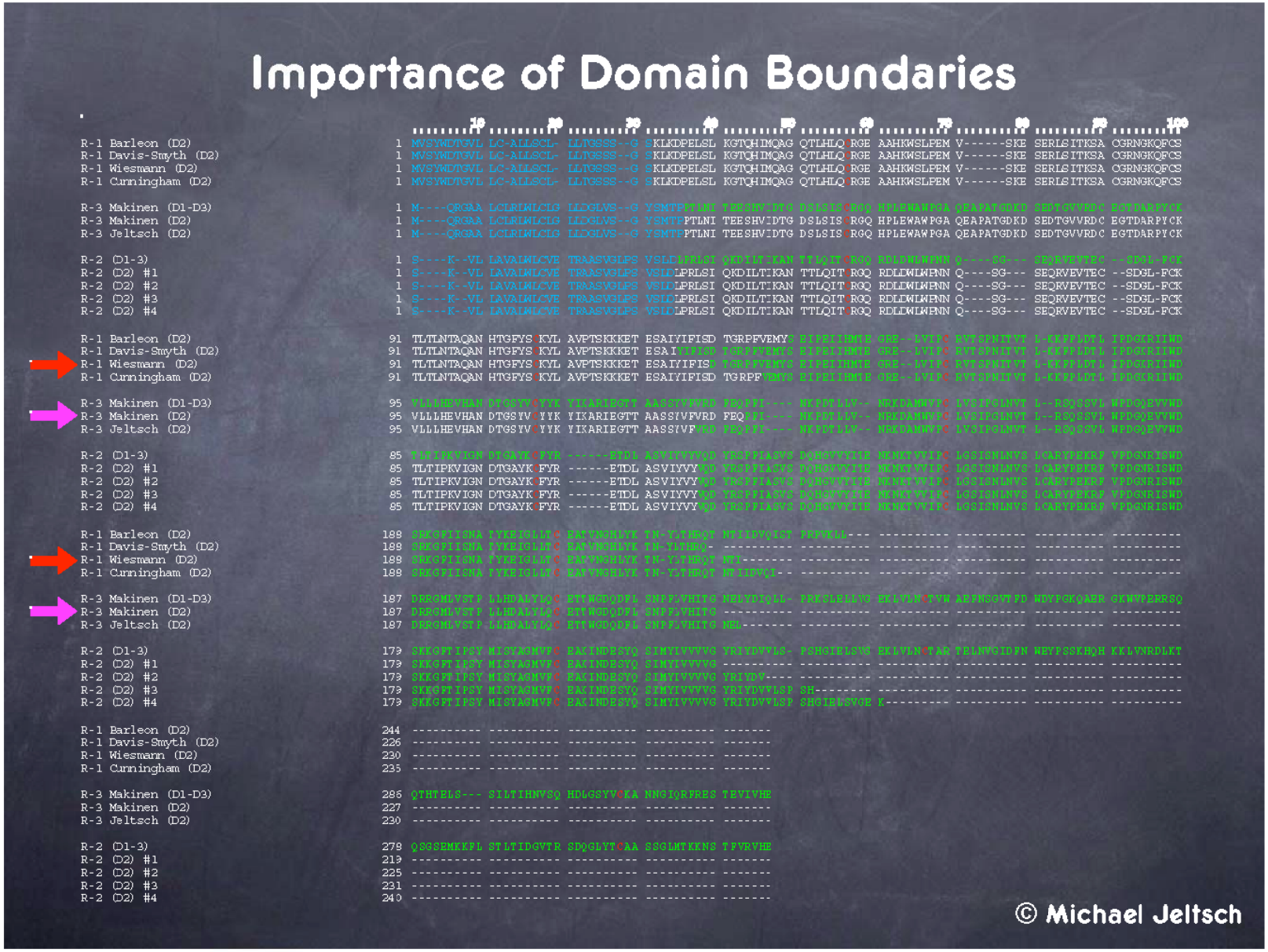
Other possible trouble sources

- *LB, ampicilin, contaminated glycerol stocks (growth directly from stocks)*


domain 1
domain 2
domain 3
domain 4
© Michael Jeltsch


VEGF
VEGF-C
SS
VEGFR-1
VEGFR-2
VEGFR-3
© Michael Jeltsch

Importance of Domain Boundaries

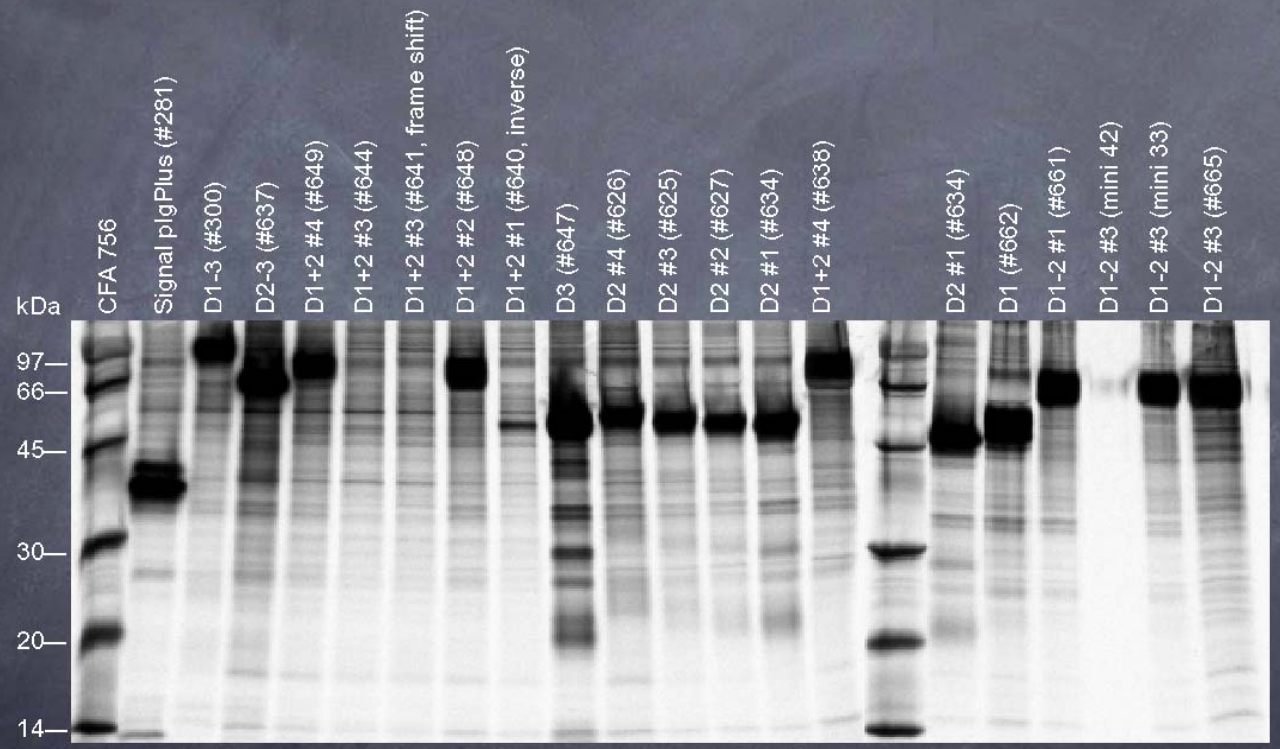


VEGFR-3 hIgGF$_c$ constructs

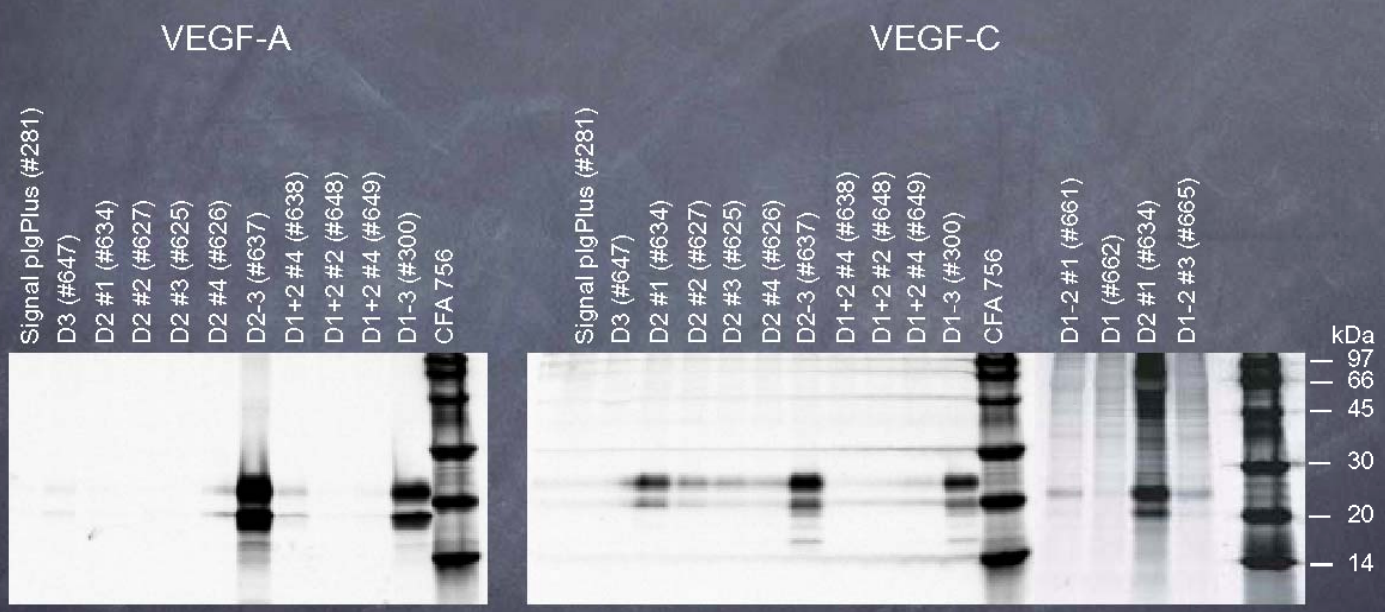
VEGFR-3 domains	mass prep no	VEGF-C binding
1 only	382	
2 only #1	457	
#2	388	
#3	376	
#4	456	no expression!
#5	455	no expression!
1+2 #2	389	✔
#3	375	✔
#4	465	no expression!
#5	464	no expression!
2+3	381	
1–3	307	✔

