

## Important research goals that I achieved during the past 2 years:

1. The neon lamps at my bench were repaired.
2. Get a bad reputation in order to work in peace.
3. Acquiring a PAGE apparatus and making 15-well combs

## Major future plans:

1. Get cardboard boxes for Eppendorf tube storage and racks for 15- and 50-ml-Falcon tubes and Eppendorf racks
2. Catch somebody in the very act of pouring boiling agarose onto a gel tray and beating him half to death to set an example and in revenge for what happened to Yassir El Maryod.

## Possible strategies to achieve goal #1:

- Ally with Birgitta and try to have a reasonable discussion.
- Bribing with the bottle Champagne I brought from Germany.
- Ordering myself and pay with my own money.

## Possible strategies to achieve goal #2:

- “The soldier-and-three-bushes”-problem.

species	Spodoptera frugiperda (fall armyworm)		Trichoplusia ni (cabbage looper)
cell lines	Sf9	Sf21	High Five
appearance	spherical, regular size	spherical, different sizes, but generally larger than Sf9 loose attachment	mix of round and spindel shape firm attachment
suspension culture	no adaptation required		adaptation required
doubling time	18-22 h~24 h		
growth media	Sf900II, TNM-FH		Ex cell 400 (monolayer), Ex cell 405 (suspension culture), TNM-FH
plaque formation	supported		not supported
protein production	1-500 mg/litre (protein-dependent)		5-10 x higher than Sf

time (d)

1. Transfection of insect cells 2-5
  - ↓
  2. Analysis of transfection supernatant 4  
(IP/Western, NTA-Ni<sup>2+</sup> affinity purification & Ag staining; functional assay)
  - ↓
  3. First amplification of selected clones 2-5
  - ↓
  4. Second amplification of selected clones 2-3
  - ↓
  5. Test infection with high titre stock 2
  - ↓
  6. Analysis of conditioned medium 2  
protein detection
  - ↓
  7. Prepare large scale stock virus 3-5  
(5 big bottles/~200 ml)
  - ↓
  8. Growing cells for large scale infection 7  
(~40 big bottles)
  - ↓
  9. Large scale infection 2  
(800 ml conditioned medium 0.8-4 mg DNDC-melSP-VEGF-C/D-H6)
  - ↓
  10. Analysis of conditioned medium 2  
like step 6
  - ↓
  11. Concentration of medium, purification & desalting 2-3
  - ↓
  12. Analysis of purified protein 3  
Quantitation: Coomassie staining & functional assay (determining the biological activity)
- total amount of time 33-43



## VEGF-CxVEGF-D heterodimer formation in HF cells

MOI

VEGF-C-FL	0	0	10	50	90	100
VEGF-D-FL-H <sub>6</sub>	0	100	90	50	10	0

IP with H<sub>5</sub>-Ab (1:1, Dianova/Qiagen)

1. Western with VEGF-C-Ab (882)
2. Silver-staining/metabolic labelling (size difference)

# Problems with baculovirus expression system

## 1. Generating clonal virus stocks

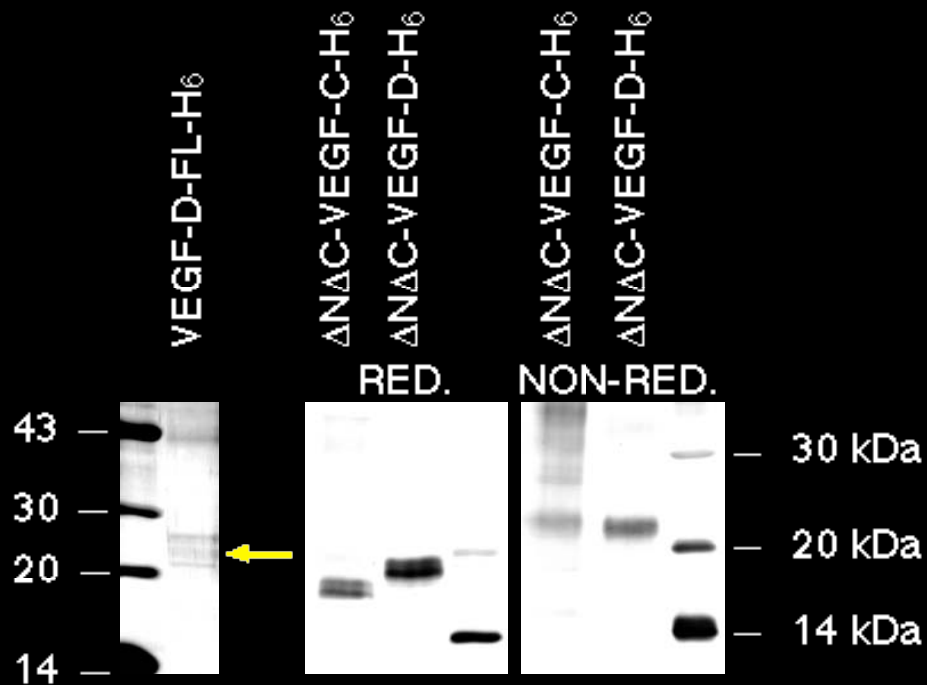
- Plaque purification
- FastBac system only: Retransformation of *E. coli* DH10B with isolated Bacmid-DNA

