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

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Abstract

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

relevance of studies based on the use of "anti-VEGFAxxxb" antibodies. Our analysis has implications for the proposed therapeutic use of isoform-specific anti-VEGFA strategies for treating cancer and retinopathies.

Classical angiogenic VEGFA isoforms

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

Non-classical anti-angiogenic VEGFAxxxb and VEGF-Ax isoforms

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

Existence of VEGFAxxxb isoforms: clinical implications

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

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

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

The Controversy: Do VEGFAxxxb isoforms really exist?

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

Absence of Evidence: Detection of *Vegfaxxxb* transcripts with flanking primers.

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

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

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

Detection of *Vegfaxxxb*-like transcripts with "isoform-specific primers": a tale of a tail.

Over time, there has been a considerable drift in the design of primers aimed at the specific identification of *Vegfa165b* that span the exon 7 – exon 8b splice site. However, in most studies, the exon 7-specific stretch has been greatly lengthened at the expense of the exon 8b-specific region (see **Figure 2A** and **Table S1**). Since exon 7 would be shared by both *Vegfa165* and *Vegfa165b*, the isoform-specificity of these reverse primers is questionable. Technically, the

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

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

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

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

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

In conclusion, the detection of "*Vegfaxxxb*-like" transcripts could be explained by the use of inadequate RT-PCR methodologies involving 5´-tailing of the primers (also see **BOX1**).

Bioinformatic analysis of alternative splicing: absence of evidence for Vegfaxxxb isoforms.

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

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

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

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

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

No *Vegfaxxxb* transcripts: detection of VEGFAxxxb proteins is an artefact.

Absence of endogenous *Vegfaxxxb* mRNA implies absence of endogenous VEGFAxxxb proteins. Consequently, studies which investigated VEGFAxxxb levels in tissues or plasma, using anti-VEGFAxxxb antibodies and ELISA kits, or those which relied exclusively on over-expression or injection of recombinant VEGFAxxxb proteins have questionable physiological relevance (see **Table S1**; e.g. [18,59-74]).

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

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

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

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

Concluding remarks

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

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Glossary

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

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

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

Text BOXES

BOX1

Further technical issues with the use of qRT-PCR

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

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

BOX2

Ruling out potential issues with mRNA secondary structure

The idea that PCR amplification of *Vegfa165b* might be difficult due to secondary structure of the mRNA in the 3′-UTR has been invoked [9] to explain why we [31,33] and other authors [13,79,80] failed to detect these transcripts. The existence of an unusually stable secondary structure seems unlikely because most regulatory splicing mechanisms are based on the recognition of short degenerate RNA motifs at the exon/intron boundaries rather than secondary structures [82,83]. Indeed, bioinformatic analysis of the ribonucleotidic sequence does not predict formation of potential hairpins that might inhibit reverse transcription (data not shown). Furthermore, classical *Vegfaxxx* isoforms are readily amplified using primers spanning the putative alternative splice site (see main text). There is therefore no evidence to support the notion that *Vegfaxxxb* isoforms, which would lack a 66bp stretch present in *Vegfaxxx* isoforms, would resist PCR amplification. Furthermore, *Vegfa165b* transcripts expressed from a recombinant construct were readily detected by RNA-seq [32] and since the library preparation 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 *Vegfaxxxb* 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 *Vegfaxxxb* 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 *Vegfaxxxb* 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 *Vegfaxxx* 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/VEGFAxxxb
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 VEGFAxxxb 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 VEGFAxxxb 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 hVEGFAxxxb 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 VEGFAxxxb
603 PCR product was obtained. For further information, see Lomet et al. [33]. G/ Schematic of the

signaling pathway elicited by VEGFA isoforms. Classical VEGFA proteins bind both VEGFR2 homodimer and the Neuropilin1 (NRP1) co-receptor while VEGFAxxxb isoforms fail to bind NRP1, impairing intracellular transduction pathways.