VEGFR-3 domain 1 replaced by corresponding domains from VEGFR-1, c-fms, PDGFR-α

Expression of VEGFR-2/IgGFc fusion proteins in 293T cells



Binding of VEGF-A & VEGF-C to VEGFR-2/IgGF$_c$ fusion proteins



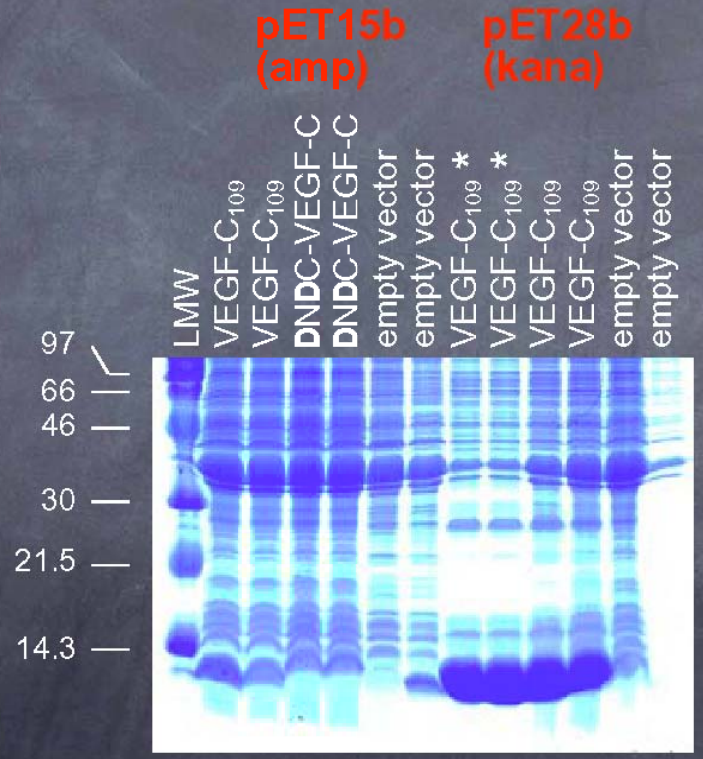
VEGFR-2 hIgGF$_c$ constructs

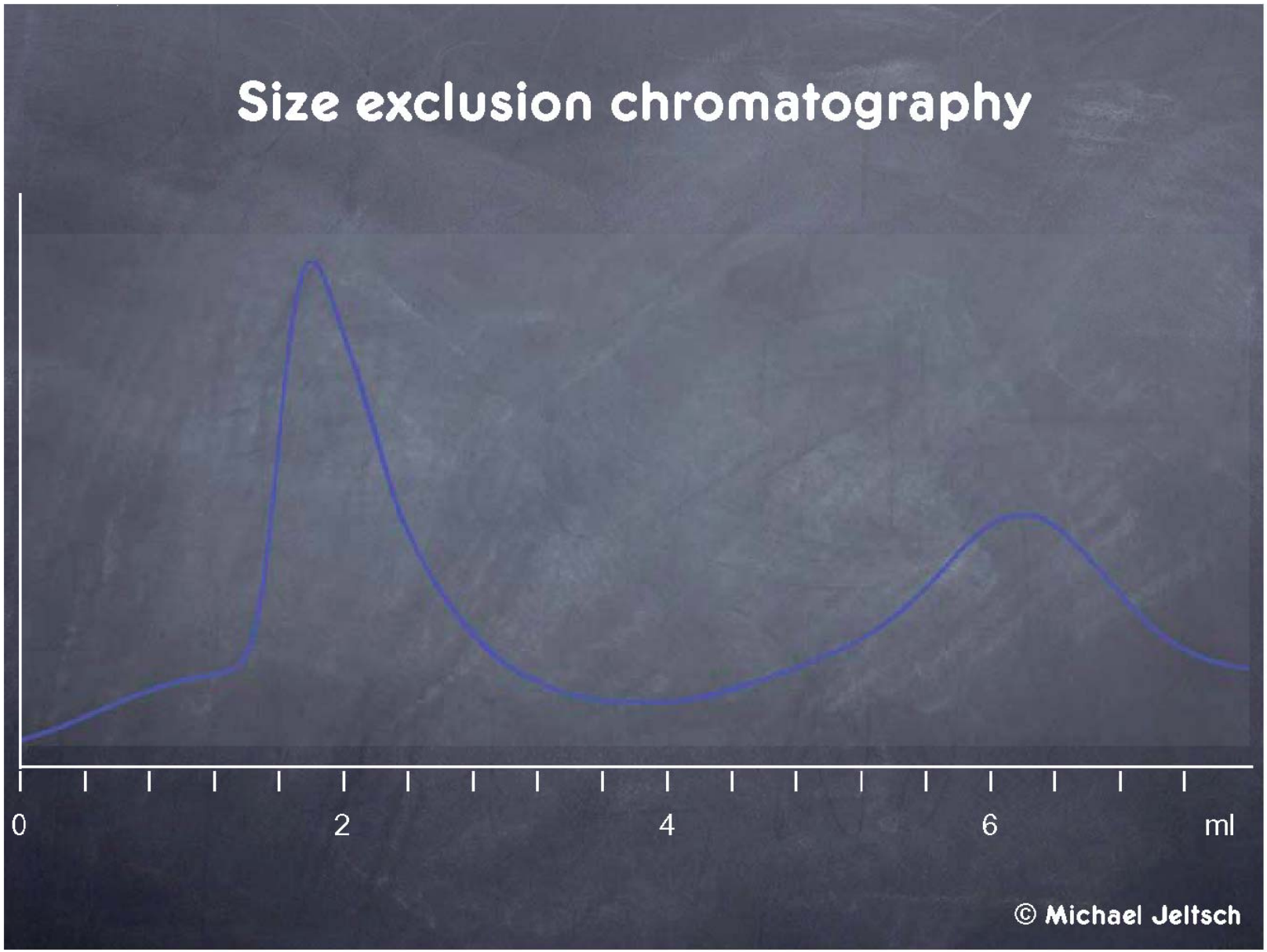
VEGFR-2 domains	mass prep no	VEGF-A binding	VEGF-C binding
1 only	662		
2 only #1	634		✔
#2	627		weak
#3	625		
#4	626		
3 only	647		
1+2 #1	661		
#2	648		
#3	663		
#4	649		
2+3	637	✔	✔
1–3	300	✔	✔

E.coli Expression

- Expression optimization
  different strains: AD494, BL21(DE3), **BL21(DE3)***
  different constructs: pET15 (amp), **pET28 (kana)**
- Inclusion body isolation by differental centrifugation & washing: **10mM β-ME, 5% glycerol, 1% NP40, 0.5‰ NaDOC**
- Solubilization: 8M urea, **6M guanidinium hydrochloride, 10mM DTT**
- Crude seperation before refolding: **size exclusion chromatography**

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




Size exclusion chromatography
0
2
4
6
ml
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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

