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1 **Debunking the myth of the endogenous anti-angiogenic *Vegfaxxb* transcripts.**

2
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16 DS edited the MS and contributed additional data analysis; all authors approved the final draft.

17

18 **Abstract**

19 In this article we critically assess evidence for the existence of a family of anti-angiogenic
20 *Vegfaxxb* transcripts, arising from the use of a phylogenetically conserved alternative distal
21 splice site within exon 8 of the *VEGFA* gene. We explain that prior evidence for *Vegfaxxb*
22 transcripts in tissues rests heavily upon flawed RT-PCR methodologies, with the extensive use of
23 5'-tailing in primer design being the main issue. Furthermore, our analysis of large RNA-seq
24 datasets (human and ovine) fails to identify a single *Vegfaxxb* transcript. Therefore, we
25 challenge the very existence of *Vegfaxxb* transcripts, which further questions the physiological

26 relevance of studies based on the use of “anti-VEGFA_{xxx}b” antibodies. Our analysis has
27 implications for the proposed therapeutic use of isoform-specific anti-VEGFA strategies for
28 treating cancer and retinopathies.

29

30 **Classical angiogenic VEGFA isoforms**

31 VEGFA is a key regulator of vascular homeostasis and therapeutic target in pathological
32 angiogenesis. The *VEGFA* gene is subject to alternative splicing, which occurs primarily in
33 exons 6 and 7 (**Figure 1**) [1-4]. In humans, the most abundant mature translated isoform is
34 VEGFA165 (i.e. this isoform comprises 165 amino acids), which lacks exon 6. Three other
35 isoforms are present in moderate/high amounts: VEGFA121, which lacks both exons 6 and 7,
36 VEGFA189, which includes exon 7 but uses an alternative 5'-donor site in exon 6 and
37 VEGFA206, which includes both exons 6 and 7 (**Figure 1B & Figure 1E**). These VEGFA
38 isoforms exist in other species, including mouse, sheep and cattle, but they are one amino acid
39 shorter than their human counterparts. Other less abundant isoforms have been described, some
40 of which are also generated through alternative splicing within exons 6/7 (VEGFA145,
41 VEGFA148, VEGFA183). The amino acid stretches encoded by exons 6 and 7 are enriched in
42 clusters of basic amino acids, which confer VEGFA with the ability to bind the extracellular
43 matrix and thus mitigate its diffusion. Consequently, VEGFA121 is highly diffusible, while
44 VEGFA189 and VEGFA206 remain bound to the extracellular matrix and VEGFA165 has
45 intermediate matrix binding characteristics [1-4]. VEGFA isoforms bind with varying affinities
46 to the tyrosine kinase receptors VEGFR1 and VEGFR2 and recruit the co-receptor Neuropilin 1
47 to activate intracellular signaling pathways as illustrated on the left side of **Figure 1G** [5,6].
48 Critically, all VEGFA isoforms promote angiogenesis, which is key to the progression of
49 tumorigenesis [7].

50

51

52

53 **Non-classical anti-angiogenic VEGFA_{xxx}b and VEGF-A_x isoforms**

54 In 2002, Bates and colleagues [8] reported a novel human *Vegfa* splice variant, generated
55 through use of an alternative distal splice site within the last exon of the *VEGFA* gene, exon 8
56 (see **Figure 1C** and **Figure 1D**). The transcript was dubbed *Vegfa165b*. Follow-up studies
57 suggested that the encoded VEGFA165b protein product belongs to an entire VEGFA_{xxx}b
58 family of proteins, which are derived from transcripts that use the distal splice site in exon 8
59 (**Figure 1E**) and bear anti-angiogenic properties [3,9-14]. From a functional standpoint,
60 VEGFA165b was shown to bind to VEGFR2 with the same affinity as VEGFA165 [15-18] but
61 failed to bind to Neuropilin 1 as illustrated on the right part of **Figure 1G** [16,17,19].
62 VEGFA165b induces VEGFR2 tyrosine phosphorylation in a dose-dependent manner, albeit less
63 efficiently and more transiently than VEGFA165 [15-17,19]. However, this impaired ability of
64 VEGFA165b to activate intracellular signaling pathways may not apply to all cell types [20,21].
65 Another isoform, dubbed VEGF-A_x, has recently been described [22]. VEGF-A_x arise from
66 programmed translational read-through, such that the stop-codon of classical VEGFA isoforms
67 leads to insertion of a Serine residue, and the in-frame stop-codon of putative VEGFA_{xxx}b
68 isoforms is used instead. In other words, VEGF-A_x and VEGFA_{xxx}b isoforms would share the
69 same C-term sequence. However, the *in vivo* existence of VEGF-A_x is disputed and *in vitro* data
70 demonstrate pro-angiogenic properties [23].

71

72 **Existence of VEGFA_{xxx}b isoforms: clinical implications**

73 Overall, VEGFA165b appears to behave as a partial agonist [16], which competes with
74 VEGFA165 for VEGFR2/Neuropilin 1 binding. This may explain the reduced angiogenic
75 potential of VEGFA165b [20,23,24]. Based on this, Harper & Bates proposed a model in which

76 the balance between endogenous levels of angiogenic VEGFA_{xxx} and anti-angiogenic
77 VEGFA_{xxxb} isoforms sets the angiogenic potential of a tissue [3]. They further proposed that
78 VEGFA_{xxxb} isoforms predominate in many normal cells and tissues where they can amount to
79 more than 50% of total VEGFA protein [10]. In normal colonic tissues in particular,
80 VEGFA_{xxxb} variants have been reported to comprise as much as 95% of all VEGFA [25]. The
81 proposed use of the alternative distal splice site within exon 8 is therefore not a rare event, and
82 its prevalence implies major physiological relevance. However, in pathological conditions such
83 as cancer and retinopathies, VEGFA_{xxxb} levels appear to be substantially downregulated.
84 Considering the crucial role of VEGFA in angiogenesis of various cancers and retinopathies
85 [1,26-28] such a finding may have broad clinical implications.

86
87 Indeed, current strategies to target VEGFA, such as the anti-VEGFA antibody bevacizumab,
88 Avastin®) or aflibercept (VEGFA-trap), would indiscriminately inhibit both angiogenic
89 VEGFA_{xxx} and anti-angiogenic VEGFA_{xxxb} isoforms. Bates *et al* showed that targeting
90 VEGFA in colorectal cancer was more efficient if endogenous levels of VEGFA_{xxxb} were low
91 and proposed that assessment of the ratio of VEGFA_{xxxb} to VEGFA_{xxx} could potentially
92 predict response to bevacizumab and other therapies directed against VEGFA [3]. Therefore,
93 modification of VEGFA splicing to alter the VEGFA_{xxxb}/VEGFA_{xxx} balance, or development
94 of compounds to target only VEGFA_{xxx} isoforms, may have therapeutic value for treating
95 cancers and retinopathies [29]. In 2009, D.O. Bates and S.J. Harper filed a patent for the “Novel
96 use of VEGFA_{xxxb}”, which covers the use of these isoforms (primarily VEGFA165b) for
97 several potential therapeutic applications.

98

99 **The Controversy: Do VEGFA_{xxxb} isoforms really exist?**

100 Despite a substantial amount of literature reporting evidence for the existence of VEGFA_{xxx}b
101 isoforms, the VEGFA scientific community is divided on this issue. We and others have yet to
102 detect these isoforms in normal and pathological tissues and have thus concluded that
103 VEGFA_{xxx}b isoforms, if they do exist, are likely not of physiological relevance. In recent
104 reviews on VEGFA signaling and disease, VEGFA_{xxx}b biology is largely disregarded [7] or
105 notably overlooked [30]. Until now, evidence for the existence of VEGFA_{xxx}b isoforms has
106 been provided through PCR-based approaches and studies using an antibody generated against
107 the sequence TCRSLTRKD encoded by putative exon 8b of the human *Vegfa* gene. Here, we
108 critically review and reassess the evidence which led to the assumption that "there is an
109 important role for VEGFA_{xxx}b isoforms in normal physiology" [10]. Our own findings [31-33]
110 and a thorough analysis of the literature has led us to question and re-evaluate the existence of
111 *Vegfa_{xxx}b* transcripts

112

113 **Absence of Evidence: Detection of *Vegfa_{xxx}b* transcripts with flanking primers.**

114 Since alternative splicing affects exons 6, 7 and 8, the best way to identify all *Vegfa* splice
115 variants is to use primers flanking these regions. Such a primer design is expected to amplify all
116 *Vegfa* isoforms. To do so, the forward primer has to be located within exons 1-5 (common to all
117 splice variants) and the reverse primer located downstream of the putative exon 8b splice site. If
118 *Vegfa_{xxx}* and *Vegfa_{xxx}b* splice variants are sister families and *Vegfa_{xxx}b* mRNAs represent a
119 large fraction of total *Vegfa* mRNA, at least in normal tissues [10], a standard RT-PCR with the
120 aforementioned primers would be expected to yield at least 6 main products/bands corresponding
121 to mRNA for the major isoforms VEGFA189, VEGFA165 and VEGFA121 and their "sister
122 bands" for VEGFA189b, VEGFA165b and VEGFA121b, albeit at different abundances. The
123 bands encoding *Vegfa_{xxx}b* isoforms would be 66bp shorter than their respective counterparts
124 (see **Figure 1D**) thereby yielding 3 "doublets" easily discernible on an agarose gel. However, in

125 their seminal paper reporting on the discovery of *Vegfa165b* [8], Bates *et al* stated that "PCR of
126 the full-length product using primers V165K (complementary to the translation initiation site of
127 the other isoforms of VEGF) and V165X (a primer downstream of the original 3'-UTR) resulted
128 in one strong band at ~670bp". *Considering the strategy used, it is difficult to explain why other*
129 *Vegfaxxx* and *Vegfaxxxb* transcripts were not identified by this initial study. Indeed, as mentioned
130 above, at least 6 bands, corresponding to the 3 "doublets" (VEGFA189/189b, VEGFA165/165b
131 and VEGFA121/121b), should have been observed by agarose electrophoresis of PCR products.
132 ~~Furthermore, nested PCR with a 3'-UTR and exon 7a primers resulted in a strong band at~~
133 ~~~130bp confirming that the full-length was VEGFA165b".~~

134
135 We and others have adopted this strategy in order to identify *Vegfaxxxb* transcripts in human,
136 mouse, sheep and cattle but failed to identify *Vegfa165b* mRNA - or other *Vegfaxxxb* products
137 [22,31,33,34]. Instead, only three bands were observed (see **Figure 1B** and **Figure 1F**) and
138 sequencing of the PCR products showed unequivocally that they encoded VEGFA189,
139 VEGFA165 and VEGFA121 [22,24,32-34]. Therefore, an unbiased and straightforward RT-PCR
140 strategy to simultaneously amplify all *Vegfa* isoforms failed to support the existence of
141 *Vegfaxxxb* mRNA. Since these flanking primers could not discriminate *Vegfaxxx* from putative
142 *Vegfaxxxb* transcripts, isoform-specific primers were developed.