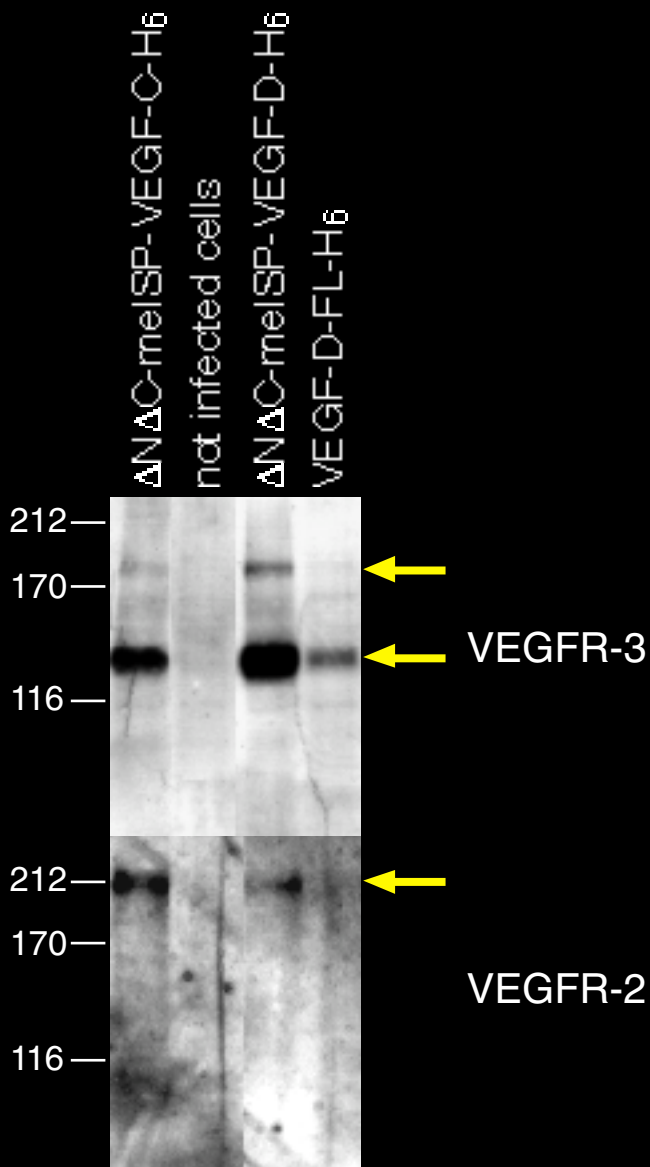
## 2. Protein-heterogeneity

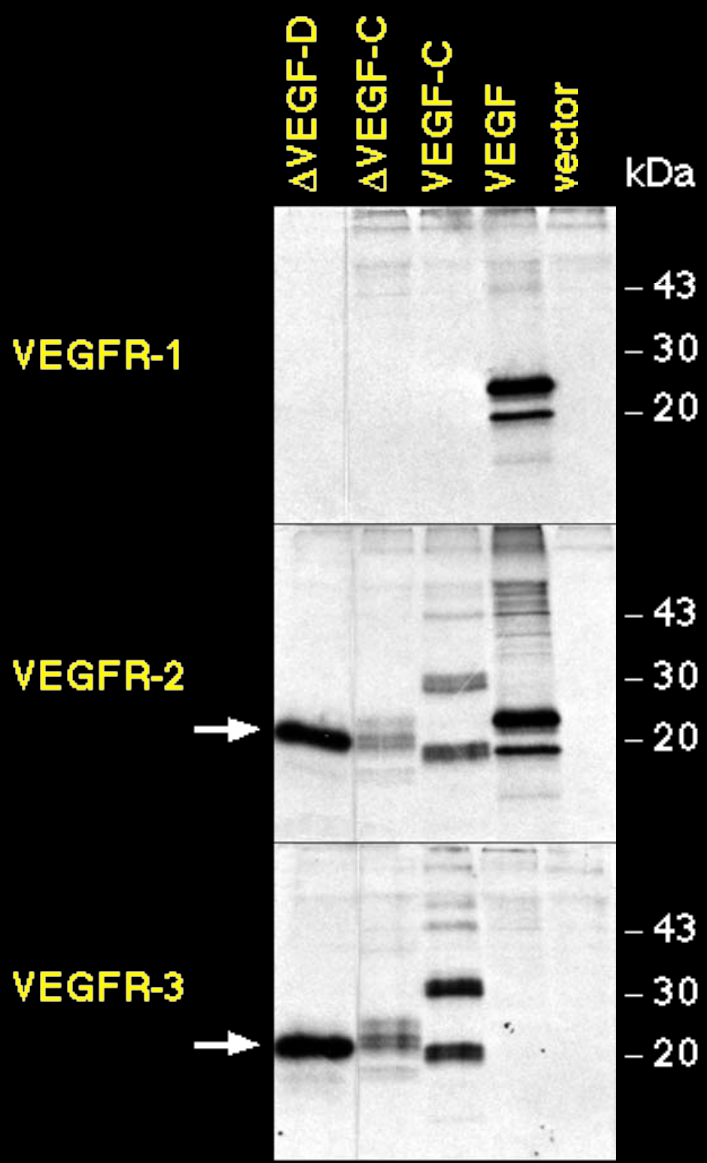
- partial proteolytic processing
- heterogenous cleavage by the signal peptidase
- differential glycosylation

## 3. Expression level

- Generation of high titre stocks: DIPs
- Drop in infectivity due to storage (UV)
- Condition of insect cells: viability, too high passage number, heat-shock protein vs. recombinant protein

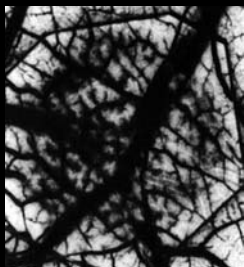








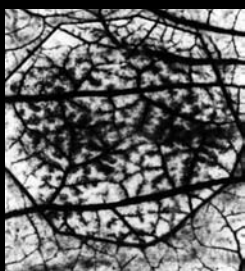
control



VEGF<sub>121</sub>



VEGF<sub>165</sub>



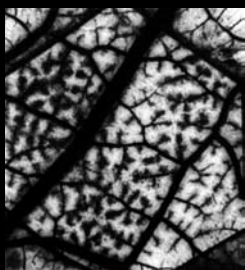
VEGF<sub>121</sub>  
x VEGF<sub>165</sub>



PIGF-1



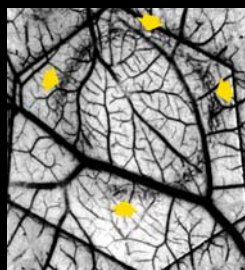
PIGF-2



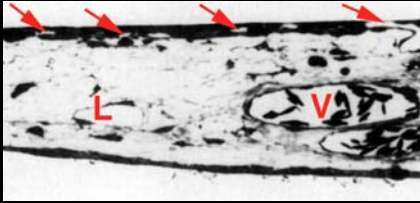
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x PIGF-1



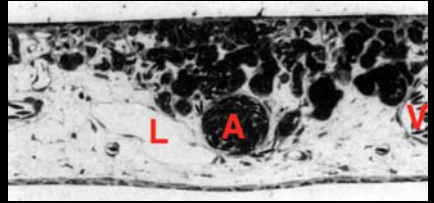
VEGF<sub>165</sub>  
x PIGF-2



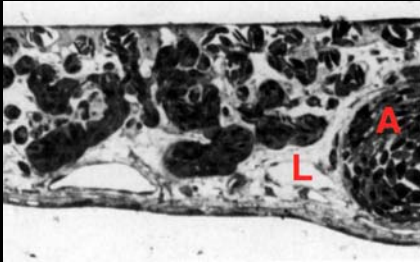
VEGF-C



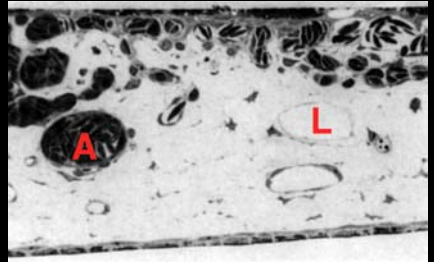
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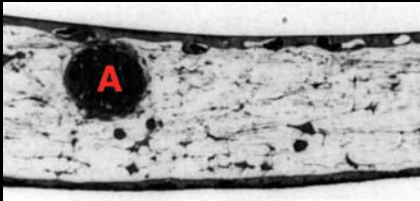
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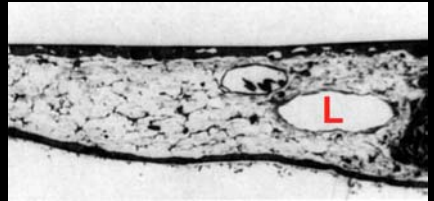
VEGF<sub>165</sub>