FoldIt Screen™

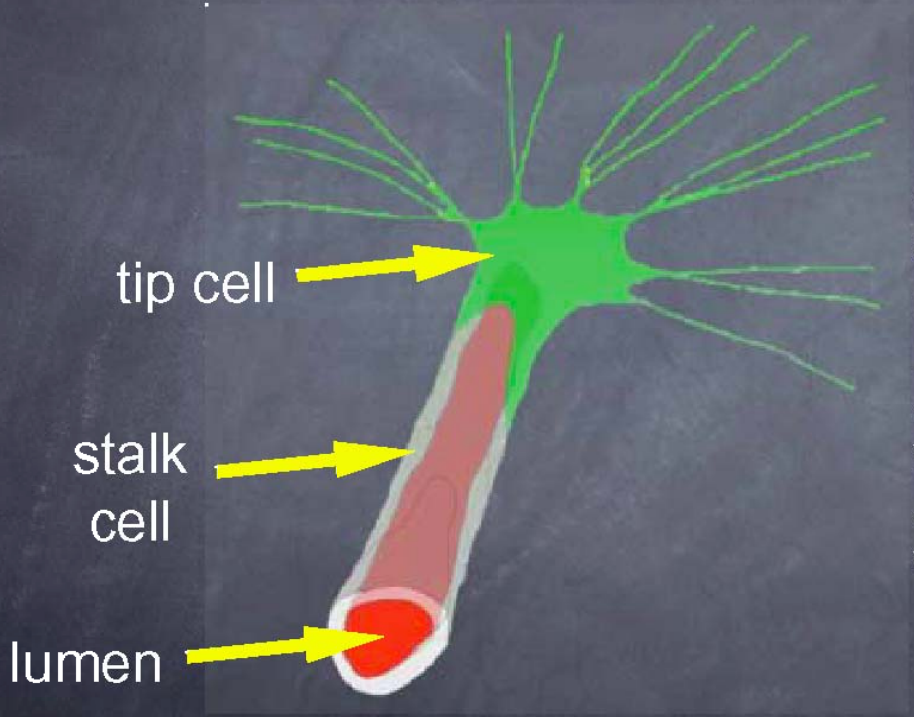
HAMPTON RESEARCH

Solutions for Crystal Growth

Formulations

Buffer	Salt	PEG 3350 (% w/v)	Guanidine HCl	Cation/Chelator	Polar & Nonpolar Additives
1.) 55 mM Tris pH 8.2	1.) 264 mM Na Chloride, 11mM K Chloride	1.) 0.055%	1.) 0 mM	1.) 1.1 mM EDTA	1.) none
2.) 55 mM MES pH 6.5	2.) 10.56 mM Na Chloride, 0.44 mM K Chloride	2.) none	2.) 550 mM	2.) 2.2 mM Mg Chloride, 2.2 mM Ca Chloride	2.) none
3.) 55 mM MES pH 6.5	3.) 10.56 mM Na Chloride, 0.44 mM K Chloride	3.) 0.055%	3.) 550 mM	3.) 1.1 mM EDTA	3.) 440 mM Sucrose, 550 mM L-Arginine
4.) 55 mM Tris pH 8.2	4.) 264 mM Na Chloride, 11mM K Chloride	4.) none	4.) 0 mM	4.) 2.2 mM Mg Chloride, 2.2 mM Ca Chloride	4.) 440 mM Sucrose, 550 mM L-Arginine
5.) 55 mM MES pH 6.5	5.) 264 mM Na Chloride, 11mM K Chloride	5.) none	5.) 0 mM	5.) 2.2 mM Mg Chloride, 2.2 mM Ca Chloride	5.) 440 mM Sucrose
6.) 55 mM Tris pH 8.2	6.) 10.56 mM Na Chloride, 0.44 mM K Chloride	6.) 0.055%	6.) 550 mM	6.) 1.1 mM EDTA	6.) 440 mM Sucrose
7.) 55 mM Tris pH 8.2	7.) 10.56 mM Na Chloride, 0.44 mM K Chloride	7.) none	7.) 550 mM	7.) 2.2 mM Mg Chloride, 2.2 mM Ca Chloride	7.) 550 mM L-Arginine
8.) 55 mM MES pH 6.5	8.) 264 mM Na Chloride, 11mM K Chloride	8.) 0.055%	8.) 0 mM	8.) 1.1 mM EDTA	8.) 550 mM L-Arginine
9.) 55 mM MES pH 6.5	9.) 264 mM Na Chloride, 11mM K Chloride	9.) 0.055%	9.) 550 mM	9.) 2.2 mM Mg Chloride, 2.2 mM Ca Chloride	9.) 440 mM Sucrose
10.) 55 mM Tris pH 8.2	10.) 10.56 mM Na Chloride, 0.44 mM K Chloride	10.) none	10.) 0 mM	10.) 1.1 mM EDTA	10.) 440 mM Sucrose
11.) 55 mM Tris pH 8.2	11.) 10.56 mM Na Chloride, 0.44 mM K Chloride	11.) 0.055%	11.) 0 mM	11.) 2.2 mM Mg Chloride, 2.2 mM Ca Chloride	11.) 550 mM L-Arginine
12.) 55 mM MES pH 6.5	12.) 264 mM Na Chloride, 11mM K Chloride	12.) none	12.) 550 mM	12.) 1.1 mM EDTA	12.) 550 mM L-Arginine
13.) 55 mM Tris pH 8.2	13.) 264 mM Na Chloride, 11mM K Chloride	13.) none	13.) 550 mM	13.) 1.1 mM EDTA	13.) none
14.) 55 mM MES pH 6.5	14.) 10.56 mM Na Chloride, 0.44 mM K Chloride	14.) 0.055%	14.) 0 mM	14.) 2.2 mM Mg Chloride, 2.2 mM Ca Chloride	14.) none
15.) 55 mM MES pH 6.5	15.) 10.56 mM Na Chloride, 0.44 mM K Chloride	15.) none	15.) 0 mM	15.) 1.1 mM EDTA	15.) 440 mM Sucrose, 550 mM L-Arginine
16.) 55 mM Tris pH 8.2	16.) 264 mM Na Chloride, 11mM K Chloride	16.) 0.055%	16.) 550 mM	16.) 2.2 mM Mg Chloride, 2.2 mM Ca Chloride	16.) 440 mM Sucrose, 550 mM L-Arginine

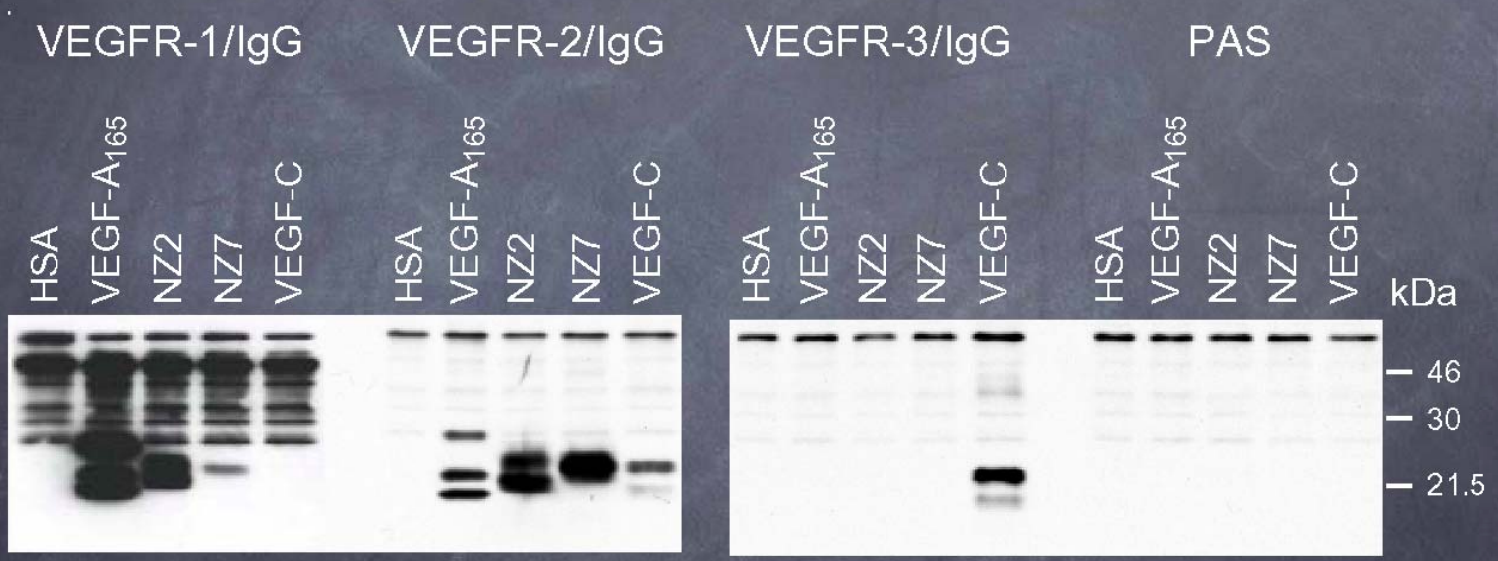
VEGF guides angiogenesis through endothelial tip-cell filopodia



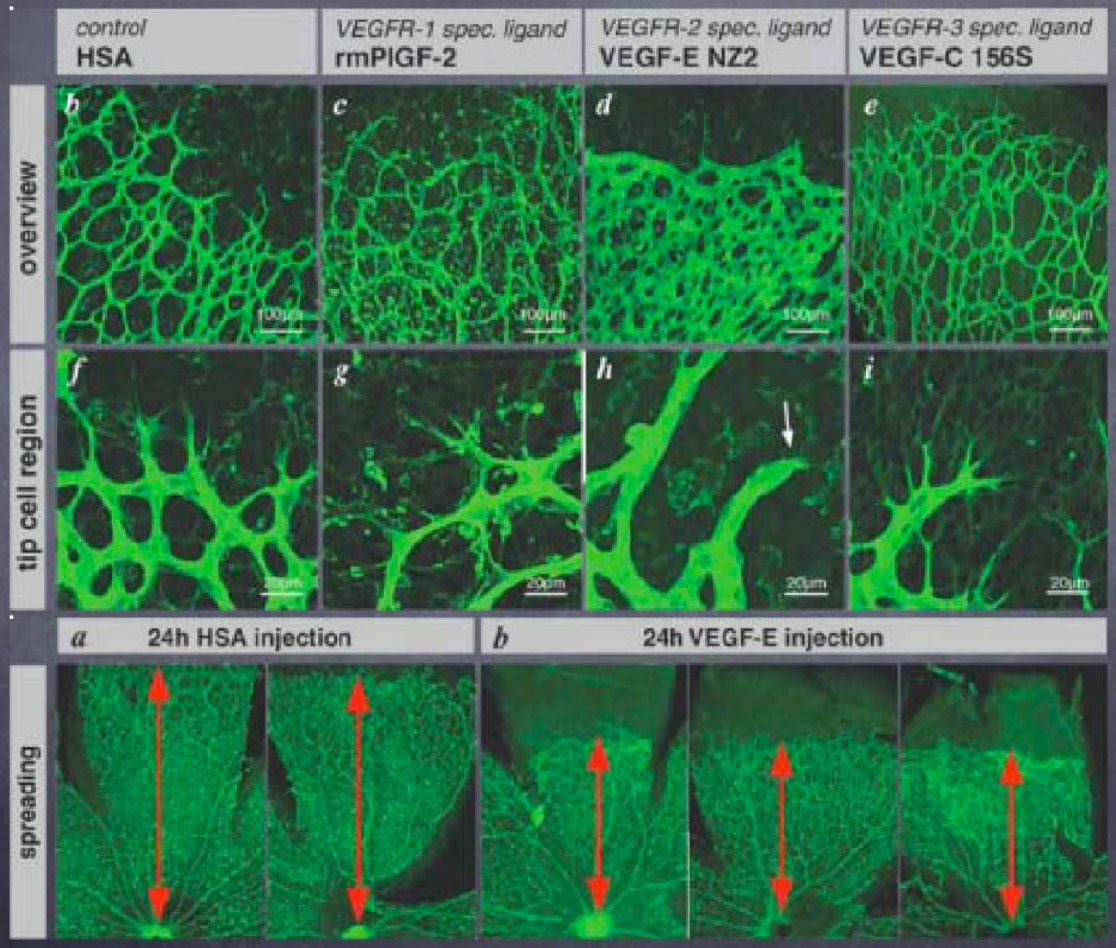
Tip cells:

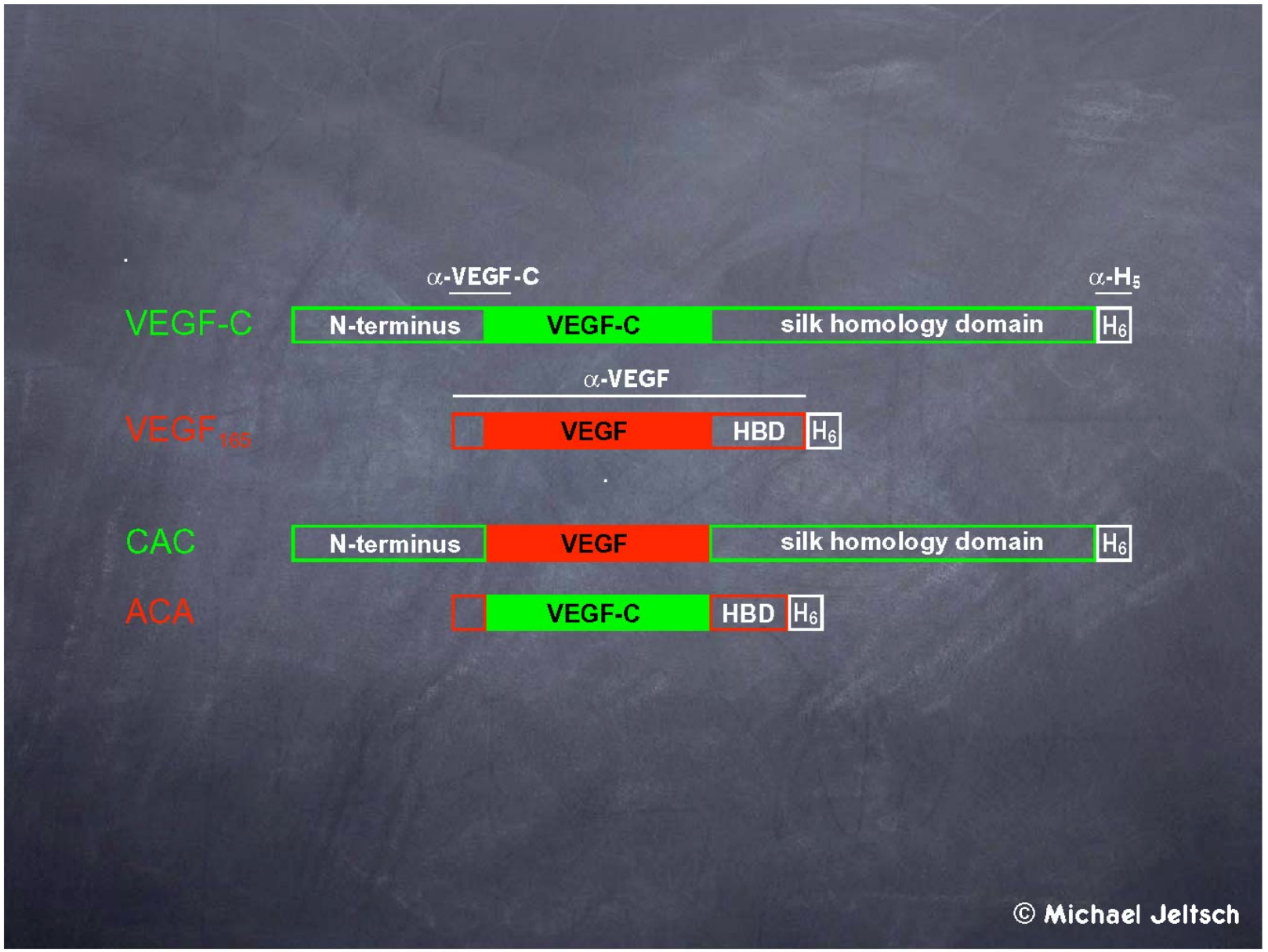
- different expression pattern compared to stalk cells (e.g. more VEGFR-2 and PDGF-B)
- no proliferative response to VEGF
- migratory response to a VEGF gradient

Binding of VEGF-E NZ2 & NZ7 to VEGFR-2/IgGF$_C$ fusion proteins



VEGFR-2 mediated tip cell migration & stalk cell proliferation




α-VEGF-C
α-H5
VEGF-C
N-terminus
VEGF-C
silk homology domain
H6
α-VEGF
VEGF165
VEGF
HBD
H6
CAC
N-terminus
VEGF
silk homology domain
H6
ACA
VEGF-C
HBD
H6
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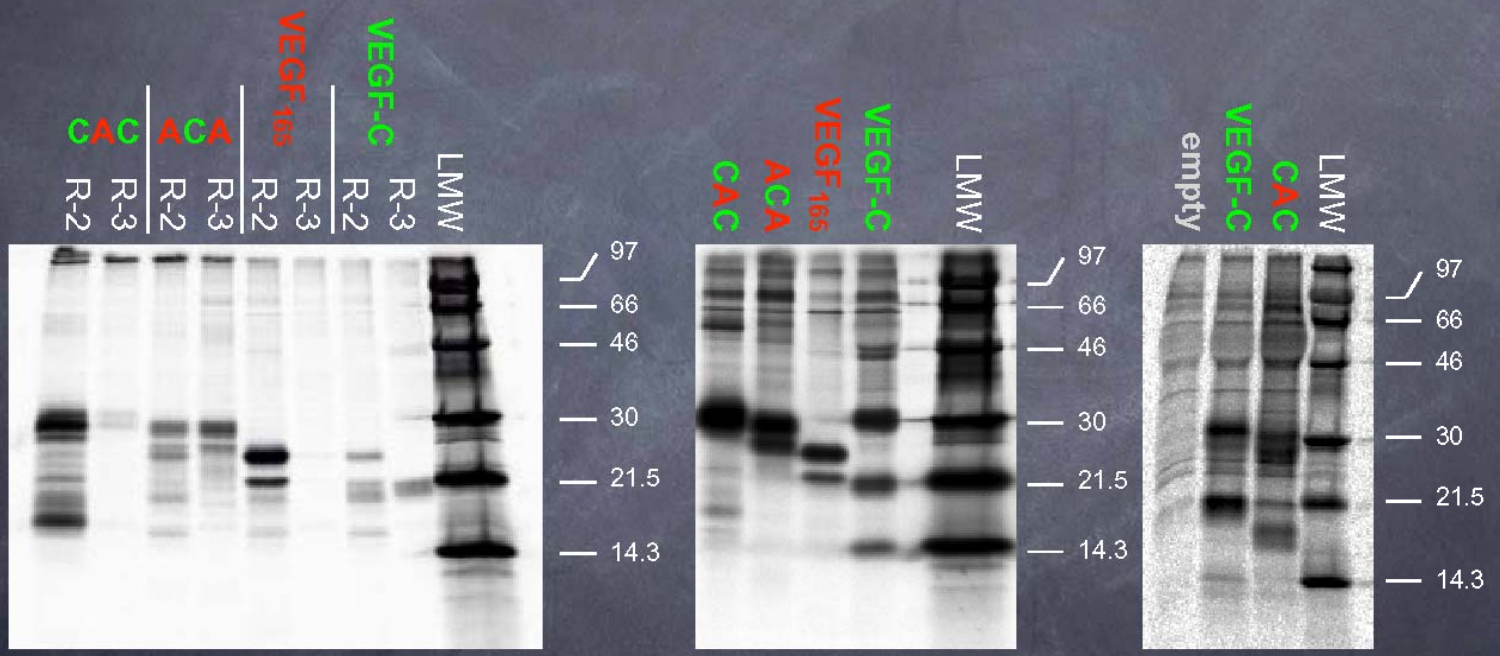
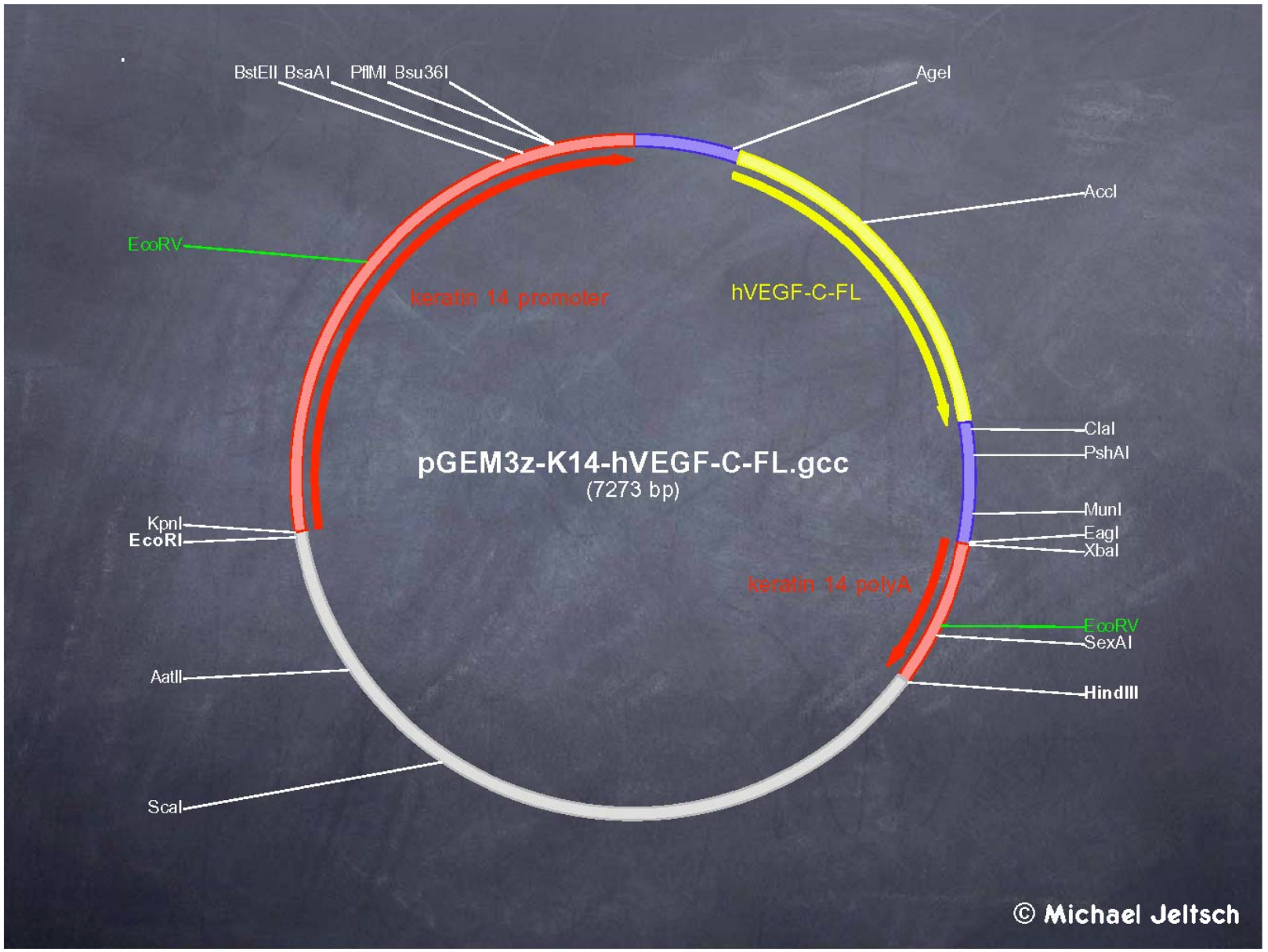
Expression & Receptor Binding of VEGF-A/VEGF-C Propeptide Domain swaps