143
144 **Detection of *Vegfaxxxb*-like transcripts with "isoform-specific primers": a tale of a tail.**
145 Over time, there has been a considerable drift in the design of primers aimed at the specific
146 identification of *Vegfa165b* that span the exon 7 – exon 8b splice site. However, in most studies,
147 the exon 7-specific stretch has been greatly lengthened at the expense of the exon 8b-specific
148 region (see **Figure 2A** and **Table S1**). Since exon 7 would be shared by both *Vegfa165* and
149 *Vegfa165b*, the isoform-specificity of these reverse primers is questionable. Technically, the

150 necessary and sufficient primer design to allow specific detection of *Vegfa165b* mRNA would
151 include the shortest possible 3'-anchor within exon 7; based on standard PCR principles (see
152 **Figure 3**) one base specific to exon 7 on the 3'-end of the primer would suffice. However, in the
153 case of the human sequence, since the AT motif would be shared by both *Vegfa165* and
154 *Vegfa165b* (see **Figure 1**) this means that the last 3bp at the 3'-end of such a primer would
155 anneal to exon 7 with the last nucleotide discriminating between *Vegfa165* and *Vegfa165b*
156 isoforms (see **Figure 2A**, Primer P1). Surprisingly, this most simple strategy has never been used
157 in the literature.

158
159 Rather than using such a “minimal and sufficient” primer design for detection of *Vegfaxxb*
160 isoforms, the design of the primer has been "adapted". The initial design, published in the
161 seminal *Vegfa165b* paper by Bates *et al* [8], comprised of 7bp complementary to either *Vegfa165*
162 or *Vegfa165b* (**Figure 2A**, Primer P2). This primer has been used in a number of subsequent
163 studies by the same group [15,35-39] and other research teams that used RT-PCR to demonstrate
164 the existence of *Vegfaxxb* transcripts [40,41]. In 2007, Ribeiro *et al* used a modified version of
165 this primer for their studies in pig [42], with 9bp complementary to either *Vegfa165* or
166 *Vegfa165b* (**Figure 2A**, Primer P3), while Baba *et al* used 8bp complementary to either
167 *Vegfa165* or *Vegfa165b* [43]. Overall, the number of bases complementary to either *Vegfa165* or
168 *Vegfa165b* has been on the rise since 2007: 13 bp in 2008 ([44]; **Figure 2A**, Primer P4), 14bp in
169 2009 (study in rat; [45]) and 15bp in 2010-2011 ([46,47]; **Figure 2A**, Primer P5). Finally, a
170 design including no less than 17bp common to both *Vegfa165* or *Vegfa165b* was proposed to
171 specifically amplify *Vegfa165b* mRNA ([48]; **Figure 2A**, Primer P6). We also note that the same
172 methodology has been used for the design of a *Vegfa165b*-specific forward primer, which
173 includes 14bp that would also anneal to *Vegfa165* ([34]; **Figure 2A**, Primer P7). This approach
174 has also been used by others to "specifically" detect *Vegfa121b* isoforms ([29,44]; see **Table S1**).

175
176 We demonstrated that this primer design is responsible for PCR artefacts, due to 5'-tailing, that
177 could be mis-interpreted as *Vegfaxxb* transcripts ([31,33]; see **Figure 3**). The conclusion of
178 these studies was that PCR products correspond to putative *Vegfaxxb* transcripts only because
179 primers were designed to include on their 5'-end a few bases of the *Vegfaxxb* sequence itself.
180 This method has been used for decades, most often to add restriction sites to aid cloning. Good
181 examples are provided by Catena *et al* [20] and Ganta *et al* [49], who flanked the 5'-end of their
182 reverse primers with an *HindIII* (or *BamHI*, respectively) restriction site followed by the entire
183 predicted sequence for the putative exon 8b and 20-23 bp complementary to either exon 5 or
184 exon 7 in order to generate synthetic expression vectors for *Vegfa121b* or *Vegfa165b* (see **Table**
185 **S1**). We demonstrated that *Vegfa* amplification by RT-PCR could be obtained when bases
186 "specific for *Vegfaxxb*" on the primer 5'-end were changed to a GGGGG or an AAAAA stretch
187 [33]. The minimal primer design we defined to be sufficient to obtain artefactual *Vegfaxxb*-like
188 PCR products perfectly matches the P4 primer described above ([33]; see **Figure 2B**). Therefore,
189 studies which claimed identification of *Vegfaxxb* transcripts using primers with ≥ 13 bp common
190 to *Vegfaxx* and putative *Vegfaxxb* mRNA do not reach required standards of evidence, and
191 their conclusions should be re-evaluated.

192
193 By 2013, the use of the questionable P4 primer design had become widespread in the literature
194 [44,50-52]. This design constitutes a significant departure from initial studies that used the P2
195 primer with 7bp complementarity to either *Vegfa165* or *Vegfa165b*. The reasons for this drift in
196 primer design are not clear. However, as early as 2006, Bates and colleagues [37] acknowledged
197 that "...RT-PCR is not quantitative and it has not been possible so far to develop isoform-
198 specific qPCR, due to the lack of exon-specific sequences". It seems surprising that primers

199 deemed suitable for specific detection of *Vegfa165b* in standard RT-PCR would not be suitable
200 for qPCR.

201 In conclusion, the detection of “*Vegfaxxb*-like” transcripts could be explained by the use of
202 inadequate RT-PCR methodologies involving 5’-tailing of the primers (also see **BOX1**).

203

204

205

206 **Bioinformatic analysis of alternative splicing: absence of evidence for *Vegfaxxb* isoforms.**

207 Using AVISPA, a tool for prediction and analysis of alternative splicing, Barash *et al* reported
208 that "prediction of other splice variations of *Vegfa*, such as the 3’ splice site variation in exon 8,
209 are currently not supported by the tool [53] ". Another splice site prediction software ([54];
210 available at http://www.fruitfly.org/seq_tools/splice.html) identified the canonical acceptor
211 splice site of exon 8 (score of 0.97) but failed to identify the putative exon 8b splice site.
212 Therefore, efficient *in silico* tools do not currently support the existence of the proposed distal
213 splice site in exon 8 of the *Vegfa* gene.

214

215 **Detection of the *Vegfaxxb* transcripts: evidence of absence by unbiased RNA-seq analysis.**

216 As detailed above, the most parsimonious explanation for the lack of *Vegfaxxb* PCR products is
217 that such transcripts do not exist. However, a theoretical possibility remains that failure to detect
218 *Vegfaxxb* transcripts results from the targeted nature of the approach (also see **BOX2**). If so, the
219 analysis of RNA datasets obtained through the use of an alternative, unbiased methodology, such
220 as RNA-seq, should clarify this matter. Indeed, “RNA-seq represents the method of choice for
221 the discovery of alternative splicing events across tissues [55]”.

222

223 Therefore, we investigated existence of *Vegfaxxb* transcripts in publicly available RNA-seq
224 datasets generated from multiple human tissues [29]. Our extensive analysis confirmed all
225 known *Vegfa* splice variants and also identified novel transcripts. In particular >40000 *Vegfa*
226 transcripts were uncovered that spanned the junction between exon 7 and exon 8 in >10 different
227 human tissues. None of these reads supported the existence of an exon 8b splice site [29].

228
229 We then analysed two independent RNA-seq datasets of hypothalamus / pituitary from castrated
230 rams [57] and ovariectomized, estradiol-implanted ewes [56]. This analysis confirmed that *Vegfa*
231 splice variants are phylogenetically conserved, as transcripts identified in sheep correspond to
232 those present in human [33]. Indeed, we also identified several novel ovine *Vegfa* transcripts that
233 correspond to those identified by Bridgett *et al* in human [29]. Crucially, we identified 2693
234 *Vegfa* transcripts that cover the junction between exon 5 and exon 8 or between exon 7 and exon
235 8. None of the reads supported the existence of an exon 8b splice site, hence the existence of
236 ovine *Vegfaxxb* transcripts [33]. These findings [33] rule out the proposed role for VEGFAxxx
237 isoforms in the control of ovine seasonal breeding proposed by Castle-Miller *et al* [58]. We
238 further note that the 43bp-long reverse primer (see **Table S1**), as provided in the erratum of the
239 aforementioned study [58] to “specifically” amplify *Vegfa165b* by qPCR, would indeed lead to
240 co-amplification of the classical *Vegfa165* isoform, as its sequence is complementary to a stretch
241 of exon 8 located downstream of the putative distal splice site. This implies that qPCR would
242 yield two amplicons, which makes the primer pair inadequate for this use and leads to questions
243 on the validity of the data published by Castler-Miller *et al* [58]. **In summary, the failure to**
244 **detect *Vegfaxxb* mRNA in human and ovine RNA-seq data strongly suggests that these**
245 **transcripts do not exist** (also see **BOX3**).