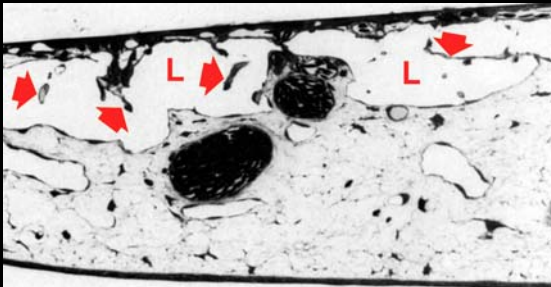
VEGF<sub>121</sub> x VEGF<sub>165</sub>



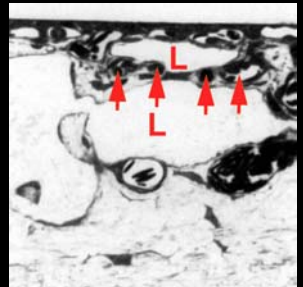
PIGF-1



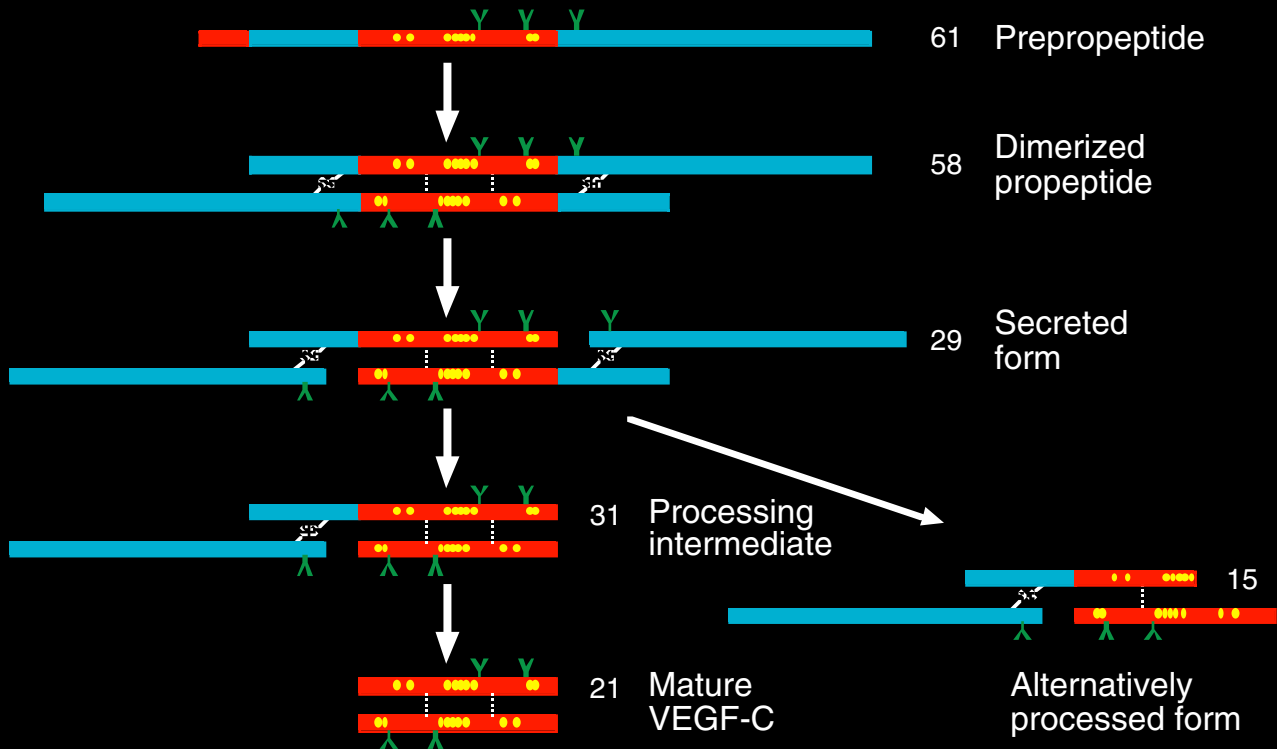
PIGF-2

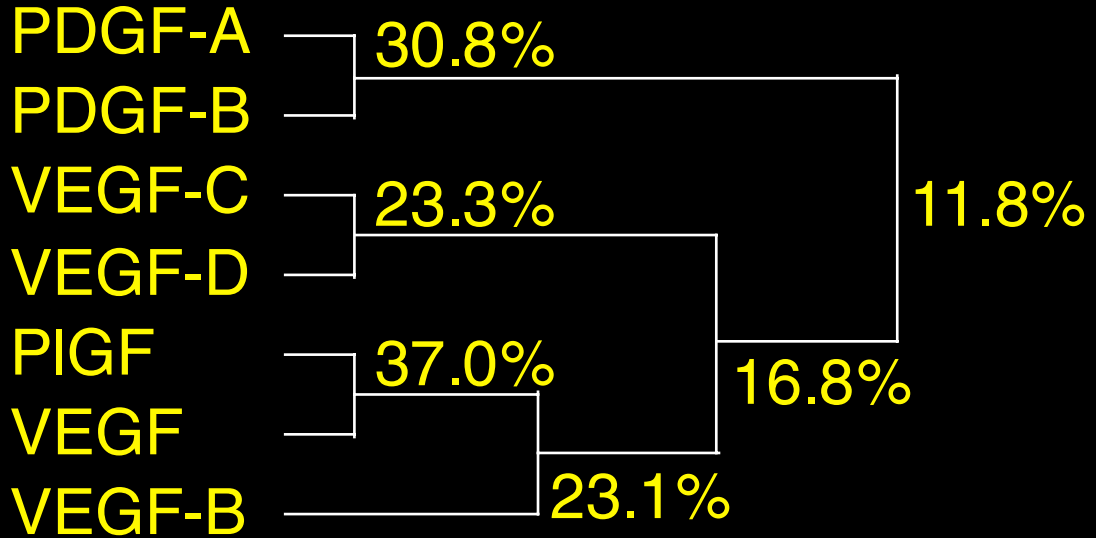


VEGF-C



VEGF-C

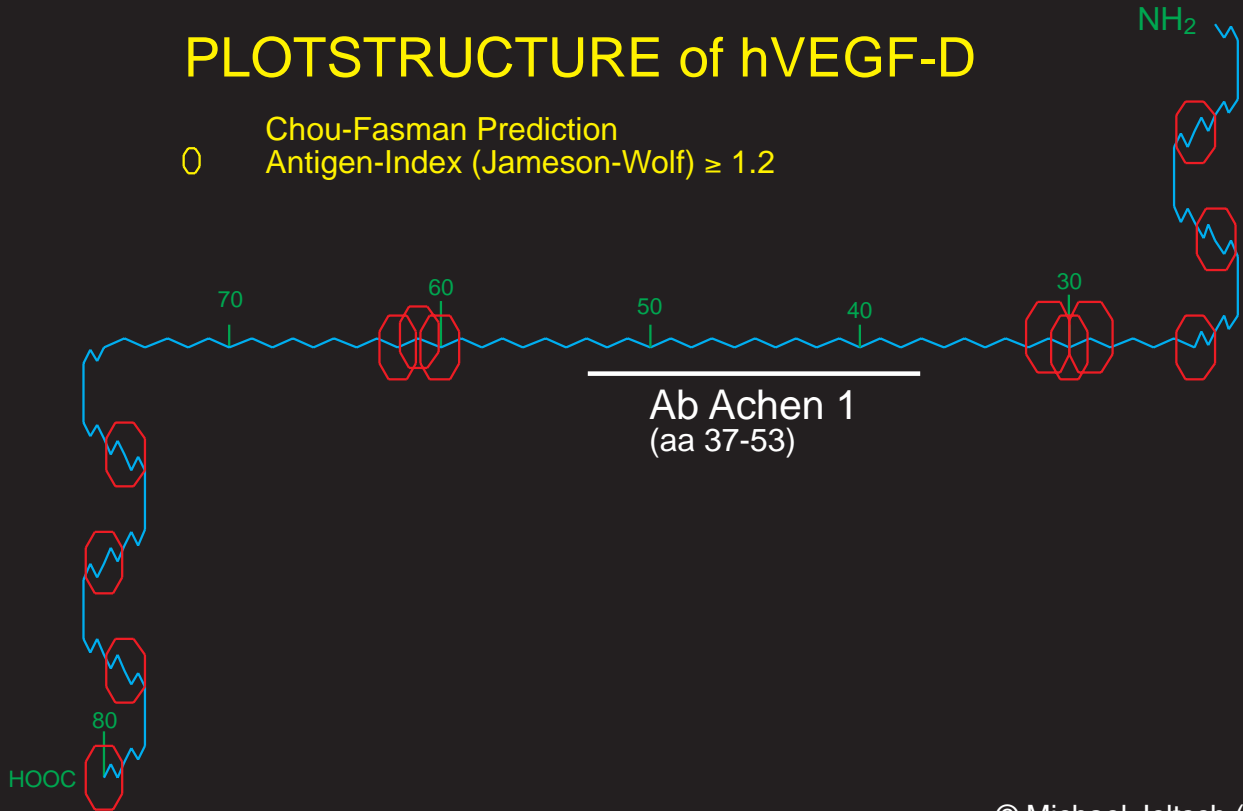


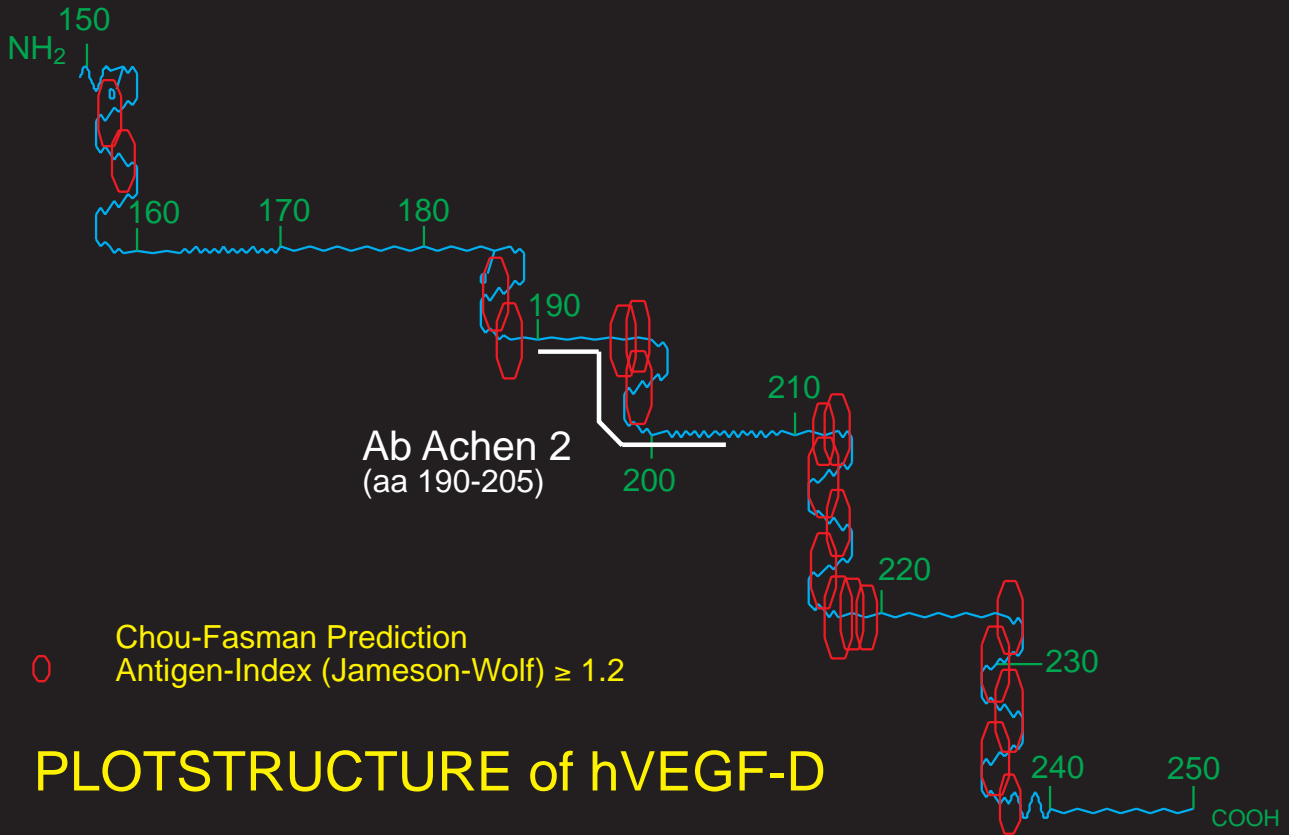


	1				50
hVEGF-C	MHLLGFFSVA	CSLLA..AAL	LPGPREAPAA	AAAF.....	ESGLDLSDAE
hVEGF-C	MHLLCFLSLA	CSLLA..AAL	IPSPREAPAT	VAAF.....	ESGLGFSEAE
hVEGF-D	..MYREWVVV	NVFMMLYVQL	VQGSSENEHGP	VK.....RSS	QSTLERS...
mVEGF-D	..MYGEWGMG	NILMMFHVYL	VQGFARSEHGP	VKDFSEFRSS	RSMLERS...
	51				100
hVEGF-C	PDAGEATAYA	SKDLEEQLRS	VSSVDELMTV	LYPEYWKMYK	CQLRKGGWQH
hVEGF-C	PDGGEVKAFE	GKDLEEQLRS	VSSVDELMSV	LYPDYWKMYK	CQLRKGGWQ.
		<u>Ab Achen 1</u>			
hVEGF-D	.....	...EQQIRA	ASSLEELLRI	THSEDWKLWR	CRRLRKSF..
mVEGF-D	.....	...EQQIRA	ASSLEELLQI	AHSEDWKLWR	CRCLKLSL..
	101		<u>Ab 882</u>		150