BstEII BsaAI PflMI Bsu36I
AgeI
AccI
EcoRV
keratin 14 promoter
hVEGF-C-FL
pGEM3z-K14-hVEGF-C-FL.gcc
(7273 bp)
ClaI
PshAI
MunI
EagI
XbaI
KpnI
EcoRI
keratin 14 polyA
EcoRV
SexAI
AatII
HindIII
ScaI
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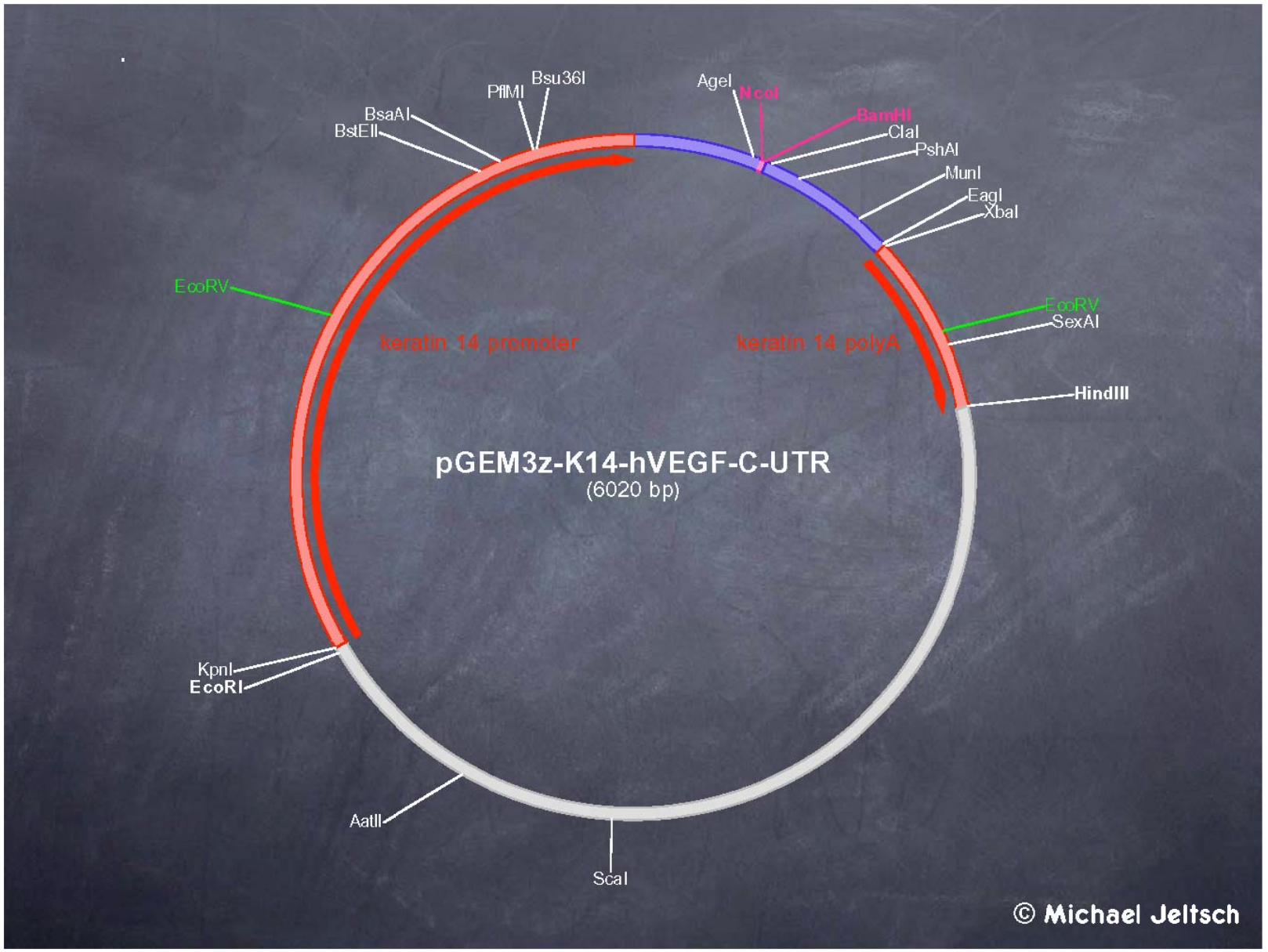
Bsu36I
PflMI
AgeI
NcoI
BsaAI
BstEII
BamHI
ClaI
PshAI
MunI
EagI
XbaI
EcoRV
EcoRV
SexAI
keratin 14 promoter
keratin 14 polyA
HindIII
pGEM3z-K14-hVEGF-C-UTR
(6020 bp)
KpnI
EcoRI
AatII
ScaI
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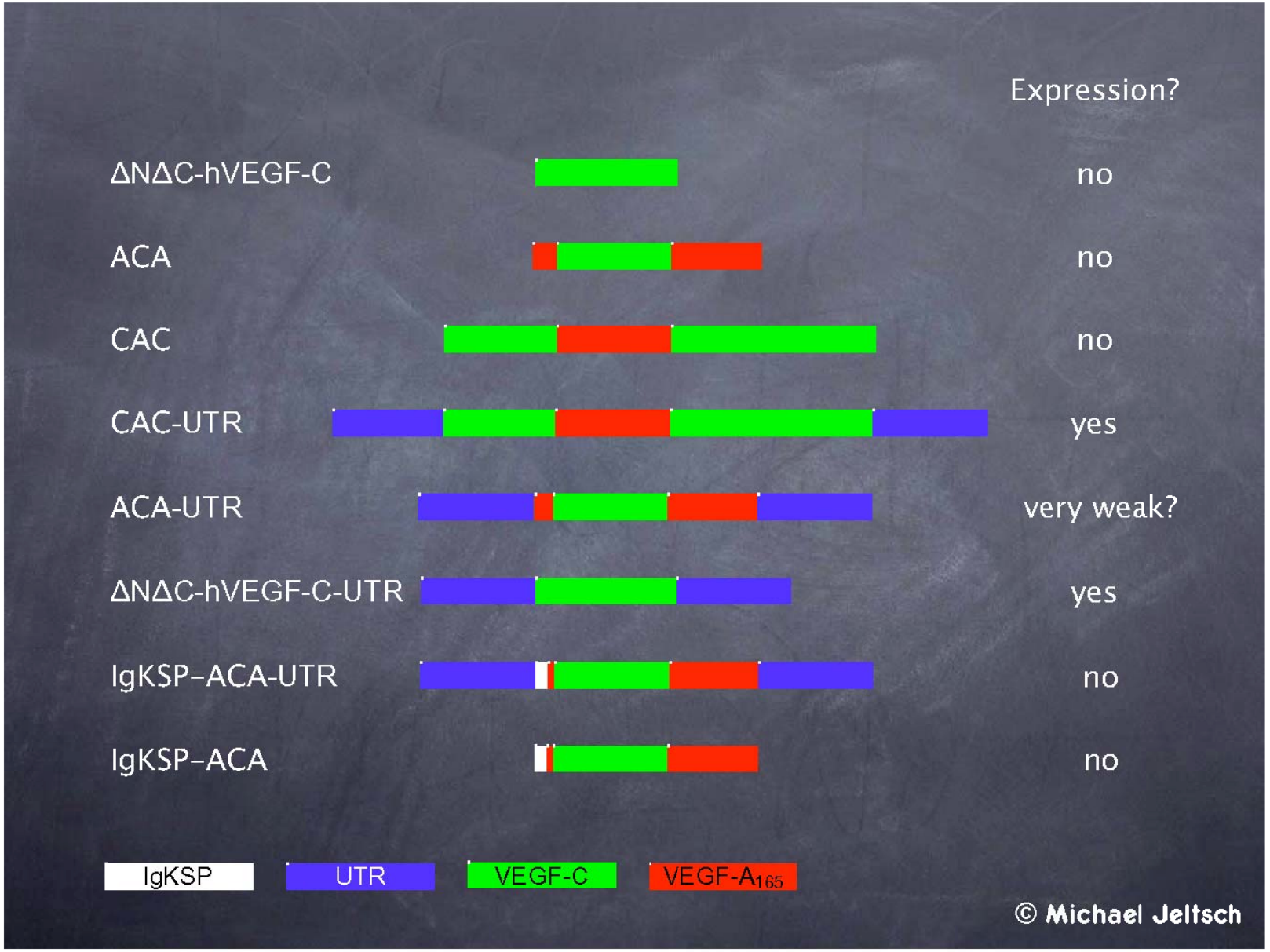
Expression?
ΔNΔC-hVEGF-C
no
ACA
no
CAC
no
CAC-UTR
yes
ACA-UTR
very weak?
ΔNΔC-hVEGF-C-UTR
yes
IgKSP–ACA-UTR
no
IgKSP–ACA
no
IgKSP
UTR
VEGF-C
VEGF-A165
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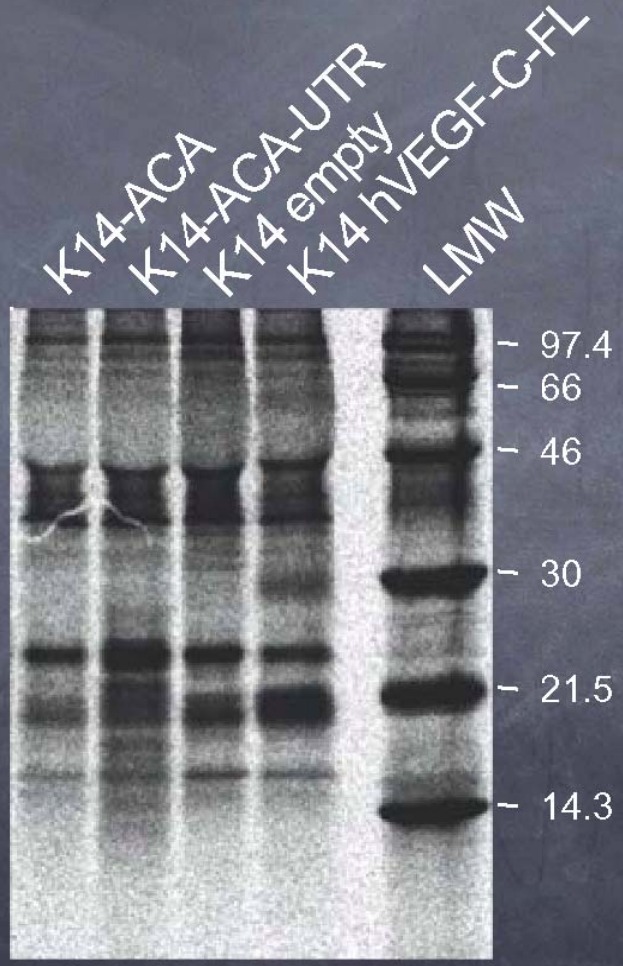
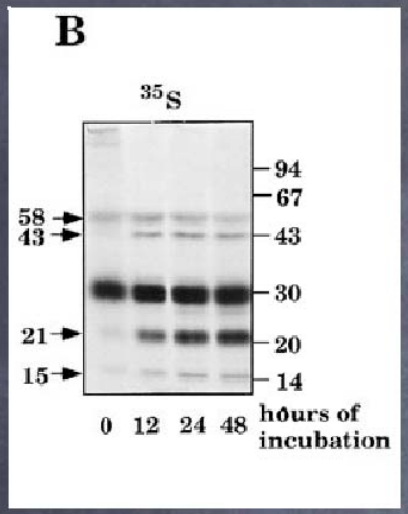
Some more disturbing results