246

247 **No *Vegfaxxb* transcripts: detection of VEGFAxxx proteins is an artefact.**

248 Absence of endogenous *Vegfaxxb* mRNA implies absence of endogenous VEGFA_{xxx}b
249 proteins. Consequently, studies which investigated VEGFA_{xxx}b levels in tissues or plasma,
250 using anti-VEGFA_{xxx}b antibodies and ELISA kits, or those which relied exclusively on over-
251 expression or injection of recombinant VEGFA_{xxx}b proteins have questionable physiological
252 relevance (see **Table S1**; e.g. [18,59-74]).

253
254 The first antibody against VEGFA_{xxx}b isoforms (MVRL56/1) was developed by Woolard *et al*
255 2004 [15] and was raised against the synthetic peptide TCRSLTRKD which corresponds to the
256 nine amino acid C-terminal sequence of human VEGFA165b (see **Figure 1C**). This peptide is
257 only six amino acids different from the peptide that corresponds to the C-terminal sequence of
258 VEGFA165 (TCRCDKPRR). This antibody was made commercially available and distributed
259 by R&D (MAB3045) and Abcam (Ab149940; [41]). A sandwich ELISA kit that utilizes the
260 MAB3045 antibody is also available via R&D (#DY3045) who also supply a recombinant
261 human VEGFA165b protein (#3045-VE-025). Interestingly, Abcam removed Ab149940 from
262 their catalog in 2015 as it "did not meet the quality criteria". Specifically, the antibody yielded
263 "low signal-to-noise ratio in immunofluorescence and detected multiple non-specific bands in
264 western-blot" (communication from Abcam). The use of MVRL56/1 for analysis in the same
265 tissue has sometimes led to opposite findings. For instance, using normal breast tissue, Catena *et*
266 *al* [20] found no VEGFA_{xxx}b staining while Qiu *et al* [75] observed strong staining. When used
267 in western-blot studies, MVRL56/1 yields multiple bands (i.e. smear) that span a broad range of
268 molecular weights. These bands have been considered to represent monomers, dimers or large
269 complexes of VEGFA_{xxx}b isoforms [36,37]. However, definitive evidence that these bands
270 correspond to various forms of VEGFA_{xxx}b is still lacking.

271

272 As summarized in **Table S1**, virtually all studies aimed at detection of VEGFA_{xxx}b proteins
273 have relied on the use of the MVRL56/1 antibody. Origene Europe also sells a different antibody
274 against hVEGFA165b C-Terminal peptide (the exact sequence of the epitope is not provided;
275 #DM3615P). Finally, an "Anti-VEGFA111b antibody" has been raised [76] using "synthetic
276 peptide fragments of the 8 amino acids CRSLTRKD". Contrary to what the authors claim, this
277 antibody would not be specific for the VEGFA111b isoform, [since the epitope corresponds to the](#)
278 [8 amino acid C-terminal sequence shared by all predicted human VEGFA_{xxx}b isoforms.](#)

279
280 Surprisingly, the original anti-human VEGFA_{xxx}b antibody (MVRL56/1) has been used to
281 demonstrate the presence of VEGFA_{xxx}b in multiple species that do not share the same C-
282 terminal sequence (see **Figure S1**). For instance, it has been used in sheep [58] for which the
283 predicted sequence for the last 9 amino acids of the C-Terminal would be TCRCLTRKD,
284 therefore slightly divergent from that in human (underlined, Cys instead of Ser). This would
285 reduce the size of the VEGFA_{xxx}b-specific epitope recognized by the anti-VEGFA_{xxx}b
286 antibody to the LTRKD sequence. Having shown that *Vegf_{xxx}b* transcripts do not exist, we
287 surmised that VEGFA_{xxx}b-like immunostaining is accounted for by cross-reactivity of the
288 antibody with one or several protein(s) unrelated to VEGFA but bearing an epitope of similar
289 sequence to that of the putative LTRKD sequence of VEGFA_{xxx}b. We searched human protein
290 databases using BLASTP, which led to the unambiguous identification of 10 proteins harboring
291 an LTRKD motif [33]. The MVRL56/1 anti-human VEGFA_{xxx}b antibody was also used to
292 detect endogenous VEGFA_{xxx}b isoforms in tissues from mouse [65,75,77] and rat [45,60],
293 species which, as already noted by us and others ([13,28]; see **Figure S1**), would present a
294 distinct CRPLTGKTD motif at the C-term of VEGFA_{xxx}b, divergent from the human sequence.
295 Specifically, we found that in mouse, this antibody detects unidentified proteins that can be
296 mistaken for VEGFA isoforms and have raised this as a serious concern [28]. These non-specific

297 proteins were present in mouse cell extracts and their conditioned media as well as mouse
298 tissues. Although the suggestion by Bates *et al* [50] that these findings might be due to
299 artefactual detection of mouse IgG could potentially be correct when testing mouse tissues, this
300 possibility is excluded in cell culture and serum-free conditioned media. Taken together, we
301 conclude that the anti-VEGFA_{xxx}b antibody might cross-react with a large number of
302 endogenous proteins (>10) across different species but none of these belong to the VEGFA_{xxx}b
303 family.

304

305

306 **Concluding remarks**

307 We have reviewed current evidence for the proposed existence of a *Vegfaxxxb* family of
308 transcripts which would yield VEGFA_{xxx}b proteins bearing anti-angiogenic properties.
309 Inadequate PCR methodology which likely led to the erroneous identification of such transcripts
310 has been identified and characterized. Multiple published studies, along with our additional
311 analyses of RNA-seq data have failed to identify any *Vegfaxxxb*-specific splicing events. Thus
312 we conclude that *Vegfaxxxb* transcripts do not exist *in vivo* and therefore challenge the view that
313 endogenous VEGFA_{xxx}b proteins have any physiological relevance. Consequently, efforts to
314 develop therapeutics to modulate VEGFA activity should not be based upon modification of a
315 splicing event that is not supported by evidence. [The story of *Vegfaxxxb* splicing reinforces the](#)
316 [importance of supporting new findings using orthogonal techniques prior to basing subsequent](#)
317 [studies upon them \(see Outstanding Questions\).](#)

318

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321

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498

499 **Glossary**

500 **Alternative splicing:** process by which splice sites in precursor (pre)-mRNA are differentially
501 selected to produce multiple mRNA and protein isoforms. This process diversifies the functional
502 characteristics of genes and drastically expands the potential repertoire of protein variants.
503 Nearly all multi-exon genes have at least one splice variant.

504 **VEGFA** (Vascular Endothelial Growth Factor A): a key member of the family of growth factors
505 which plays a prominent role in angiogenesis - the growth of blood vessels - both in health and
506 disease (tumorigenesis and retinopathies).

507 **5'-tailing**: addition of nucleotides on the 5'-end of a PCR primer. These nucleotides are not
508 complementary to the target mRNA but do not impair PCR processivity and aid further cloning
509 of the PCR amplicon.

510

511

512

513 **Text BOXES**

514 **BOX1**

515 **Further technical issues with the use of qRT-PCR**

516 Since primers for standard PCR were not suitable for qPCR, Bates and co-workers developed an
517 alternative method, which we might call "indirect subtractive strategy" [25]. This requires the
518 use of two distinct primer pairs. The first pair consists of "an exon 7b forward primer and a 3'-
519 UTR Primer (both pan-VEGF)", the sequences of which were not provided. The second pair of
520 primers consists of "an exon 7a forward primer and a reverse primer specific for exon 8a that do
521 not detect *Vegfaxxb* isoforms". The authors then assume that subtracting what is found with the
522 second primer pair (*Vegfaxx* only) to what is found with the first primer pair (total *Vegfa*:
523 *Vegfaxx* + *Vegfaxxb*) yields the amount of *Vegfaxxb*. This is an unusual strategy, which is
524 likely to be flawed for a number of reasons. The primary concern is that these are two different
525 pairs of primers, with different sequences, hence divergent optimal T_m 's, which amplify
526 fragments of different lengths and nucleotide composition and therefore have differing
527 efficiencies. The use of qPCR is based on the principle that the number of copies doubles at
528 each cycle. This rule is used as a proxy to determine the initial number of copies. Using the

529 values of the slopes for the calibration curves (see Figure 1 in [25]) for both primer pairs allows
530 calculation of an amplification factor (which should ideally be ~2), which informs on the
531 efficiency of the qPCR assay. The efficiency should be ~100%, but values between 85-115% are
532 usually judged acceptable, providing the dissociation curves are good [78]. The calculated
533 efficiency values for the primer pairs used by Varey *et al* [25] are 83.7% and 71.8%. The
534 meaning of this is twofold. First, neither primer pair is appropriate for use in qPCR. Second,
535 different efficiencies are enough to generate a difference when data obtained with the two primer
536 pairs are compared. A broadly similar technique, but with different primers, was used by the
537 same team in a later study [79]. Other issues with RT-PCR and primer design were also found,
538 which are detailed in **Supplemental text**.

539 **BOX2**

540 **Ruling out potential issues with mRNA secondary structure**

541 The idea that PCR amplification of *Vegfa165b* might be difficult due to secondary structure of
542 the mRNA in the 3'-UTR has been invoked [9] to explain why we [31,33] and other authors
543 [13,79,80] failed to detect these transcripts. The existence of an unusually stable secondary
544 structure seems unlikely because most regulatory splicing mechanisms are based on the
545 recognition of short degenerate RNA motifs at the exon/intron boundaries rather than secondary
546 structures [82,83]. Indeed, bioinformatic analysis of the ribonucleotidic sequence does not
547 predict formation of potential hairpins that might inhibit reverse transcription (data not shown).
548 Furthermore, classical *Vegfaxxx* isoforms are readily amplified using primers spanning the
549 putative alternative splice site (see main text). There is therefore no evidence to support the
550 notion that *Vegfaxxxb* isoforms, which would lack a 66bp stretch present in *Vegfaxxx* isoforms,
551 would resist PCR amplification. Furthermore, *Vegfa165b* transcripts expressed from a
552 recombinant construct were readily detected by RNA-seq [32] and since the library preparation
553 involved both RT and PCR steps, neither are inherently blocked by the transcript secondary

554 structure. Therefore, the inability to detect an abundant mRNA such as *Vegfa165b* at least in
555 normal tissues by classical RT-PCR can not be explained by the secondary structure of the
556 mRNA.