hVEGF-C	NREQANLNSR	TEE..TIKFA	AAHYNTEILK	SIDNEWRTQ	CMPREVCIDV
hVEGF-C	...QPTLNTR	TGD..SVKFA	AAHYNTEILK	SIDNEWRTQ	CMPREVCIDV
		<u>Ab 928 &amp; 929</u>			
hVEGF-D	...TSMDSR	SASHRSTRFA	ATFYDIETLK	VIDEEWQRTQ	CSPRETCEVV
mVEGF-D	...ASMSDR	SASHRSTRFA	ATFYDTETLK	VIDEEWQRTQ	CSPRETCEVV
	151				200
hVEGF-C	GKEFGVATNT	FFKPPCVSVY	RCGGCCNSEG	LQCMNTSTSY	LSKTLFEITV
hVEGF-C	GKEFGAATNT	FFKPPCVSVY	RCGGCCNSEG	LQCMNTSTGY	LSKTLFEITV
hVEGF-D	ASELGKSTNT	FFKPPCVNVF	RCGGCCNEES	LICMNTSTSY	ISKQLFEISV
mVEGF-D	ASELGKTTNT	FFKPPCVNVF	RCGGCCNEEG	VMCMNTSTSY	ISKQLFEISV
	201		↓C1HIS	↓C2HIS	250
hVEGF-C	PLSQGPKPVT	ISFANHTSCR	CMSKLDVYRQ	VHSIIRRSIP	.ATLPQCOAA
hVEGF-C	PLSQGPKPVT	ISFANHTSCR	CMSKLDVYRQ	VHSIIRRSIP	.ATLPQCOAA
		<u>Ab Achen 2</u>			
			↓C1HIS	↓C2HIS	
hVEGF-D	PLTSVPELVP	VKVANHTGCK	CLPTAP..RH	PYSIIRRSIQ	IPPEEDRCSHS
mVEGF-D	PLTSVPELVP	VKIANHTGCK	CLPTGP..RH	PYSIIRRSIQ	TPEEDECPHS
	251				300
hVEGF-C	NKTCPTNYMW	NNHICRCLAQ	EDFMFSSDAG	DDSTDGFHDI	CGPNKELDEE