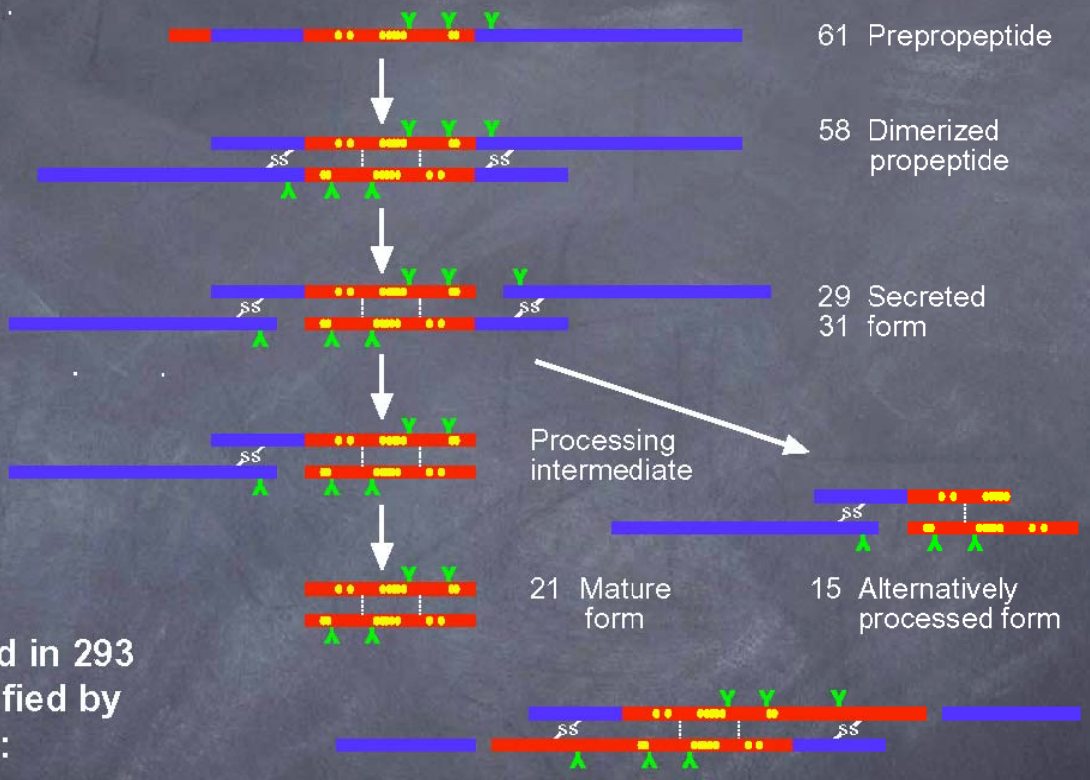
IP:
polyclonal
anti-hVEGF-A
antiserum
&
polyclonal
anti-hVEGF-C VHD
antiserum



VEGF-C

- What is known about the different forms of VEGF-C and why we still lack important knowledge
- 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?