557 **BOX3**

558 **Further RNA-seq evidence for absence of *Vegfaxxb* mRNAs**

559 Recently developed long read RNA sequencing technologies (e.g. PacBio Iso-Seq and Oxford
560 Nanopore Technology) can measure full length mRNA transcripts [85]. Both individual
561 alternative splicing events and the co-occurrence of multiple events in transcripts such as *Vegfa*
562 are detected. To determine whether data generated with this newly available approach could
563 detect *Vegfaxxb* mRNAs we analysed a publicly available dataset [85]. Full length transcripts
564 encoding all the commonly reported exon 5 and exon 6 isoforms (VEGFA121, VEGFA165,
565 VEGFA189) were present but no evidence of splicing to generate *Vegfaxxb* was detected
566 (**Supplemental Figure S2**). A notable feature apparent from this full length transcript data is the
567 retention of the intron between exons 3 and 4 in almost 20% of transcripts (which is supported
568 by previous EST data). The regulatory potential of such intron retention events has been widely
569 discussed [86,87]. Our recent analysis of RNA-seq data from tumours grown from fibrosarcomas
570 expressing VEGFA188 [88] has also failed to identify reads corresponding to the exon 7-8b
571 splice site, even though endogenous expression of multiple *Vegfxxx* isoforms could be detected
572 from the stromal cells (**Supplemental Figure S3**). To better understand the role of alternative
573 splicing in the regulation of *Vegfa* expression, research should be focused upon such events that
574 are supported by strong experimental evidence.

575

576 **Figure Legends**

577 **Figure 1:** Structure and sequence of the human *Vegfa* gene and VEGFAxxx/VEGFAxxx
578 isoforms. A/ Schematic of the human *Vegfa* gene locus. The gene is comprised of 8 exons. B/

579 Schematic of the longest human VEGFA protein (VEGF206, see GenBank accession number
580 NM_001171623). The stop-codon (TGA, in red) is located in exon 8 and the sequence of the last
581 9 amino acids at the C-terminus of the protein is provided below. The location of flanking PCR
582 primers O14I/O16I is also indicated (see panel F). C/ Schematic of the putative human
583 VEGFA206b protein (GenBank accession number NM_001033756). The stop-codon (TGA, in
584 red) is located in exon 8 – renamed exon 8b due to the usage of a distal splice site – and the
585 sequence of the last 9 amino acids at the C-terminus of the protein is provided below. Note that
586 only the sequence of the last 6 amino acids at the C-terminus differ between VEGFA and
587 VEGFAb isoforms. D/ Nucleotide sequence (from nt 1716 to nt 1808) of the 5'-end of human
588 *Vegfa* exon 8 and deduced amino acid sequences corresponding to the C-term of VEGFAxxx and
589 VEGFAxxx isoforms ; corresponding nucleotide triplets are underlined. The reference sequence
590 used for nucleotide annotation is Genbank NM_001171623. The usual splice site (mauve and
591 underlined) leading to VEGFAxxx isoforms (as shown in B) and the putative alternative splice
592 site (green and underlined) leading to VEGFAxxx isoforms (as shown in C) are shown. The
593 respective stop codons are also shown (red and bold). Also note that exon 8 and exon 8b would
594 share an AT dinucleotide sequence at their 5'-end (grey boxes and bold). E/ Schematic of the
595 four most abundant classical hVEGFA splicing isoforms and of their corresponding sister
596 isoforms of the proposed hVEGFAxxx family : both families differ only by the alternative use
597 of either exon 8a or 8b, as described in panels B and C using VEGFA206 as an example.
598 Adapted from Bridgett *et al* [32]. F/ Agarose gel electrophoresis of PCR products obtained using
599 flanking primers O14I/O16I and cDNA from ovine medio-basal hypothalamus (see panel B for
600 location). Gel extraction, cloning and sequencing of the three bands revealed products encoding
601 ovine homologs of human VEGFA206 and VEGFA189 (562 and 544bp ; sizes too close to be
602 separated on the gel), VEGFA165 (490bp) and VEGFA121 (358bp). No single VEGFAxxx
603 PCR product was obtained. For further information, see Lomet *et al.* [33]. G/ Schematic of the

604 signaling pathway elicited by VEGFA isoforms. Classical VEGFA proteins bind both VEGFR2
605 homodimer and the Neuropilin1 (NRP1) co-receptor while VEGFA_{xxx}b isoforms fail to bind
606 NRP1, impairing intracellular transduction pathways.

607
608 **Figure 2:** Issues with RT-PCR primer design: how 5'-tailing led to erroneous identification of
609 *Vegfaxxxb* transcripts. A/ Schematic of the putative junction between exon 7 (blue) and the
610 putative exon 8b (green and black) of *Vegfaxxxb* transcripts and location of the PCR primers
611 used to identify *Vegfaxxxb* transcripts. Note that exon 7 and the AT dinucleotide (green) at the
612 end of exon 8 sequences would be common to both classical *Vegfaxxx* and *Vegfaxxxb* isoforms.
613 The number of non-isoform specific nucleotides is provided in blue; note that this number has
614 substantially increased (from 7bp to 17bp) throughout the years (primers P1 through to P7).
615 Sequences in orange represent 5'-tailing. Primers P1-P6 are reverse primers while primer P7 is a
616 forward primer, as indicated by the arrows. B/ Minimal sequence requirement to obtain PCR
617 amplification of ovine *Vegfaxxxb*-like transcripts through 5'-tailing as defined in Lomet *et al*
618 [33]. The error-prone primer consists of 11 nucleotides in exon 7 and 2-3 nucleotides of exon 8
619 (in green, shared by isoforms), then any nucleotide can be added on the 5'-end of the primer
620 providing the number is sufficient (6 or more) to reach a T_m compatible with the PCR
621 methodology (see **Figure 3**). Note that this primer design corresponds to primer P4.

622
623 **Figure 3:** Basic principles of PCR and how 5'-tailing is achieved. A/ Principle of standard PCR.
624 After reverse transcription, cDNAs are submitted to 30-40 cycles of denaturation/annealing and
625 extension during which a pair of primers (forward and reverse) allow efficient amplification of
626 the target sequence they flank. B/ To anneal to the cDNA, the melting temperature (T_m) of the
627 primer must be higher than the annealing temperature. C/ The T_m of the primer depends on its
628 nucleotide composition and its length, with long GC-rich primers having higher T_ms than short

629 AT-rich primers. A series of 3 efficient primers is shown, the one on the top is the shortest. D/
630 The shortest efficient reverse primer as defined in C can be flanked on its 5'-end with a large
631 stretch of additional non-specific extra-bases (for instance restriction sites for further cloning
632 purposes), which do not anneal to the target and do not impair PCR efficiency and processivity:
633 this is 5'-tailing. E/ The additional non-specific 5'-tail is added to the amplicon as the PCR
634 progresses. At the end of the 30-40 cycles, all amplicons are flanked with the additional, non-
635 specific stretch of the reverse primer.

636

637 **Supplementary Material**

638 Includes Supplemental Text, Supplemental table S1, Supplemental Figures S1-S3

| Authors | Journal | Issue:Page | Year | Species/Tissue | PCR | IHC WB ELISA | Recomb. prot Over-exp |
|------------------------------|-----------------------------------|--------------|------|----------------------------|--|---|-----------------------------|
| Bates <i>et al</i> | <i>Cancer Res</i> | 62:4123-4131 | 2002 | Human / Kidney | VEGF165b exon 8b/ 7 - Reverse primer : 5'-TCAGTCTTTCTGGTGAGAGATCTGCA-3' ----- Amplification of full-length VEGF165b with a reverse primer located downstream in the 3'UTR. | | Home made |
| Cui <i>et al</i> | <i>Am J Physiol Renal Physiol</i> | 286:F767-773 | 2004 | Human / Kidney | Same primers as Bates <i>et al</i> 2002. | | |
| Woolard <i>et al</i> | <i>Cancer Res</i> | 64:7822-7835 | 2004 | Various | Same primers as Bates <i>et al</i> 2002. | Production monoclonal antibody hVEGF165b | Home made |
| Perrin <i>et al</i> | <i>Diabetologia</i> | 48:2422-2427 | 2005 | Human / Eye | Same primers as Bates <i>et al</i> 2002. | Home made ELISA | |
| Konopatskaya <i>et al</i> | <i>Mol Vis</i> | 12:626-632 | 2006 | Mouse / Eye | No | | 3045-VE-025 |
| Bates <i>et al</i> | <i>Clin Sci (Lond)</i> | 110:575-585 | 2006 | Human / Placenta | Same primers as Bates <i>et al</i> 2002. | MAB3045 Home made ELISA | 3045-VE-025 |
| Cebe Suarez <i>et al</i> | <i>Cell Mol Life Sci</i> | 63:2067-2077 | 2006 | Human cell line (HUVEC) | No | MAB3045 | Home made |
| Ribeiro <i>et al</i> | <i>Mol Reprod Dev</i> | 74:163-171 | 2007 | Swine / Ovary | VEGF164b exon 8b/ 7 - Reverse primer : 5'-TCCTGGTGAGAGATCTGCAAG-3' Note : level << VEGF164 (2 ¹² fold) | | |
| Pritchard-Jones <i>et al</i> | <i>Br J Cancer</i> | 97:223-230 | 2007 | Human / Melanoma tissue | VEGF165b HindIII RS - exon 8b/ 7 - Reverse primer : 5'-TTAAGCTTTCAGTCTTTCTGGTGAGACTGCA-3' Note : GAT missing from oligo ; see Bates <i>et al</i> 2002 Invalid primer. | MAB3045 | |
| Schumacher <i>et al</i> | <i>J Am Soc Nephrol</i> | 18:719-729 | 2007 | Human / Kidney | VEGF both isoforms BamHI RS – 3'UTR Reverse primer : 5'-ATGGATCCGTATCAGTCTTTCCT-3' Note : short primer, theoretical fusion T° = 41°C | | |
| Ergorul <i>et al</i> | <i>Mol Vision</i> | 14:1517-1524 | 2008 | Rat / Retina | No | Ab14994 | |
| Qiu <i>et al</i> | <i>Faseb J</i> | 22:1104-1112 | 2008 | Mouse / Various tissues | Primers use to « detect specifically the transgene » Forward : 5'-TCAGCGCAGCTACTGCCATC-3' Reverse : 5'-GTGCTGGCCTTGGTGAGGTT-3' Note : Forward is within exon3 and Reverse is within exon4 → actually detect all Vegfa. | MAB3045 | Yes – TG mice |