hVEGF-C	NKTCPTNYVW	NNYMCRCLAQ	QDFIFYSNVE	DDSTNGFHDV	CGPNKELDED
hVEGF-D	KKLCPIDMLW	DSNKCKCVLQ	EENPL.AGTE	DHS.....	.....HLQE.
mVEGF-D	KKLCPIDMLW	DNTKCKCVLQ	DETPL.PGTE	DHS.....	.....YLQE.
	301				350
hVEGF-C	TCQCVCRAGL	RPASCGPHKE	LDRNSQCVC	KNKLFPSQCG	ANREFDENTC
hVEGF-C	TCQCVCCKGL	RPSSCGPHKE	LDRDSCQVC	KNKLFNSCG	ANREFDENTC
hVEGF-D	.....	.....	.....	.....PALCG	PHMMFDEDRC
mVEGF-D	.....	.....	.....	.....PTLCG	PHMTFDEDRC
	351				400
hVEGF-C	QCVCCKRTCPR	NQPLNPGKCA	C.ECTESPQK	CLKGKGFHH	QTCSC.....
hVEGF-C	QCVCCKRTCPR	NQPLNPGKCA	C.ECTENTQK	CFLGKGFHH	QTCSC.....
		<u>Ab Achen 3</u>			
hVEGF-D	ECVCKTPCPK	DLIQHPKNCS	CFECKESLET	CCQKHLFHP	DTCSCEDRPC
mVEGF-D	ECVCKAPCPG	DLIQHPNCS	CFECKESLES	CCQKHKIFHP	DTCSCEDRPC
	401			↓C3HIS	
hVEGF-C	.YRRPCTNRQ	KACEPGFSYS	EEVCRCVPSY	WKRPMMS*	
hVEGF-C	.YRRPCANRL	KHCDPGLSFS	EEVCRCVPSY	WKRPHLM*	
hVEGF-D	FHTRPCASGK	TACAKHCRFP	KEKRAAQGPH	SRKNP*..	
mVEGF-D	FHTRTCASRK	PACGKHWRFP	KETR.AOGLY	SQENP*..	