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

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

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

Supplementary Material

Includes Supplemental Text, Supplemental table S1, Supplemental Figues S1-S3

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

					Invalid primers → Invalid screening of TG mice.		
Bevan *et al*	*Nephron Physiol*	110:57-67	2008	Human / Kidney	No	MAB3045	
Kawamura *et al*	*Cancer Res*	68:4683-4692	2008	Cell lines	No		Home made
Varey *et al*	*Br J Cancer*	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 Vegfxxxb isoforms ». Then subtraction would yield Vegfxxxb. Invalid method.	MAB3045	Yes
Diaz *et al*	*Int J Cancer*	123 :1060-1067	2008	Human / Colon	Same primers as Bates *et al* 2002.	MRVL56/1	
Nowak *et al*	*J Cell Sci*	121:3487-3495	2008	Cell lines	VEGF both isoforms BamHI RS – 3'UTR 5'-ATGGATCC**GTATCAGTCTTTCCTGG**-3'	MAB3045 & clone 264610/1	Home made
Rennel *et al*	*Br J Cancer*	98:1250-1257	2008	Human / Prostate Mouse	VEGF165b HindIII RS - **exon 8b**/ 7 5'-TTAAGCTT**TCAGTCTTTCCTGGTGAGAG**ATCTGCA-3'	Home made ELISA	Home made
Rennel *et al*	*Eur J Cancer*	44:1883-1894	2008	Cell lines Mouse	No	MAB3045	Home made
Miller-Kasprzak and Jagodinski	*Biomed Pharmacother*	62:158-163	2008	Human / Lung cell line	VEGF121b exon 5/**8b** - Forward primer : 5'-GAAA**ATCTCTCACCAGGAAA**-3' Note : one A missing from oligo. VEGF165b **exon 8b**/ 7 - Reverse primer : 5'-**GTGAGAG**ATCTGCAAGTACG-3'	MAB3045	
Artac *et al*	*Biol Reprod*	81:978-988	2009	Rat / Ovary	VEGF165b **exon 8b**/ 7 - Reverse primer : 5'-**GGTGAGAGG**TCTGCAAGTACGTT-3'	MAB3045	
Bills *et al*	*Clin Sci (Lond)*	116:265-272	2009	Human / Plasma	No	MAB3045 Home made ELISA	
Rennel *et al*	*Br J Cancer*	101:1183-1193	2009	Human / Colon	VEGF121b exon 5/**8b** - Forward primer : 5'-GAAAA**ATCTCTCACCAGGAAA**-3'	MAB3045	DY3045
Baltes-Breitwisch *et al*	*Reproduction*	140:319-329	2010	Rat / Testes	Same primers as Artac et al. 2009.	Ab14994	
Catena *et al*	*Molecular Cancer*	9 :320	2010	Human cell lines	VEGF121b – HindIII RS – **exon 8b**/ exon5 Reverse primer : 5'-TTAAGCTTTCAGTCTTTCCTGGTGAGAGATTTTTCTT GTCTTGCTCTATC - 3' VEGF165b – HindIII RS – **exon 8b**/ exon7 Reverse primer : 5'-TTAAGCTTTCAGTCTTTCCTGGTGAGAGATCTGCAA GTACGTTCGTTTAACTC - 3'	MAB3045	Yes

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

					5'-**GTGAGAG**ATCTGCAAGTACG-3'		
Beazley-Long *et al*	*Am J Pathol*	183:918-929	2013	Human / Brain Rat / Brain	No	MAB3045	3045-VE-025
Gu *et al*	*Biochem Biophys Res Comm*	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 *et al*	*Angiogenesis*	16:353-371	2013	HEK293 cells	VEGF111b **exon8b**/4– Reverse primer : 5'-**TCCTGGTGAGAG**ATCTGCATTCAC-3' VEGF121b **exon8b**/5– Reverse primer : 5'-**GTCTTTCCTGGTGAGAGAG**TTTTCTT-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 *et al*	*Neurobiol Dis*	71:245-259	2014		Strategy similar to Varey et al 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 *et al*	*Nat Med*	20:1464-1471	2014	Human	hVEGF165b **exon 8b**/7 - Reverse primer : 5'-**GTGAGAG**ATCTGCAAGTACG-3' --- mVEGF165b **exon 8b**/7 - Reverse primer : 5'-**CTTTCCGGTGAGAGGT**CTGC-3'		3045-VE-025
Hulse *et al*	*Clin Sci*	129:741-756	2015	Cell culture Rat	No		3045-VE-025
Oltean *et al*	*J Am Soc Nephrol*	26:1889-1904	2015	Human / Kidney	VEGF165b **exon 8b**/7 - Reverse primer : 5'-**GTGAGAG**ATCTGCAAGTACG-3'	DY3045	
Vencappa *et al*	*Am J Transl Res*	7:1032-1044	2015	Cell culture Mouse	No	Ab14994	3045-VE-025
Li *et al*	*J Translat Med*	13 :164	2015	Human cell lines	Same primers as Gu et al 2013	Same as	Yes