| | | | | | | | |
|--------------------------------|----------------------------|----------------|------|---------------------------|---|--------------------------------|-----------|
| | | | | | Invalid primers → Invalid screening of TG mice. | | |
| Bevan <i>et al</i> | <i>Nephron Physiol</i> | 110:57-67 | 2008 | Human / Kidney | No | MAB3045 | |
| Kawamura <i>et al</i> | <i>Cancer Res</i> | 68:4683-4692 | 2008 | Cell lines | No | | Home made |
| Varey <i>et al</i> | <i>Br J Cancer</i> | 98:1366-1379 | 2008 | Human / Colon | Original indirect subtractive strategy : Use of « An exon 7b forward primer and a 3'UTR Primer (both pan-VEGF) » - Sequences not provided Then use of « an exon 7a forward primer and a reverse primer specific for exon 8a that did not detect Vegfxxx isoforms ». Then subtraction would yield Vegfxxx. Invalid method. | MAB3045 | Yes |
| Diaz <i>et al</i> | <i>Int J Cancer</i> | 123 :1060-1067 | 2008 | Human / Colon | Same primers as Bates <i>et al</i> 2002. | MRVL56/1 | |
| Nowak <i>et al</i> | <i>J Cell Sci</i> | 121:3487-3495 | 2008 | Cell lines | VEGF both isoforms BamHI RS – 3'UTR 5'-ATGGATCCGTATCAGTCTTCTCTGG-3' | MAB3045 & clone 264610/1 | Home made |
| Rennel <i>et al</i> | <i>Br J Cancer</i> | 98:1250-1257 | 2008 | Human / Prostate Mouse | VEGF165b HindIII RS - exon 8b/ 7 5'-TTAAGCTTTCAGTCTTCTCTGGTGAGAGATCTGCA-3' | Home made ELISA | Home made |
| Rennel <i>et al</i> | <i>Eur J Cancer</i> | 44:1883-1894 | 2008 | Cell lines Mouse | No | MAB3045 | Home made |
| Miller-Kasprzak and Jagodinski | <i>Biomed Pharmacother</i> | 62:158-163 | 2008 | Human / Lung cell line | VEGF121b exon 5/8b - Forward primer : 5'-GAAAATCTCTCACCAGGAAA-3' Note : one A missing from oligo. VEGF165b exon 8b/ 7 - Reverse primer : 5'-GTGAGAGATCTGCAAGTACG-3' | MAB3045 | |
| Artac <i>et al</i> | <i>Biol Reprod</i> | 81:978-988 | 2009 | Rat / Ovary | VEGF165b exon 8b/ 7 - Reverse primer : 5'-GGTGAGAGGCTGCAAGTACGTT-3' | MAB3045 | |
| Bills <i>et al</i> | <i>Clin Sci (Lond)</i> | 116:265-272 | 2009 | Human / Plasma | No | MAB3045 Home made ELISA | |
| Rennel <i>et al</i> | <i>Br J Cancer</i> | 101:1183-1193 | 2009 | Human / Colon | VEGF121b exon 5/8b - Forward primer : 5'-GAAAATCTCTCACCAGGAAA-3' | MAB3045 | DY3045 |
| Baltes-Breitwisch <i>et al</i> | <i>Reproduction</i> | 140:319-329 | 2010 | Rat / Testes | Same primers as Artac <i>et al</i> . 2009. | Ab14994 | |
| Catena <i>et al</i> | <i>Molecular Cancer</i> | 9 :320 | 2010 | Human cell lines | VEGF121b – HindIII RS – exon 8b/ exon5 Reverse primer : 5'-TTAAGCTTTCAGTCTTCTCTGGTGAGAGATTTTCTTGTCTTGCTCTATC - 3' VEGF165b – HindIII RS – exon 8b/ exon7 Reverse primer : 5'-TTAAGCTTTCAGTCTTCTCTGGTGAGAGATCTGCAAGTACGTTCTGTTAACTC - 3' | MAB3045 | Yes |

| | | | | | | | |
|---------------------------|-----------------------------------|----------------|------|---|---|---|-----------------------|
| Hua <i>et al</i> | <i>Invest Ophthalmol Vis Sci</i> | 51:4282-4288 | 2010 | Human / Eye | No | MAB3045 | |
| Magnussen <i>et al</i> | <i>Invest Ophthalmol Vis Sci</i> | 51:4273-4281 | 2010 | Mouse / Eye | No | MAB3045 | 3045-VE-025 |
| Nowak <i>et al</i> | <i>J Biol Chem</i> | 285:5532-5540 | 2010 | Cell lines | Same as Nowak <i>et al</i> 2008 | MAB3045 | |
| Peiris-Pagès <i>et al</i> | <i>J.Pathol</i> | 222:138-147 | 2010 | Cell lines | Same as Nowak <i>et al</i> 2008 | MAB3045 | DY3045 3045-VE-025 |
| Qiu <i>et al</i> | <i>J Am Soc Nephrol</i> | 21:1498-1509 | 2010 | Cell lines Mouse | Same invalid primers as Qiu <i>et al</i> 2008 to « detect specifically the transgene » → Invalid screening of TG mice. | Home made ELISA & clone 264610/1 | TG-mice DY3045 |
| Merdzhanova <i>et al</i> | <i>Oncogene</i> | 29: 5392-5403 | 2010 | Mouse & Human Cell lines | VEGF165b exon 8b/ 7 - Reverse primer : 5'- TGGTGAGAGATCTGCAAGTACGTT -3' | | |
| Zhao <i>et al</i> | <i>Exp Eye Res</i> | 93:921-926 | 2011 | Mouse / Retina | No | Ab14994 | |
| Amin <i>et al</i> | <i>Cancer Cell</i> | 20:768-780 | 2011 | Cell lines | Same primer as Nowak <i>et al</i> 2008. | | |
| Xu <i>et al</i> | <i>Gene</i> | 487:143-150 | 2011 | Human-Cat-Rabbit- Rat/ Various tissues | Human : VEGF165b exon 8b/ 7 Reverse primer : 5'- TGGTGAGAGATCTGCAAGTACGTT -3' Cat : VEGF165b exon 8b/ 7 Reverse primer : 5'- TGGTGAGAGGCTCTGCAAGTACGTT -3' Rabbit : VEGF165b exon 8b/ 7 Reverse primer : 5'- CGGTGAGAGGCTCTGCAAGTACGTT -3' Rat : VEGF165b exon 8b/ 7 Reverse primer : 5'- GGTGAGAGGCTCTGCAAGTACGTT -3' | | |
| Manetti <i>et al</i> | <i>Circ Res</i> | 109:e14-26 | 2011 | Human / Skin | Same primer as Rennel <i>et al.</i> 2008 | Ab14994 | |
| Baba <i>et al</i> | <i>Dev Dyn</i> | 241:595-607 | 2012 | Human / Eye | VEGF165b exon 8b/ 7 - Reverse primer : 5'- TCCTGGTGAGAGATCTGCAA -3' | Ab14994 | |
| Oltean <i>et al</i> | <i>Am J Physiol Renal Physiol</i> | 303:F1026-1036 | 2012 | Mouse / Kidney | Same invalid primers as Qiu <i>et al</i> 2008 to « detect specifically the transgene » → Invalid screening of TG mice. | | TG-mice |
| Qiu <i>et al</i> | <i>Reproduction</i> | 143:501-511 | 2012 | Marmoset / Ovary Mouse | No | Home made ELISA & clone 264610/1 | |
| Clifford <i>et al</i> | <i>J Immunol</i> | 189:819-831 | 2012 | Human / Cell lines | VEGF165b exon 8b/ 7 - Reverse primer : 5'- AGAGAGATCTGCAAGTACGTTCCG -3' VEGF189b exon 8b/ 7 - Reverse primer : 5'- GTGAGAGATCTGCAAGTACG -3' | | |
| Bates <i>et al</i> | <i>PLoS One</i> | 8:e68399 | 2013 | Human / Cell lines | VEGF165b exon 8b/7 - Reverse primer : | MAB3045 | |