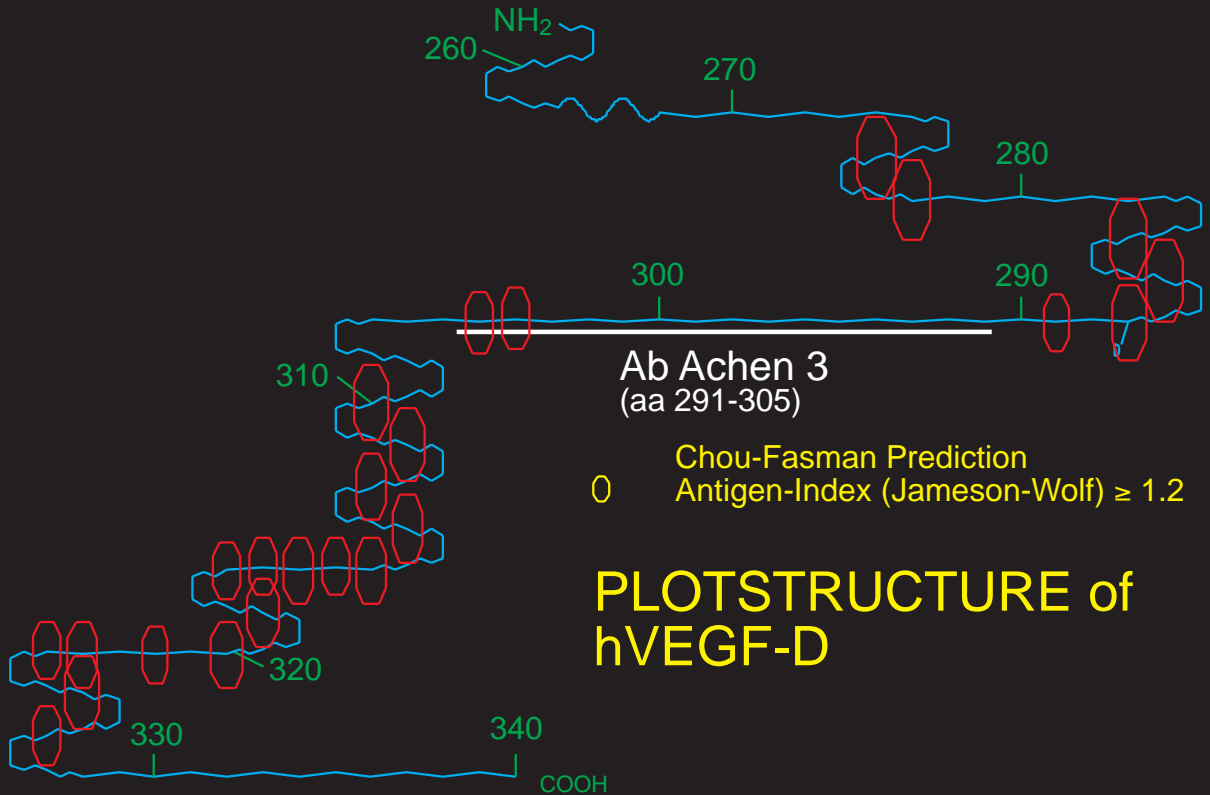
# PLOTSTRUCTURE of hVEGF-D

- Chou-Fasman Prediction
- Antigen-Index (Jameson-Wolf)  $\geq 1.2$

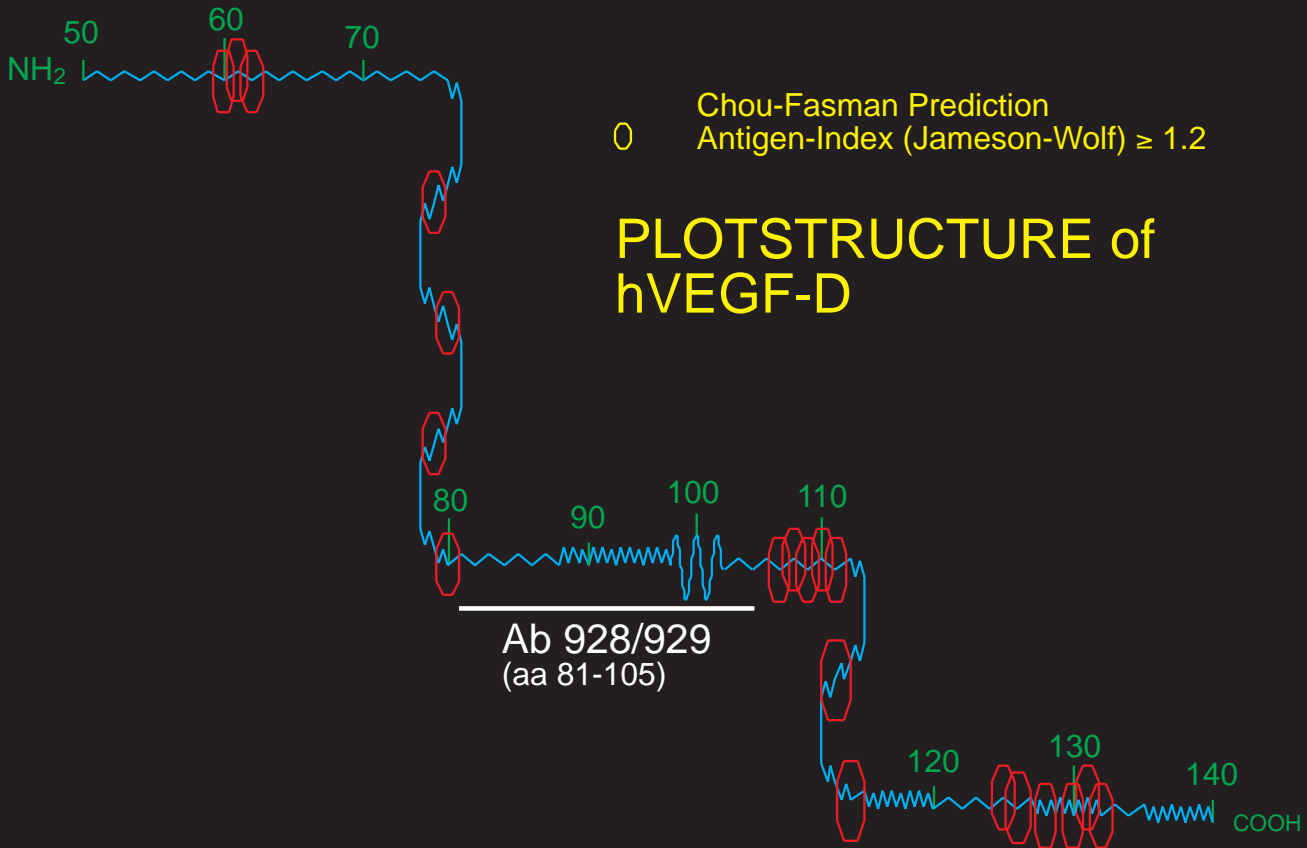




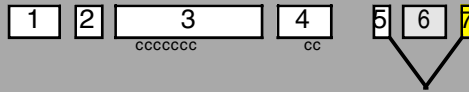
## PLOTSTRUCTURE of hVEGF-D



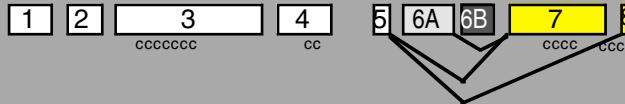
## PLOTSTRUCTURE of hVEGF-D



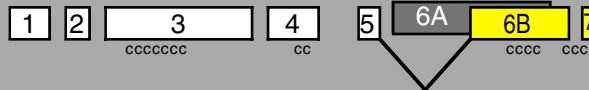
PIGF



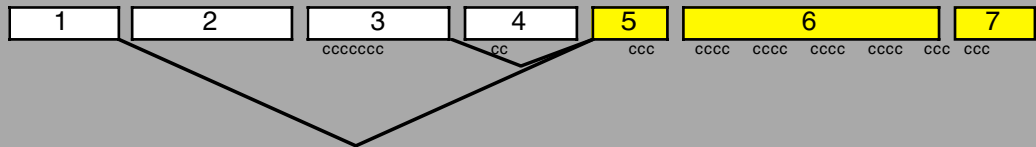
VEGF



VEGF-B



VEGF-C



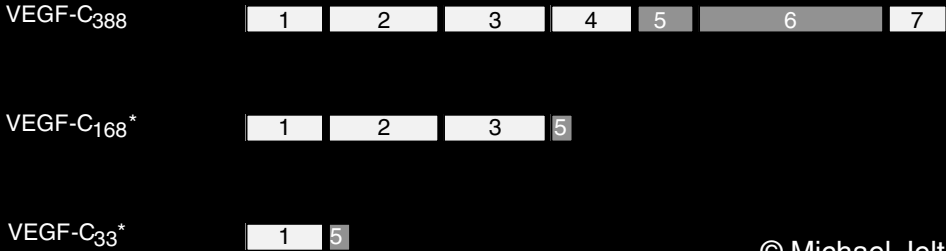
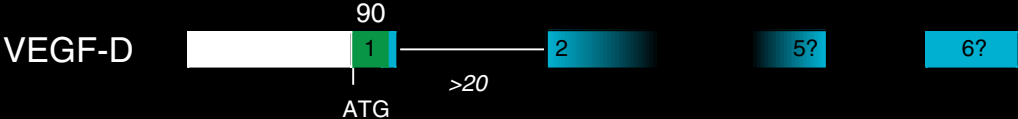
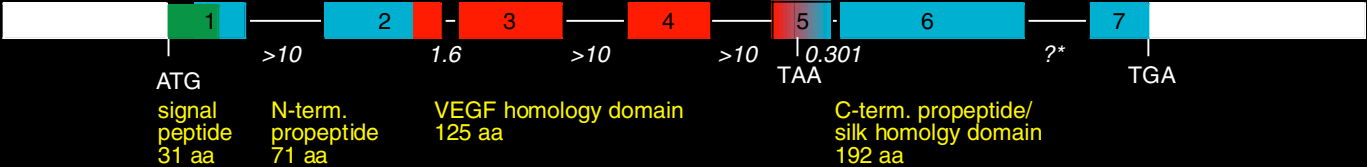
ccccccc    cc    8 conserved cysteine residues of the cystine knot consensus sequence (PDGF family subtype):  
 C(24-26X)C(5X)CXXCC(6X)C(32-36)CXC

cccc    4 conserved cysteines of the BR3P consensus sequence:  
 C(10-14X)CXCXC

ccc    3 conserved cysteines of an incomplete BR3P consensus sequence:  
 C(10-14X)CXC

# VEGF-C

aa	49	71	64	51	36	111	37
nt	147	214	191	152	107	334	112

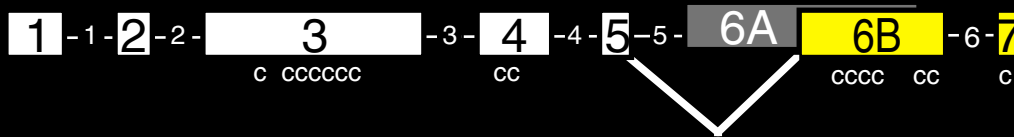


## Organization of the human VEGF-B gene

<i>mistake</i>	<i>Grimmond et al.</i>	<i>Olofsson et al.</i>	<i>Dima's genomic VEGF-B clone</i>
length of intron 1	564 bp	~760 bp	565 bp
length of intron 2	313 bp	275 bp	312 bp
exon 3-intron 3 donor	CAGgtcctgggca	CAGgtactgggca	like Olofsson's data
length of intron 3	246 bp	244 bp	244 bp
intron 4-exon 5 acceptor	tactttccagACC	tacttttcagACC	like Olofsson's data
exon 6B-intron 6 donor	CAGgtgaggcgtc	CAGgtaggtttg	like Grimmond's data
length of intron 6	~800 bp	736 bp	700 bp

### Additional information obtained while investigating the above mentioned differences:

- length of intron 4: 756 bp  $\pm$  2 bp
- length of intron 5: 197 bp (186) and 298 bp (167)
- mistakes in promoter sequence: a PstI site (nt 1030) and a BssHII site (nt 1084) are present in the promoter, GGG instead of GG at nt 1040