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

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

α-VEGF-C/
α-H5
mVEGFR-2
hVEGFR-2
hVEGFR-3
ΔNΔC
wild type
"cleavage-inhibited"
"uncleavable"
46
30
21
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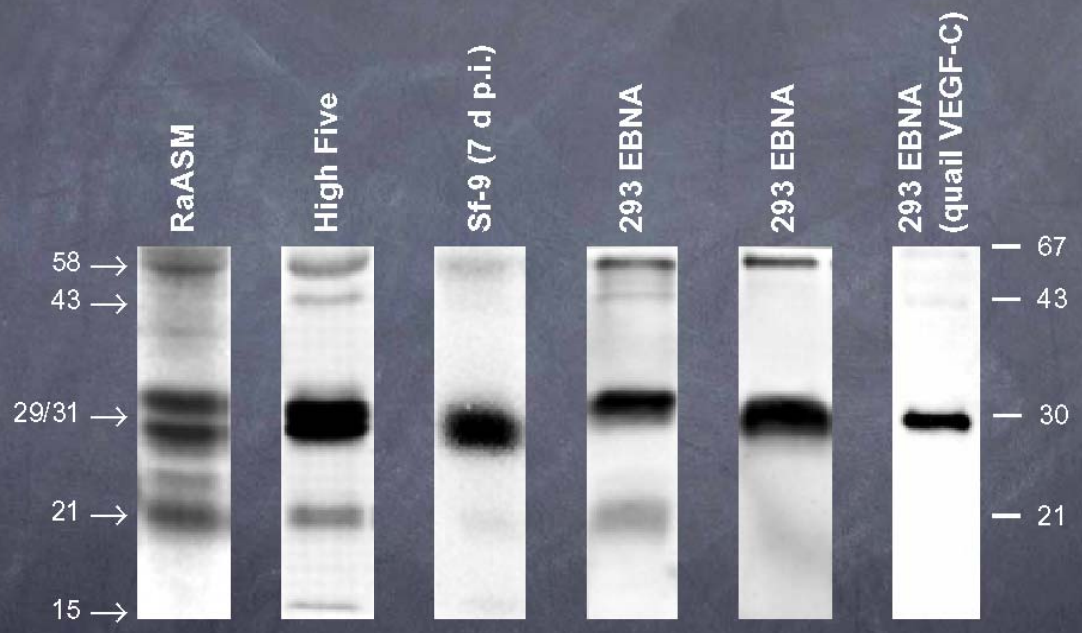
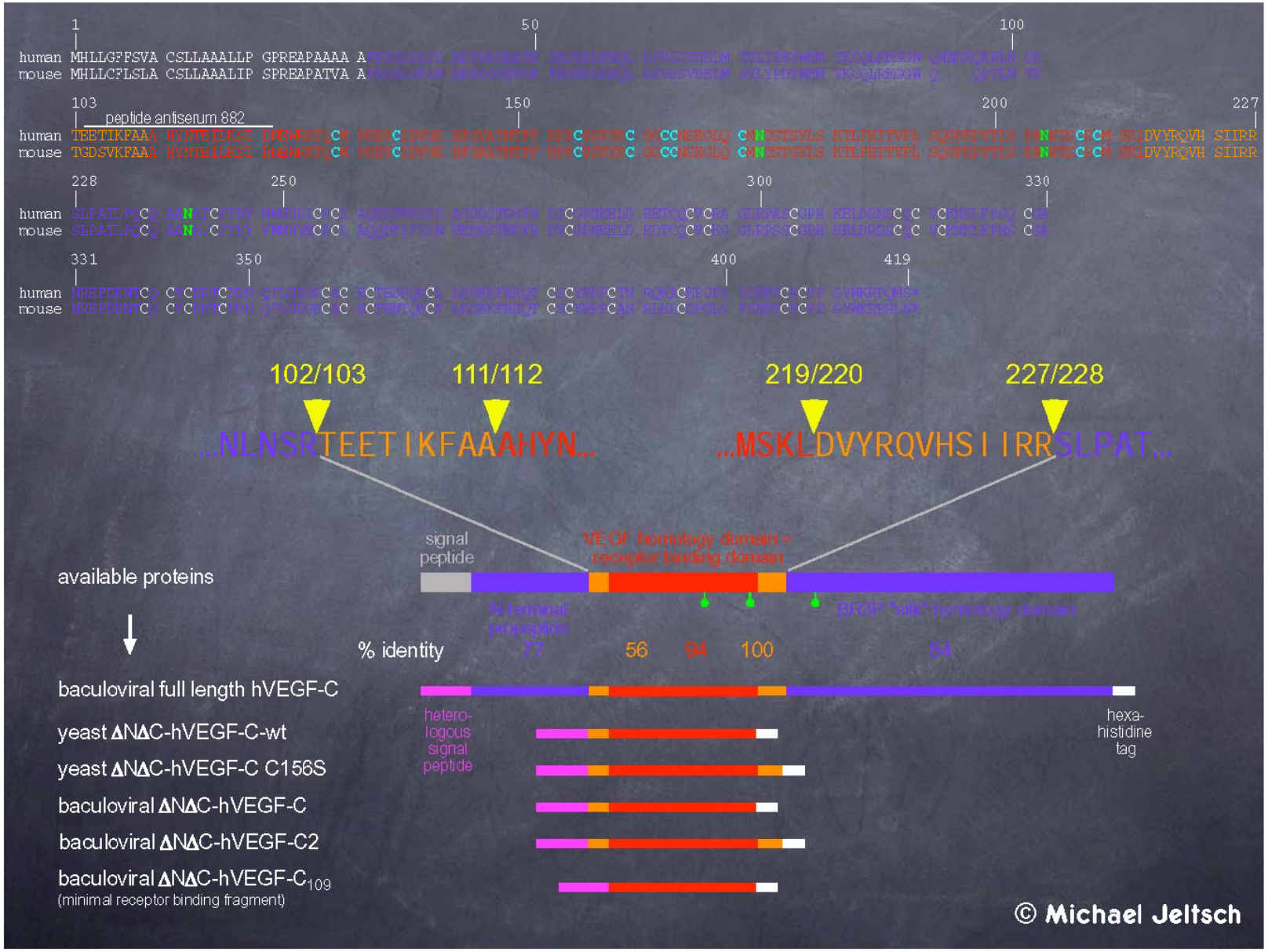
kDa	form	VEGFR-2	VEGFR-3
58	full length	+/-	+
43	alternatively C-terminally processed	++	++
29/31	main/secreted	+	+++
21	mature	++	+++
15	N-terminal propeptide + partial VHD	-	-

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




peptide antiserum 882
102/103
111/112
219/220
227/228
...NLNSRTEETIKFAAAHYN...
...MSKLDVYRQVHSIIRRSLPAT...
available proteins
signal peptide
VEGF homology domain = receptor binding domain
N-terminal propeptide
ERGP "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
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- 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 (CAM)
- full length (CAM, recent experiments)

Transgenic mice/Adenoviruses/AAV

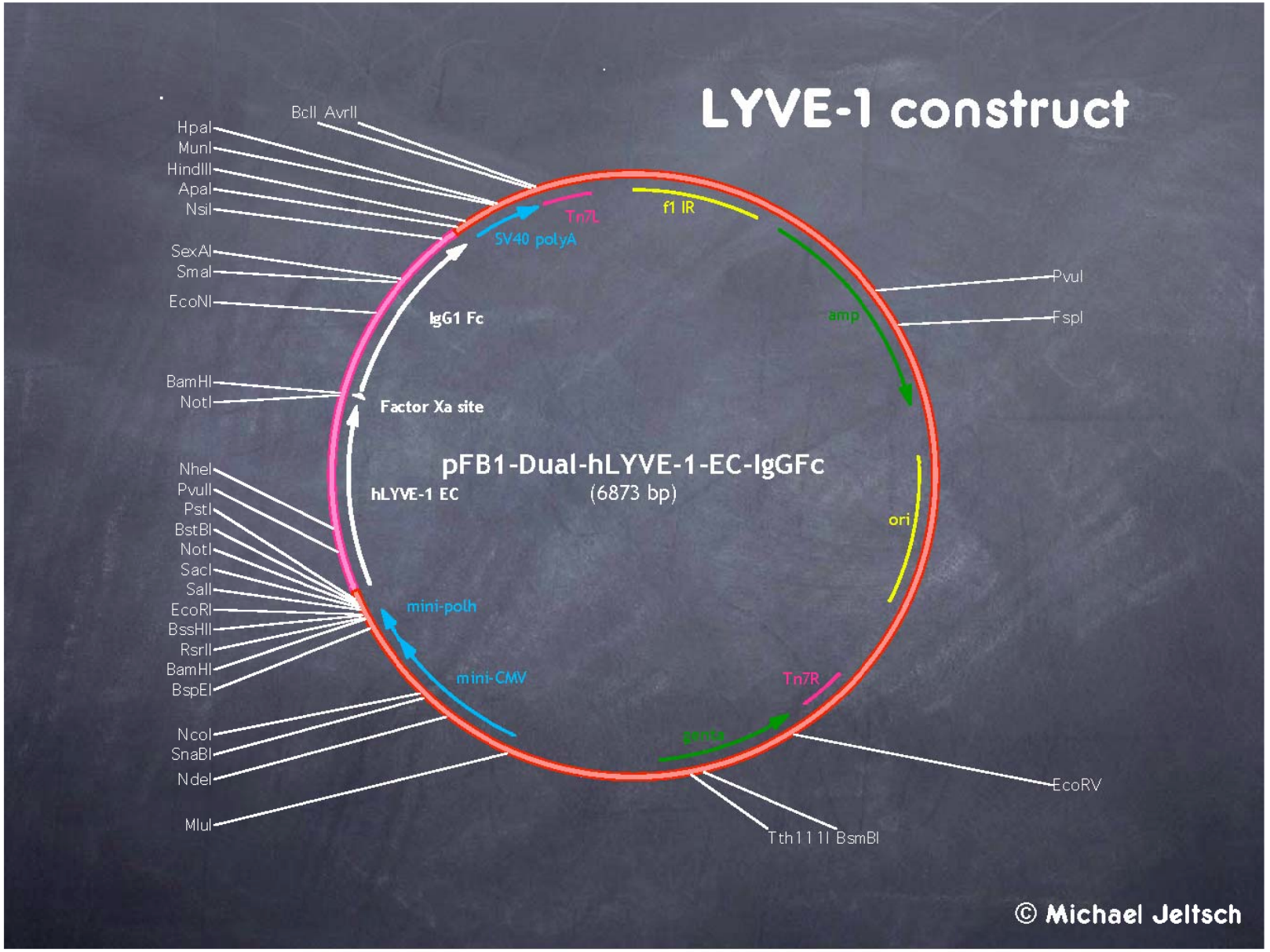
- Adenovirus: NH_2-TEETIK…MSKL-COOH ?
- Adenovirus: full length
- ???????