| | | | | | | | |
|---------------------------|---------------------------------|--------------|------|------------------------------|--|--|-------------|
| | | | | | 5'- GTGAGAGATCTGCAAGTACG -3' | | |
| Beazley-Long <i>et al</i> | <i>Am J Pathol</i> | 183:918-929 | 2013 | Human / Brain Rat / Brain | No | MAB3045 | 3045-VE-025 |
| Gu <i>et al</i> | <i>Biochem Biophys Res Comm</i> | 441:18-24 | 2013 | Human / Cell lines | VEGF111b – « Reverse primer » : 5' – AATGCAGATGTGACAAGCCGAG – 3' Actually a forward primer at junction exon4/8a . Invalid → Identification of Vegf111b invalid. VEGF165b HindIII RS - exon 8b/ 7 - Reverse primer : 5'-TTAAGCTT TCAGTCTTTCCTGGTGAGAGATCTGCA -3' | MRVL56/1 & Home Made αVEGF111b | |
| Delcombel <i>et al</i> | <i>Angiogenesis</i> | 16:353-371 | 2013 | HEK293 cells | VEGF111b exon8b/4 – Reverse primer : 5'- TCCTGGTGAGAGATCTGCATTCAC -3' VEGF121b exon8b/5 – Reverse primer : 5'- GTCTTTCCTGGTGAGAGAGTTTCTT -3' Note : primer issue → AT missing between exon8b and exon5. Invalid. VEGF165b exon8b – Reverse primer : 5'- CGATCGTTCTGTATCAGTCTTTCCT -3' Note : internal to exon 8 → PCR product = classical mVEG Invalid. | | |
| Hulse <i>et al</i> | <i>Neurobiol Dis</i> | 71:245-259 | 2014 | | Strategy similar to Varey <i>et al</i> 2008 but different Primers ; « primers specific for VEGF-A165a » are: Forward primer exon 7: 5'-GTTCAGAGCGGAGAAAGCAT-3' Reverse primer exon 8a : 5'- TCACA TCTGCAAGTACGTTCG -3' → This primer covers exon8a/7 | Ab14994 | DY3045 |
| Kikuchi <i>et al</i> | <i>Nat Med</i> | 20:1464-1471 | 2014 | Human | hVEGF165b exon 8b/7 - Reverse primer : 5'- GTGAGAGATCTGCAAGTACG -3' ----- mVEGF165b exon 8b/7 - Reverse primer : 5'- CTTCCGGTGAGAGGTCTGC -3' | | 3045-VE-025 |
| Hulse <i>et al</i> | <i>Clin Sci</i> | 129:741-756 | 2015 | Cell culture Rat | No | | 3045-VE-025 |
| Oltean <i>et al</i> | <i>J Am Soc Nephrol</i> | 26:1889-1904 | 2015 | Human / Kidney | VEGF165b exon 8b/7 - Reverse primer : 5'- GTGAGAGATCTGCAAGTACG -3' | DY3045 | |
| Vencappa <i>et al</i> | <i>Am J Transl Res</i> | 7:1032-1044 | 2015 | Cell culture Mouse | No | Ab14994 | 3045-VE-025 |
| Li <i>et al</i> | <i>J Translat Med</i> | 13 :164 | 2015 | Human cell lines | Same primers as Gu <i>et al</i> 2013 | Same as | Yes |

| | | | | | | | |
|----------------------------|-------------------------------|----------------|------|------------------------|---|---------------------------------------|---------------|
| | | | | | | Gu <i>et al</i> 2013 | |
| Hulse <i>et al</i> | <i>Neurobiol Dis</i> | 96:186-200 | 2016 | Rat | No | Ab14994 | |
| Castle-Miller <i>et al</i> | <i>Proc Natl Acad Sci USA</i> | 114:E2517-2523 | 2017 | Sheep / Pituitary | Multiple issues with primers leading to an erratum. VEGF _{Axxx} -specific reverse primer : 5'-CGGCGGCTATGGGTCGTTCTGTGTCAGTCTTTCCT GGTGAGAC-3' 43bp. Unusual. Targets a sequence downstream of putative distal splice located in exon 8. Non-isoform specific. ➔ qRT-PCR data invalid. | Home made Clone56/8 & DY3045 | |
| Ved <i>et al</i> | <i>Clin Sci (Lond)</i> | 131:1225-1243 | 2017 | Rat / Retina | No | Home made Clone56/8 | Yes |
| Pruszko <i>et al</i> | <i>EMBO reports</i> | 18:1331-1351 | 2017 | Human cell lines | VEGF121b exon 8b/5 - Reverse primer : 5'- CTTTCCTGGTGAGAGATTTTCTTGTC -3' VEGF165b exon 8b/7 - Reverse primer : 5'- CCTGGTGAGAGATCTGCAAGTAC -3' | Ab14994 | |
| Hueso <i>et al</i> | <i>Scientific Reports</i> | 7 :9962 | 2017 | Human Serum / Heart | No | MAB3045 | MBS109074 |
| Chesnokov <i>et al</i> | <i>PeerJ</i> | 6:e4915 | 2018 | Human / Tissues | VEGF _{xxx} exon 7/8b - Forward primer : 5'- ACGTACTTGCAGATCTCACCA -3' Underlined bases correspond to either end of exon7 OR exon 8. | | |
| Blochowiak <i>et al</i> | <i>Adv Clin Exp Med</i> | 27:83-90 | 2018 | Human / Parotid | No | DM3615P | |
| Boudria <i>et al</i> | <i>Oncogene</i> | AOP | | Mice / Cell lines | No | Yes | Yes |
| Stevens <i>et al</i> | <i>Nephron</i> | 139:51-62 | 2018 | Mice | No | No | Yes / TG mice |

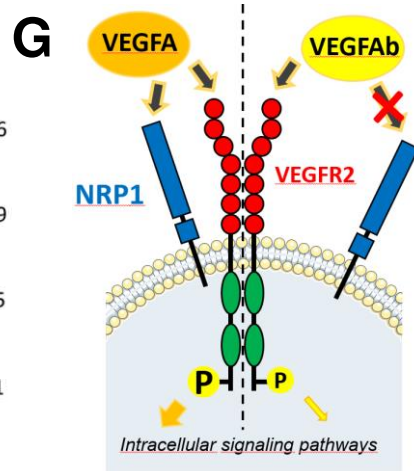
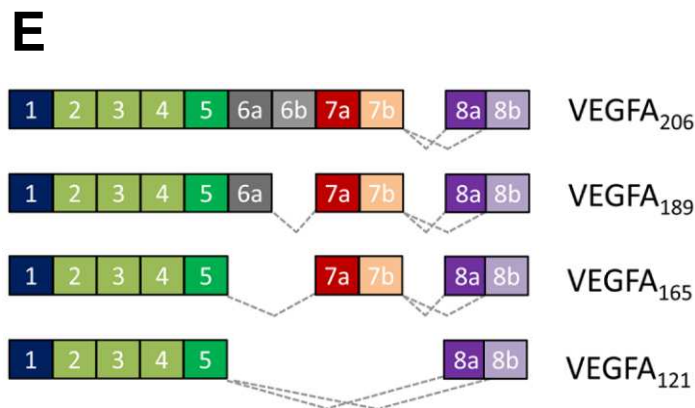
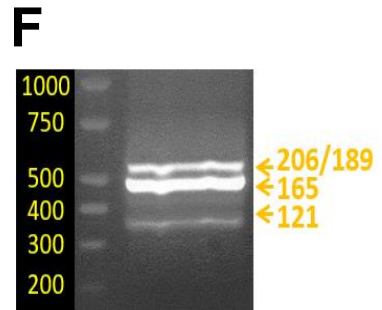
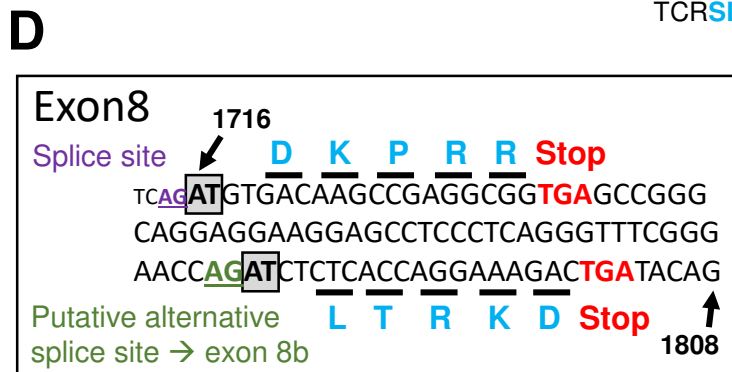
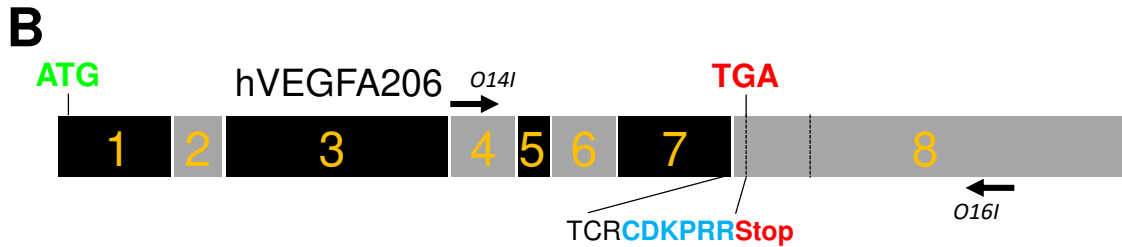
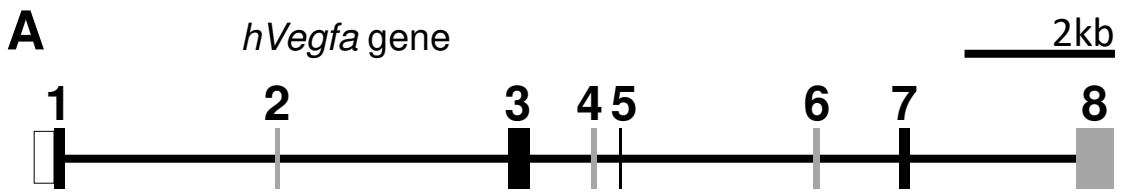