## Copy number

- Founder mice (F0) ⇒ apparent copy number reflects both actual copy number per diploid genome and the fraction of cells containing the transgene; approx. 20-30% of all founders are believed to be mosaics (Wilkie et al., 1986)
- Apparent copy number of founder mice has to be confirmed by Southern of the F1 and F2 generation, before PCR analysis alone is sufficient
- Southern blot (evaluation by eye in comparison with a standard curve) or dot blot (szintillation counting)
- Copy number does often, but not always correlate with transgene expression level! ⇒ quantitation of transgene product or phenotype
- PCR analysis is prone to produce false positives, whereas Southern and dot blot analysis is prone to produce false negatives, especially with low copy numbers
- Determination of DNA concentration (RNA, uncompletely dissolved DNA; measurement after digestion is more accurate than before digestion due to the high viscosity of undigested DNA; duplicate measurements are recommended)
- Internal standard is possible by probing for an endogenous gene (any single copy gene)
- Southern blot results are not as reliable than dot blot results (more handling steps increase variance)
- Controls: Standard curve of 1, 10 and 100 copies of the TGU mixed with neg. tail DNA

## Homozygosity

- Homozygosity is reached by backcrossing and this term should not be used for mice who inherited the transgene from two different founders; most “heterozygous” transgenes are hemizygous
- Approx. 5-15% of all transgene integration events produce recessive lethal mutations (Hogan et al., 1994)

### Identifying homozygous mice

*Methods requiring the knowledge of the genomic flanking sequences (require integration into non-repetitive DNA)*