Mouse Imunizations

- 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 Pyy): 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)

Baculoviruses work

- Baculoviruses with Dual Insect/ Mammalian Promoter
- Protein Production and Purification


LYVE-1 construct
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
ori
IgG1 Fc
Factor Xa site
pFB1-Dual-hLYVE-1-EC-IgGFc
hLYVE-1 EC
(6873 bp)
mini-polh
mini-CMV
Tn7R
gentR
EcoRV
Tth111I BsmBI
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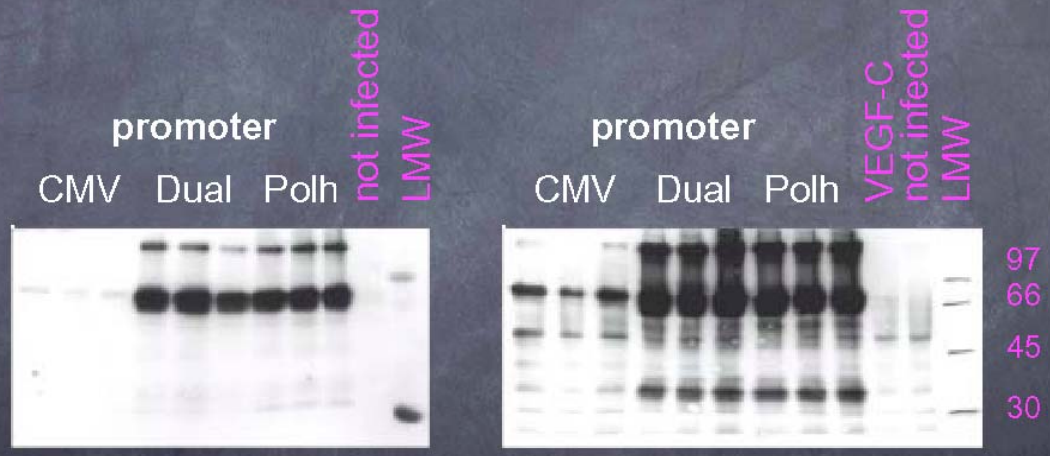
Expression of hLYVE-1-EC-hIgGFc in insect cells (HF)

straight Western: anti-hIgG

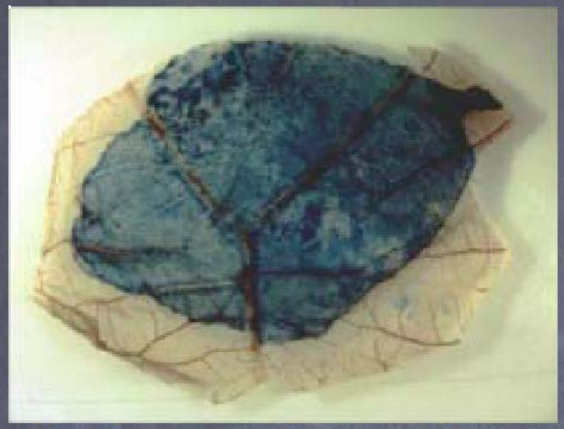
IP: PAS (from 1 ml sup)
Western: anti-hIgG



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

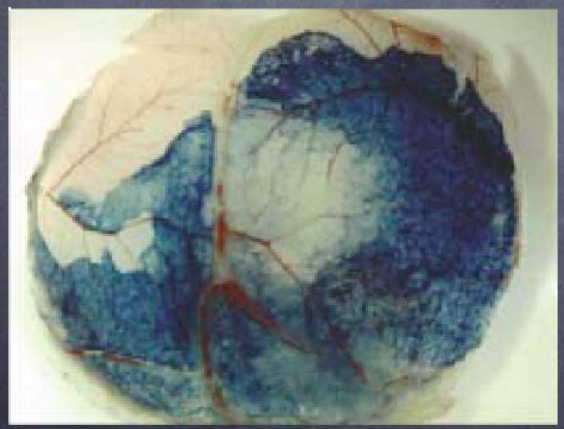
293T Cos-1

lacZ baculo-infected d 13 CAM

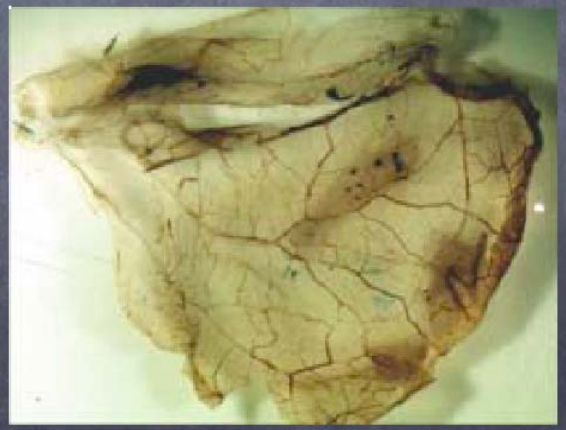


Dual promoter lacZ

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

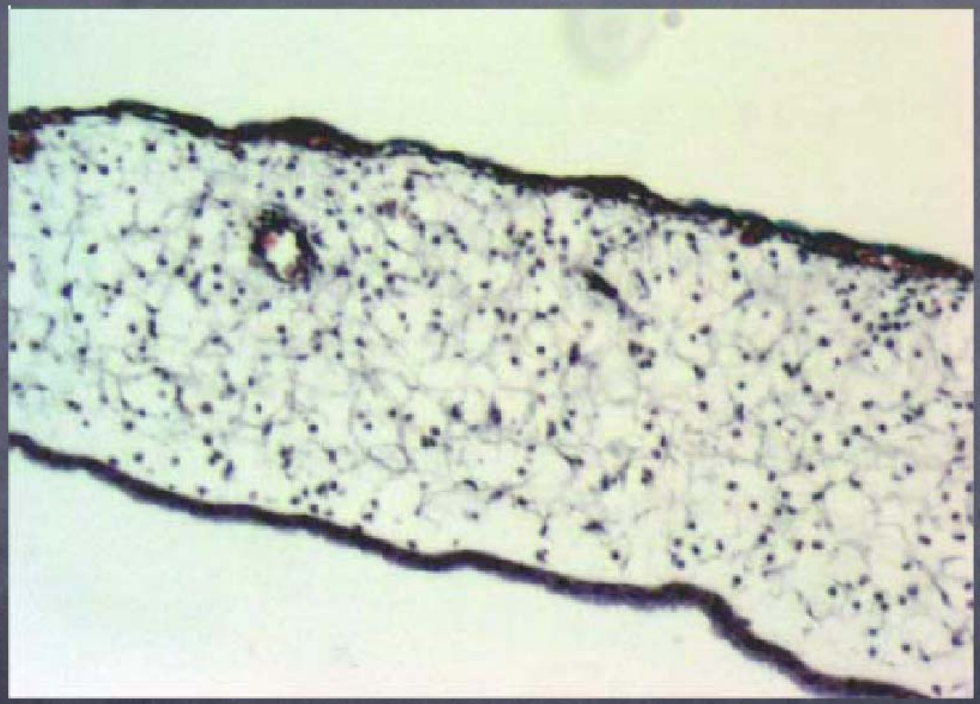


Dual promoter lacZ

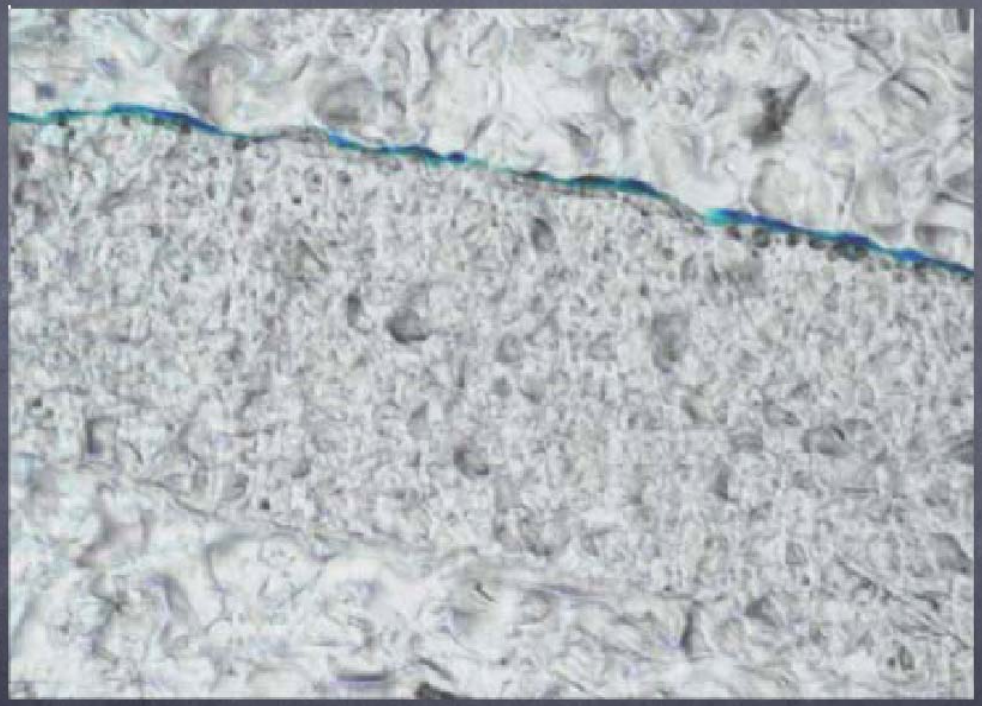


Polh promoter VEGF-C

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 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 using approx. 500 ml conditioned medium

Small vs. medium 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 in progress (problems of air-lift fermenter: stock virus quantity & quality, cell viability & cell lines, monitoring, control)
- Stable expression for large scale expression might be easier (Drosophila & InsectSelect)