Figure 1

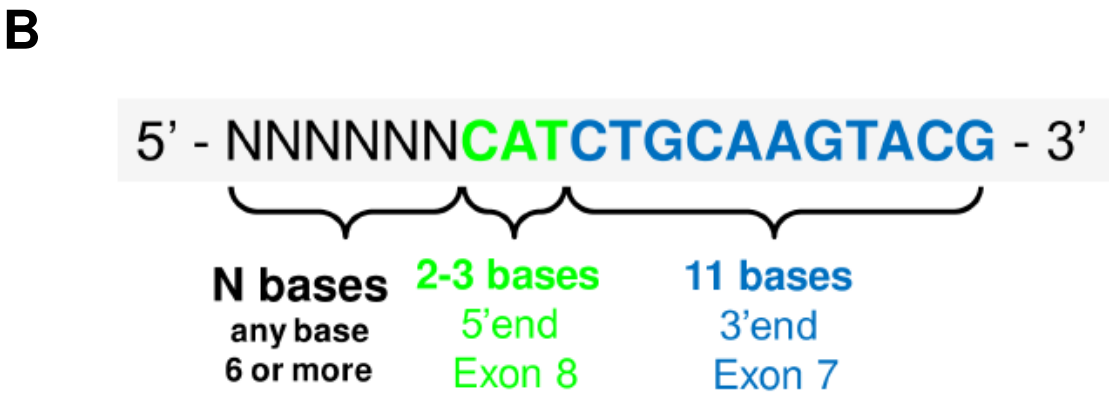
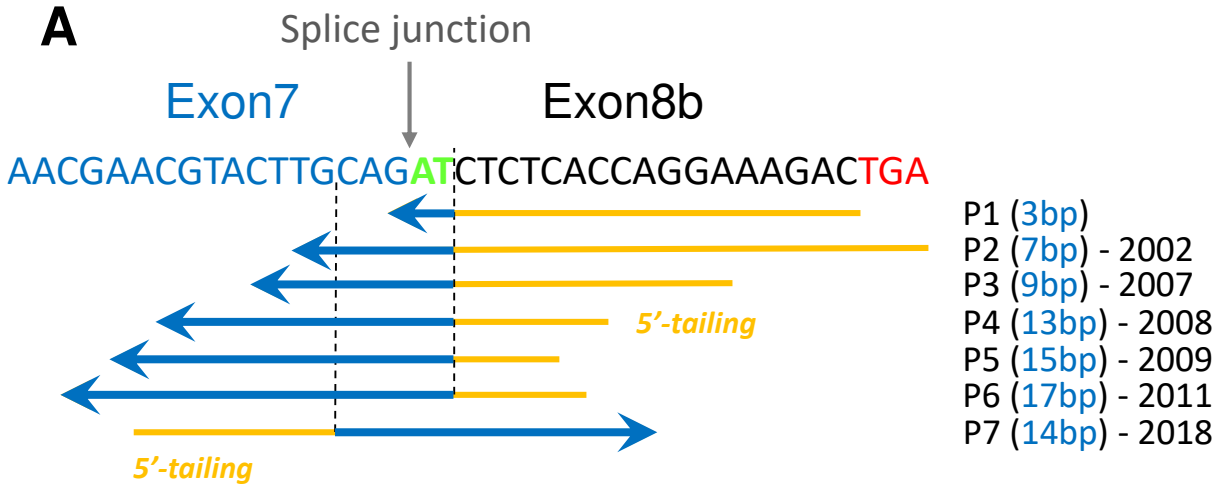


Figure 2

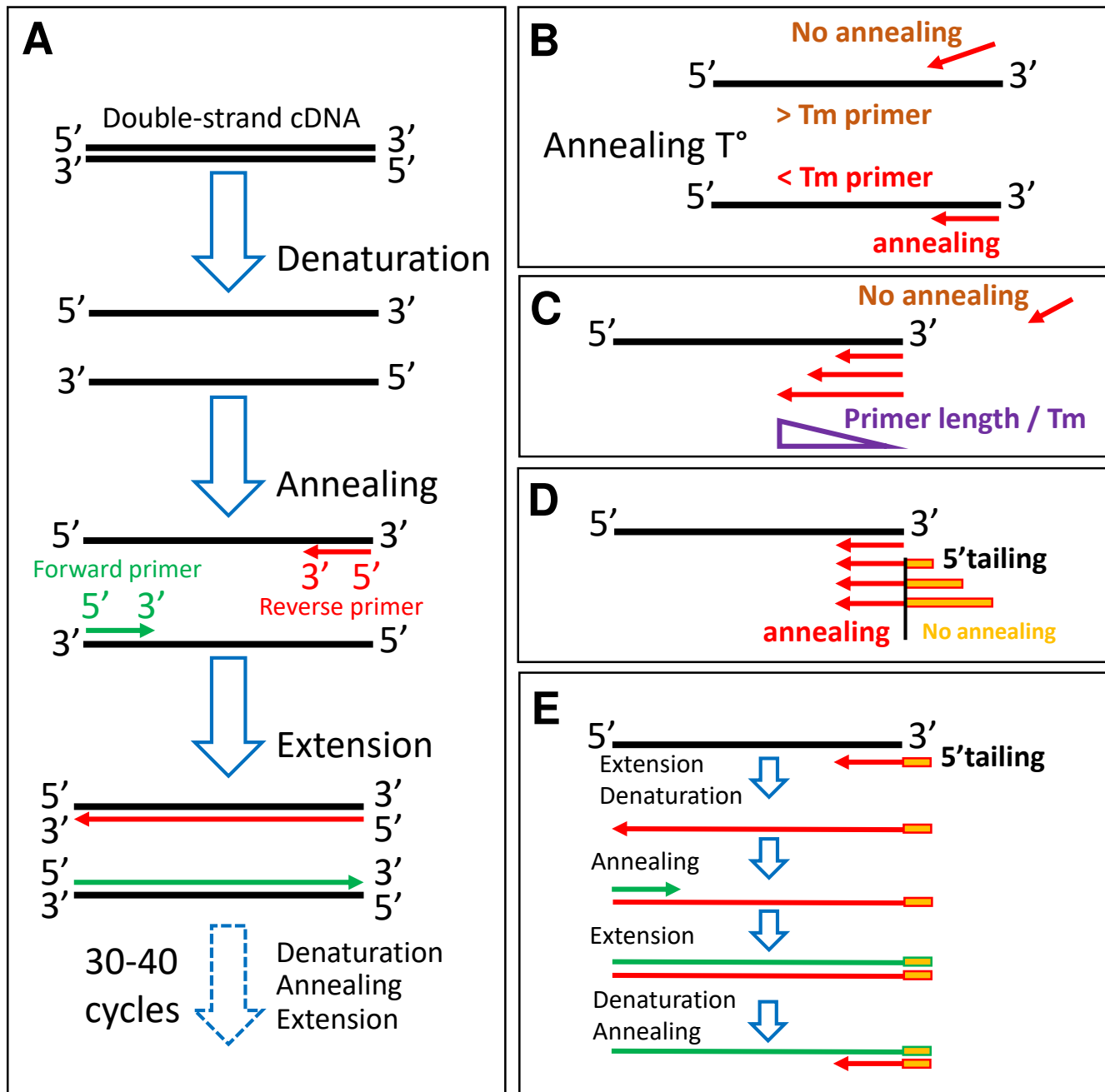


Figure 3

Supplemental Text

Debunking the myth of the endogenous anti-angiogenic *Vegfaxxb* transcripts.

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Other methodological issues with RT-PCR

We noted further inconsistencies in the literature and not-readily-explainable RT-PCR findings. First, to identify *Vegfa165b*, Bates *et al* [1] used primers designed to detect *Vegfa148*, a transcript identified from kidney by the same team [2] and recently shown to be enriched in some tissues, including kidney [3]. The pair of primers used by Bates *et al* [1] consisted of a forward primer located within exon 7a and a reverse primer located in the 3'-UTR, downstream of the putative alternative 8b splice site (i.e. flanking primers; **Table S1**). This should yield at least two PCR products of 185bp and 151bp, corresponding to *Vegfa165* and *Vegfa148*. Should the *Vegfa165b* transcript exist, one would expect an extra PCR product at 120bp. Hence, 3 bands should be discernible on the gel, with the one corresponding to *Vegfa148* located in between those for *Vegfa165* and *Vegfa165b*. This was not the case. This is puzzling considering these primers were in the first instance "designed to detect *Vegfa148*". The same issue applies to the study by Schumacher *et al* [4] who used the same primers. Furthermore, *Vegfaxxb* transcripts were not identified by

Whittle *et al* [2], who used a pair of *ad hoc* primers for this (forward primer in exon 7a and reverse primer downstream of the putative 8b splice site).

In follow-up studies from the team led by Bates and Harper, DNA rulers were often missing and sequencing of the PCR products was not reported [e.g.; 5-8], and therefore the identity of PCR products could not be confirmed. Rather, to validate the existence of an entire family, the authors used assertions such as "size compatible with *Vegfa165b*" [9], "bands corresponding to VEGFAxxx isoforms (VEGFA165b, VEGFA189b, etc.) were seen ..." [6] but unfortunately not shown, or "multiple isoforms were detected, with bands seen consistent with VEGFA121b (108 bp), VEGFA145b (219 bp) and VEGFA165b (240 bp)". In particular, Cui *et al* [9] used isoform-specific reverse primers to amplify either *Vegfa165* or *Vegfa165b*. These primers were used in combination with the same forward primer, located in exon 4. Therefore RT-PCR for *Vegfa165* is expected to yield at least 3 major bands. Considering the "sister family of *Vegfxxx*", RT-PCR for *Vegfa165b* should yield all transcripts that include exon 7b (at least *Vegfa189b* and *Vegfa165b*). However, only products of "sizes compatible with" the presence of *Vegfa189* and *Vegfa165b* were evident on the gel (see [9]; their Fig.3).

Finally, primers used to "detect specifically the transgene" [10,11] in a transgenic mouse model, which over-expresses *Vegfa165b* in the mammary gland, are located within exon3 (forward primer) and exon4 (reverse primer). Therefore, this primer pair will detect all *Vegfa* isoforms (see **Table S1**), which casts doubts on this mouse model.