						Gu *et al* 2013	
Hulse *et al*	*Neurobiol Dis*	96:186-200	2016	Rat	No	Ab14994	
Castle-Miller *et al*	*Proc Natl Acad Sci USA*	114:E2517-2523	2017	Sheep / Pituitary	Multiple issues with primers leading to an erratum. VEGFAxxxb-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 *et al*	*Clin Sci (Lond)*	131:1225-1243	2017	Rat / Retina	No	Home made Clone56/8	Yes
Pruszko *et al*	*EMBO reports*	18:1331-1351	2017	Human cell lines	VEGF121b **exon 8b**/5 - Reverse primer : 5'-**CTTTCCTGGTGAGAG**ATTTTTCTTGTC-3' VEGF165b **exon 8b**/7 - Reverse primer : 5'-**CCTGGTGAGAG**ATCTGCAAGTAC-3'	Ab14994	
Hueso *et al*	*Scientific Reports*	7 :9962	2017	Human Serum / Heart	No	MAB3045	MBS109074
Chesnokov *et al*	*PeerJ*	6:e4915	2018	Human / Tissues	VEGFxxxb exon 7/**8b** - Forward primer : 5'-ACGTACTTG**CAGATCTCTCACCA**-3' Underlined bases correspond to either end of exon7 OR exon 8.		
Blochowiak *et al*	*Adv Clin Exp Med*	27:83-90	2018	Human / Parotid	No	DM3615P	
Boudria *et al*	*Oncogene*	AOP		Mice / Cell lines	No	Yes	Yes
Stevens *et al*	*Nephron*	139:51-62	2018	Mice	No	No	Yes / TG mice