1. PCR with flanking primers
2. Southern blot with a genomic flanking probe

*Methods not requiring the knowledge of the genomic flanking sequences*

1. Test breeding
2. In situ hybridization to interphase nuclei
3. Transgene quantitation by Southern or dot blot
4. Transgene product quantitation

## Identifying mice with transgenes from two different founders

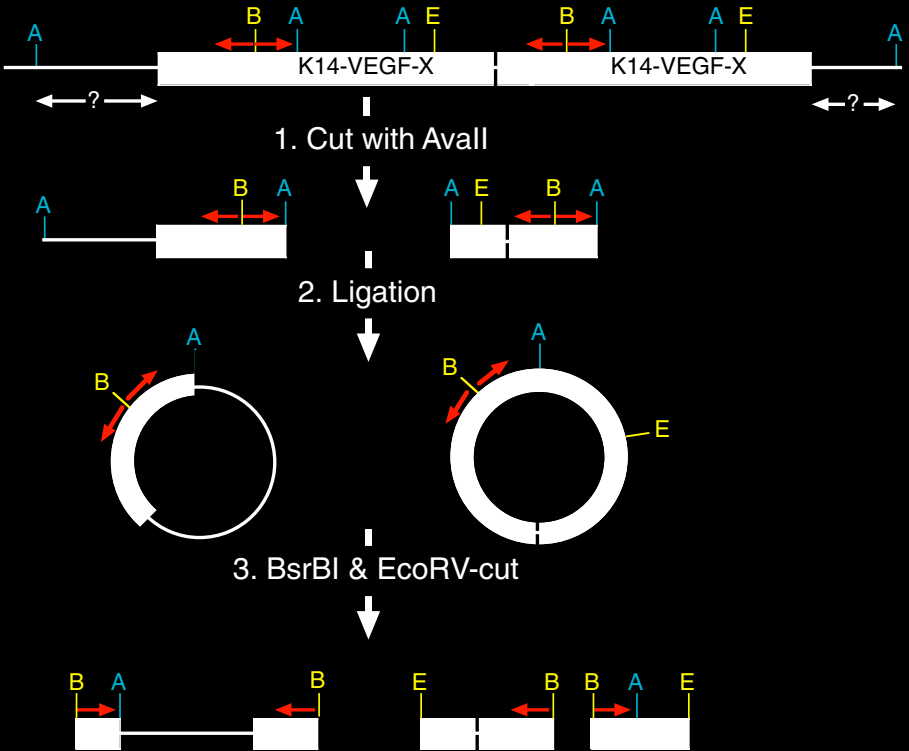
*Methods requiring the knowledge of the genomic flanking sequences*

1. PCR with flanking primers
2. Southern blot with a genomic flanking probe

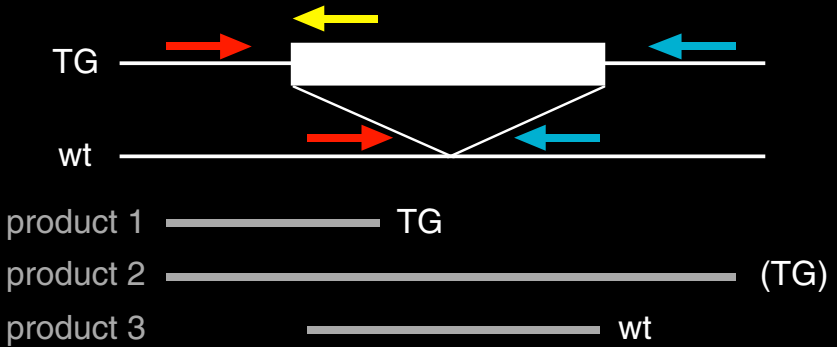
*Methods not requiring the knowledge of the genomic flanking sequences*

1. Southern blot and junction fragments: first and last fragments of a transgene array are often not suitable to identify transgenics with two integration sites
2. Test breeding
3. In situ hybridization to interphase nuclei
4. Transgene quantitation by Southern or dot blot: only possible, if the copy number of the two different transgenes is similar

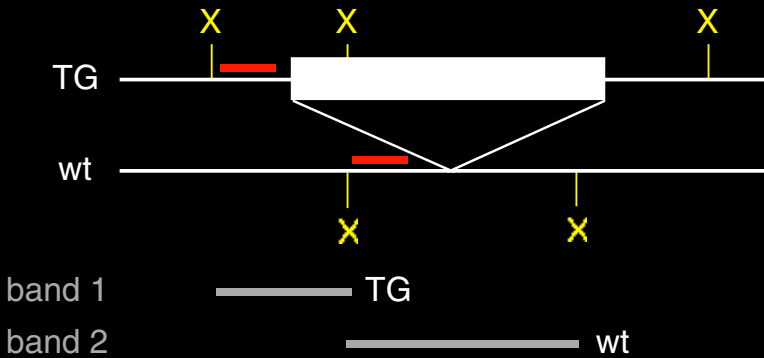
E = EcoRV (blunt-cutter)  
B = BsrBI (blunt-cutter)  
A = Avall  
→ = primer



## PCR



## Southern blot



## Expression pattern of VEGF-C and VEGF-D: A comparison

<i>transcript source(s)</i>	<i>hVEGF-D</i> 2.2 kb <i>Yamada et al., 1997</i>	<i>mVEGF-D</i> 2.4 & 3.7 kb <i>Yamada et al., 1997</i>	<i>hVEGF-C</i> 2.0 & 2.4 kb <i>Joukov et al., 1996</i>	<i>mVEGF-C</i> 2.0 & 2.4 kb <i>Kukk et al., 1996</i>
<i>lung</i>	+++	+++	+++	+++
<i>heart</i>	+++	+	+++	+++
<i>small intestine</i>	++	n.d.	+++	n.d.
<i>skeletal muscle</i>	+++	+	+	+
<i>colon</i>	++	n.d.	+	n.d.
<i>pancreas</i>	+	n.d.	+	n.d.
<i>kidney</i>	-	+	-	+
<i>liver</i>	+	n.d.	-	+
<i>placenta</i>	-	n.d.	+++	n.d.
<i>brain</i>	-	+	-	+
<i>PBL</i>	-	n.d.	-	n.d.
<i>ovary</i>	+	n.d.	+++	n.d.
<i>testis</i>	+	+	+	-
<i>prostate gland</i>	+	n.d.	+	n.d.
<i>thymus</i>	-	n.d.	-	n.d.
<i>spleen</i>	+	+	+	-
<i>fetal kidney</i>	+	n.d.	+++	n.d.
<i>fetal liver</i>	-	n.d.	-	n.d.
<i>fetal lung</i>	+++	n.d.	+++	n.d.
<i>fetal brain</i>	-	n.d.	-	n.d.