1 Bates, D.O. *et al.* (2002) VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. *Cancer Res* 62, 4123-4131

2 Whittle, C. *et al.* (1999) Heterogeneous vascular endothelial growth factor (VEGF) isoform mRNA and receptor mRNA expression in human glomeruli, and the identification of VEGF148 mRNA, a novel truncated splice variant. *Clin Sci (Lond)* 97, 303-312

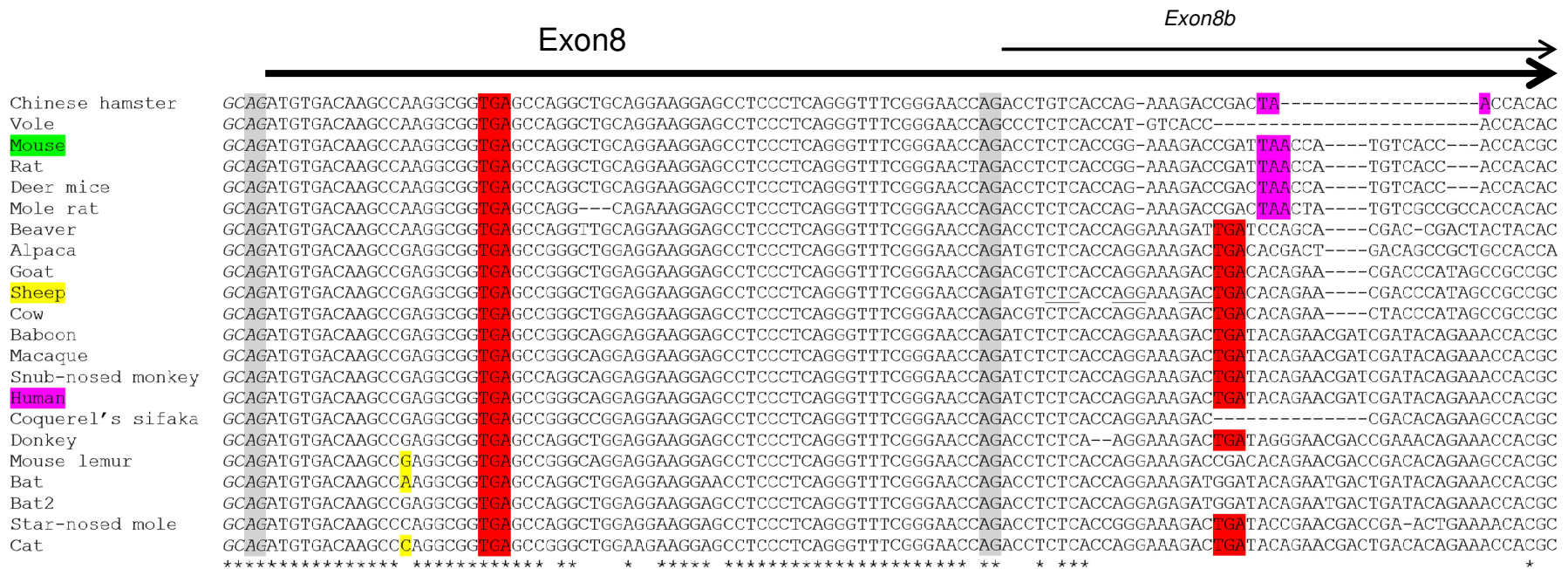
- 3 Bridgett, S. *et al.* (2017) RNA-Sequencing data supports the existence of novel VEGFA splicing events but not of VEGFAxxx isoforms. *Sci Rep* 7, 58-017-00100-3
- 4 Schumacher, V.A. *et al.* (2007) Impaired glomerular maturation and lack of VEGF165b in Denys-Drash syndrome. *J Am Soc Nephrol* 18, 719-729
- 5 Woolard, J. *et al.* (2004) VEGF165b, an inhibitory vascular endothelial growth factor splice variant: mechanism of action, in vivo effect on angiogenesis and endogenous protein expression. *Cancer Res* 64, 7822-7835
- 6 Perrin, R.M. *et al.* (2005) Diabetic retinopathy is associated with a switch in splicing from anti- to pro-angiogenic isoforms of vascular endothelial growth factor. *Diabetologia* 48, 2422-2427
- 7 Bates, D.O. *et al.* (2006) The endogenous anti-angiogenic family of splice variants of VEGF, VEGFxxx, are down-regulated in pre-eclamptic placentae at term. *Clin Sci (Lond)* 110, 575-585
- 8 Peiris-Pages, M. *et al.* (2010) Balance of pro- versus anti-angiogenic splice isoforms of vascular endothelial growth factor as a regulator of neuroblastoma growth. *J Pathol* 222, 138-147
- 9 Cui, T.G. *et al.* (2004) Differentiated human podocytes endogenously express an inhibitory isoform of vascular endothelial growth factor (VEGF165b) mRNA and protein. *Am J Physiol Renal Physiol* 286, F767-73
- 10 Qiu, Y. *et al.* (2008) Mammary alveolar development during lactation is inhibited by the endogenous antiangiogenic growth factor isoform, VEGF165b. *Faseb j* 22, 1104-1112
- 11 Qiu, Y. *et al.* (2010) Overexpression of VEGF165b in podocytes reduces glomerular permeability. *J Am Soc Nephrol* 21, 1498-1509

Antibodies and ELISA kits (see text and Supplemental Table1):

- MAB3045 from R&D : Monoclonal Mouse IgG₁ Clone # 56-1 ; directed against human VEGF_{165b} synthetic peptideTCRSLTRKD.
https://www.rndsystems.com/products/human-vegf-165b-antibody-56-1_mab3045
Note : another Ab (raised against the same 9 amino acids) from clone #264610/1 was used in Nowak et al 2008 – no further information could be found.
Note : for Castle-Miller et al 2017 and Ved et al 2017 another clone (56/8) was used. No validation provided.
- DM3615P from Origene : Monoclonal Mouse IgG1 Clone #7F17 ; directed against human recombinant Human VEGF-165b C-terminal peptide
<https://www.acris-anticorps.fr/antibodies/primary-antibodies/vegf165b-dm3615p.htm>
- DY3045 from R&D : human VEGF_{165b} Solid Phase Sandwich ELISA
https://www.rndsystems.com/products/human-vegf-165b-duoset-elisa_dy3045#product-details
- 3045-VE-025 : Recombinant human VEGFA165b protein
https://www.rndsystems.com/products/recombinant-human-vegf-165b-protein_3045-ve
- Ab14994 (clone MRVL56/1) from Abcam : mouse monoclonal antibody – discontinued since 2015.
<https://www.abcam.com/vegf165b-antibody-mrvl561-ab14994.html>
- MBS109074 form MyBioSource : hVEGFA165b ELISA kit
https://www.mybiosource.com/prods/ELISA-Kit/Human/Vascular-Endothelial-Growth-Factor-165B-VEGF-165B/VEGF-165B/datasheet.php?products_id=109074

List of additional references only cited in Supplemental Table1 :

- 1 Amin, E.M. *et al.* (2011) WT1 mutants reveal SRPK1 to be a downstream angiogenesis target by altering VEGF splicing. *Cancer Cell* 20, 768-780
- 2 Baltes-Breitwisch, M.M. *et al.* (2010) Neutralization of vascular endothelial growth factor antiangiogenic isoforms or administration of proangiogenic isoforms stimulates vascular development in the rat testis. *Reproduction* 140, 319-329
- 3 Hueso, L. *et al.* (2017) Dynamics and implications of circulating anti-angiogenic VEGF-A165b isoform in patients with ST-elevation myocardial infarction. *Sci Rep* 7, 9962-017-10505-9
- 4 Li, X. *et al.* (2015) VEGF111b, a C-terminal splice variant of VEGF-A and induced by mitomycin C, inhibits ovarian cancer growth. *J Transl Med* 13, 164-015-0522-0
- 5 Nowak, D.G. *et al.* (2008) Expression of pro- and anti-angiogenic isoforms of VEGF is differentially regulated by splicing and growth factors. *J Cell Sci* 121, 3487-3495
- 6 Nowak, D.G. *et al.* (2010) Regulation of vascular endothelial growth factor (VEGF) splicing from pro-angiogenic to anti-angiogenic isoforms: a novel therapeutic strategy for angiogenesis. *J Biol Chem* 285, 5532-5540
- 7 Qiu, Y. *et al.* (2010) Overexpression of VEGF165b in podocytes reduces glomerular permeability. *J Am Soc Nephrol* 21, 1498-1509
- 8 Stevens, M. *et al.* (2018) Vascular Endothelial Growth Factor-A165b Restores Normal Glomerular Water Permeability in a Diphtheria-Toxin Mouse Model of Glomerular Injury. *Nephron* 139, 51-62



Canonical splice site

Alternative splice site

CCA/CCG/CCC/CCT = Proline.
22 seq, all identical = CDKPRR

| | Putative VEGFAxxx sequences |
|----------------------|---|
| Chinese hamster | PVTRKTD |
| Deer mice/Mole rat | PLTRKTD |
| Mouse/Rat | PLTGKTD |
| Star-nosed mole | PLTGKD |
| Sheep/Alpaca | CLTRKD |
| Goat/Cow | RLTRKD |
| Human/Baboon/Macaque | SLTRKD |
| Beaver/Cat | PLTRKD |
| Vole | PLTMSPPHRHRHRQNSP |
| Bat | PLTRKDGyRMTDTETTPLPpHHH |
| Bat2 | PLTRRDGyRMTDTETTPLPpHHHHHRQNNP |
| Mouse | PLTRKDRHRTDTEATPLPpHRHRHRQNR |
| Donkey | PLKERLIGNDRNRNHAATTSPTSEQSLIQKPEMKEETAQSTLGPGETPAEAFPPG |
| Coquerel's sifaka | PLTRKDRHRSHAAATTPSPSTEQSLDQKPEMKEEETLRRALWVRRARLRQKHSRAGDQARSLLLELDSPFrfSCC |
| | : |

Figure S1