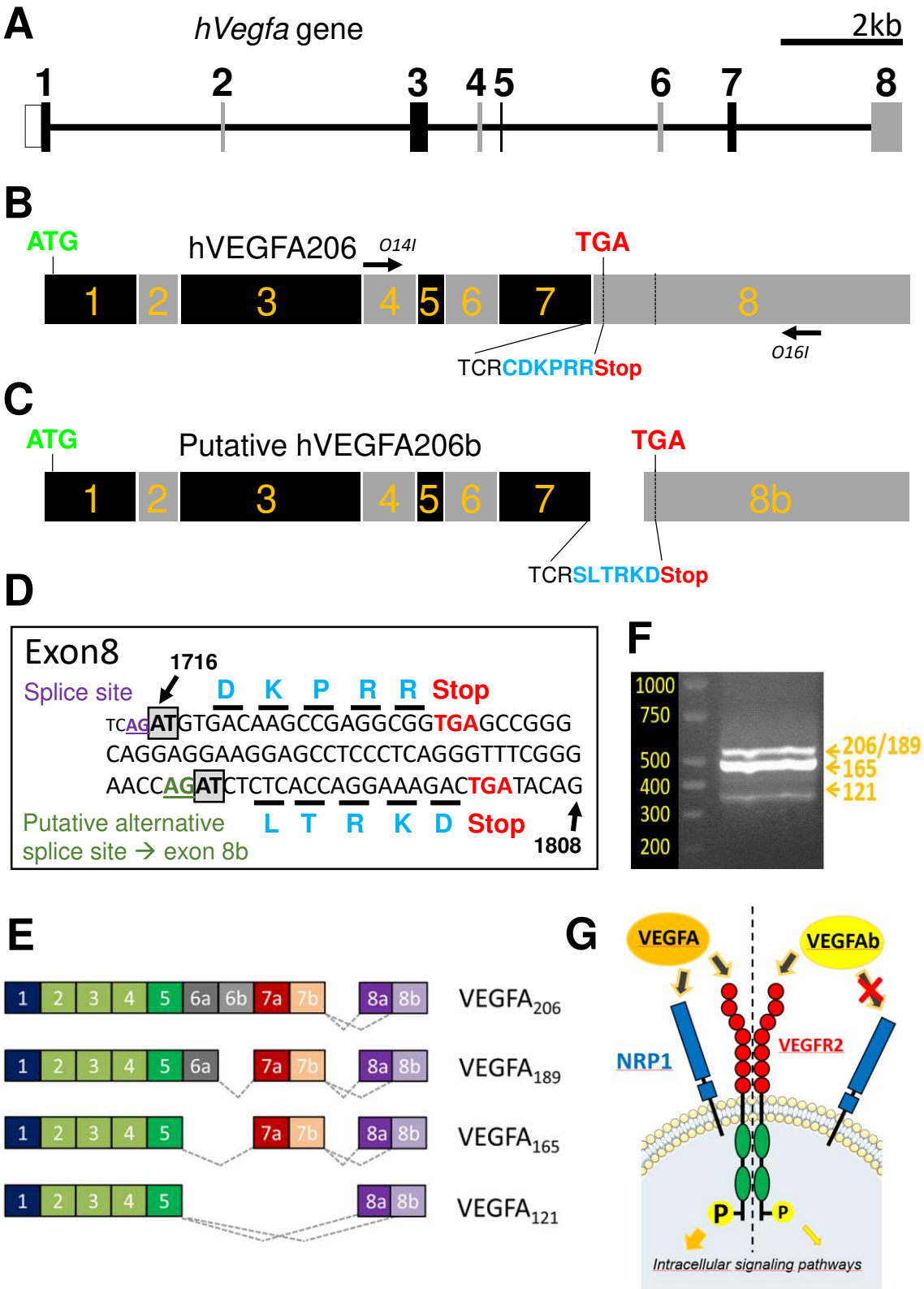
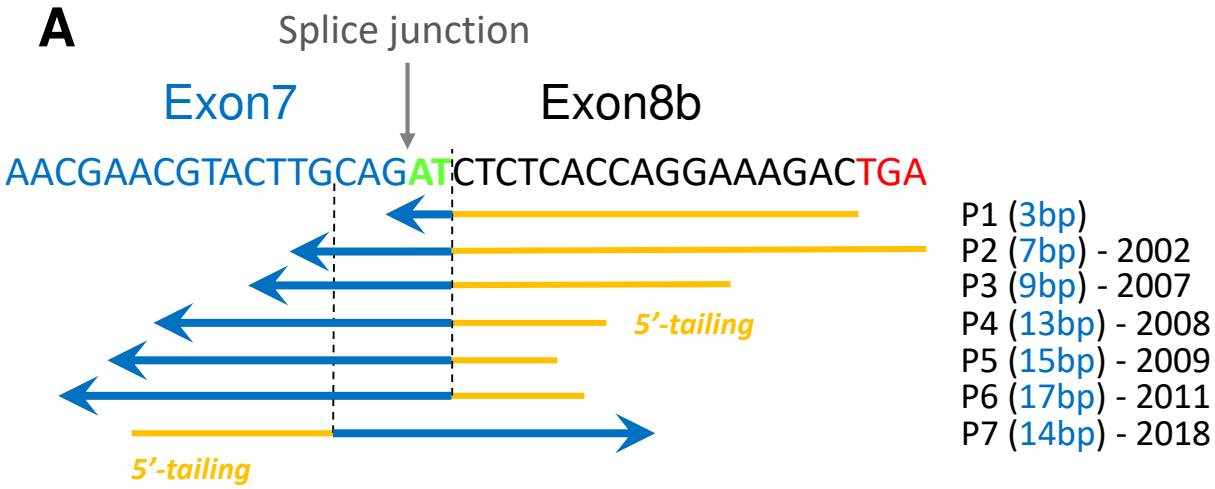

A
hVegfa gene
2kb
1 2 3 4 5 6 7 8
B
ATG
hVEGFA206
O14I
TGA
1 2 3 4 5 6 7 8
O16I
TCRCDKPRRStop
C
ATG
Putative hVEGFA206b
TGA
1 2 3 4 5 6 7 8b
TCRSLTRKDStop
D
Exon8
1716
Splice site
D K P R R Stop
TCAGATGTGACAAGCCGAGGCGGTGAGCCGGG
CAGGAGGAAGGAGCCTCCCTCAGGGTTTCGGG
AACCAGATCTCTCACCAGGAAAGACTGATACAG
Putative alternative splice site → exon 8b
L T R K D Stop
1808
E
1 2 3 4 5 6a 6b 7a 7b 8a 8b
VEGFA206
1 2 3 4 5 6a 7a 7b 8a 8b
VEGFA189
1 2 3 4 5 7a 7b 8a 8b
VEGFA165
1 2 3 4 5 8a 8b
VEGFA121
F
1000
750
500
400
300
200
206/189
165
121
G
VEGFA
VEGFAb
NRP1
VEGFR2
P
P
Intracellular signaling pathways

Figure 1


A
Splice junction
Exon7
Exon8b
AACGAACGTACTTGCAGATCTCTCACCAGGAAAGACTGA
5'-tailing
5'-tailing
P1 (3bp)
P2 (7bp) - 2002
P3 (9bp) - 2007
P4 (13bp) - 2008
P5 (15bp) - 2009
P6 (17bp) - 2011
P7 (14bp) - 2018

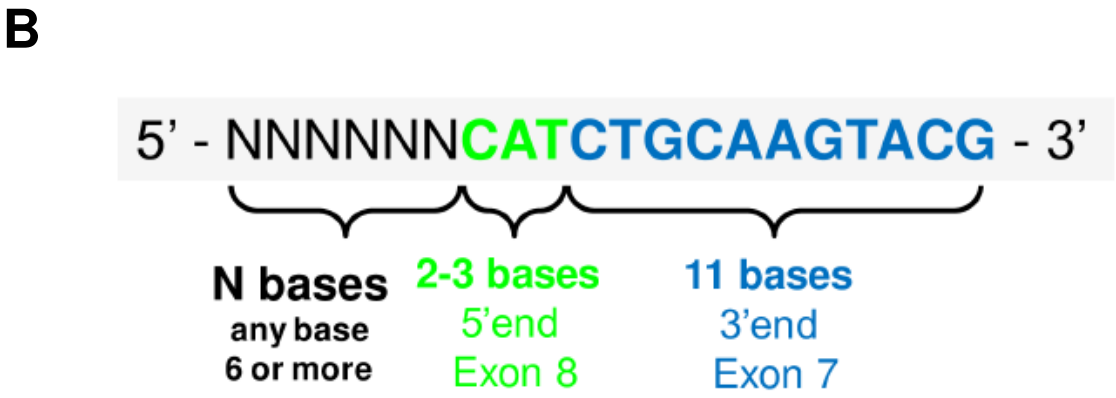

B
5' - NNNNNNCATCTGCAAGTACG - 3'
N bases
any base
6 or more
2-3 bases
5'end
Exon 8
11 bases
3'end
Exon 7

Figure 2

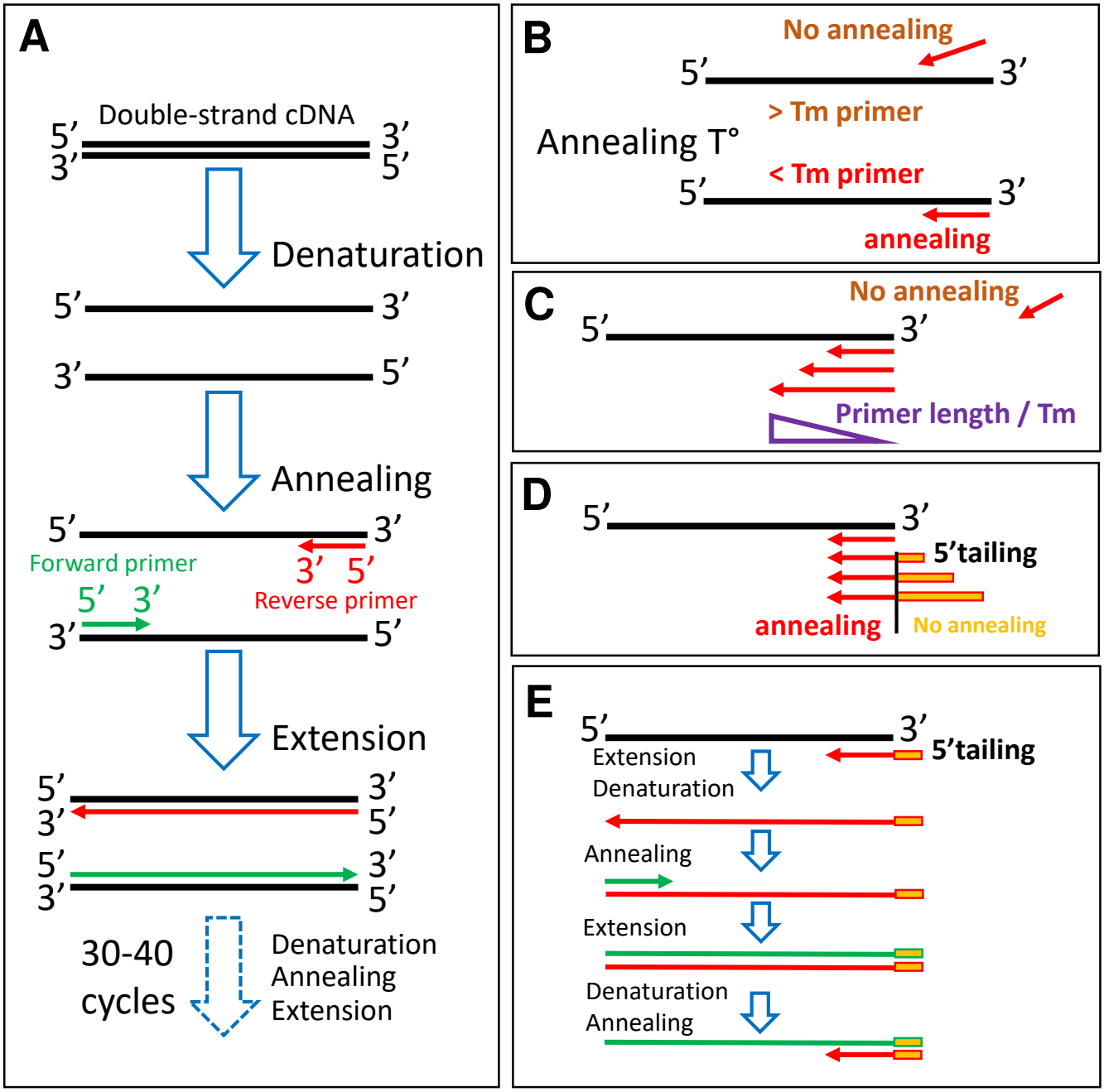

A
Double-strand cDNA
5'
3'
3'
5'
Denaturation
5'
3'
3'
5'
Annealing
5'
3'
Forward primer
3' 5'
5' 3'
Reverse primer
3'
5'
Extension
5'
3'
3'
5'
5'
3'
3'
5'
30-40
cycles
Denaturation
Annealing
Extension
B
No annealing
5'
3'
> Tm primer
Annealing T°
< Tm primer
5'
3'
annealing
C
No annealing
5'
3'
Primer length / Tm
D
5'
3'
5'tailing
annealing
No annealing
E
5'
3'
5'tailing
Extension
Denaturation
Annealing
Extension
Denaturation
Annealing

Figure 3

Supplemental Text

Debunking the myth of the endogenous anti-angiogenic *Vegfaxxxb* 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, *Vegfaxxxb* 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 VEGFAxxxb 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 *Vegfaxxxb*", 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 VEGFAxxxb 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, VEGFxxxb, 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 IgG_1 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

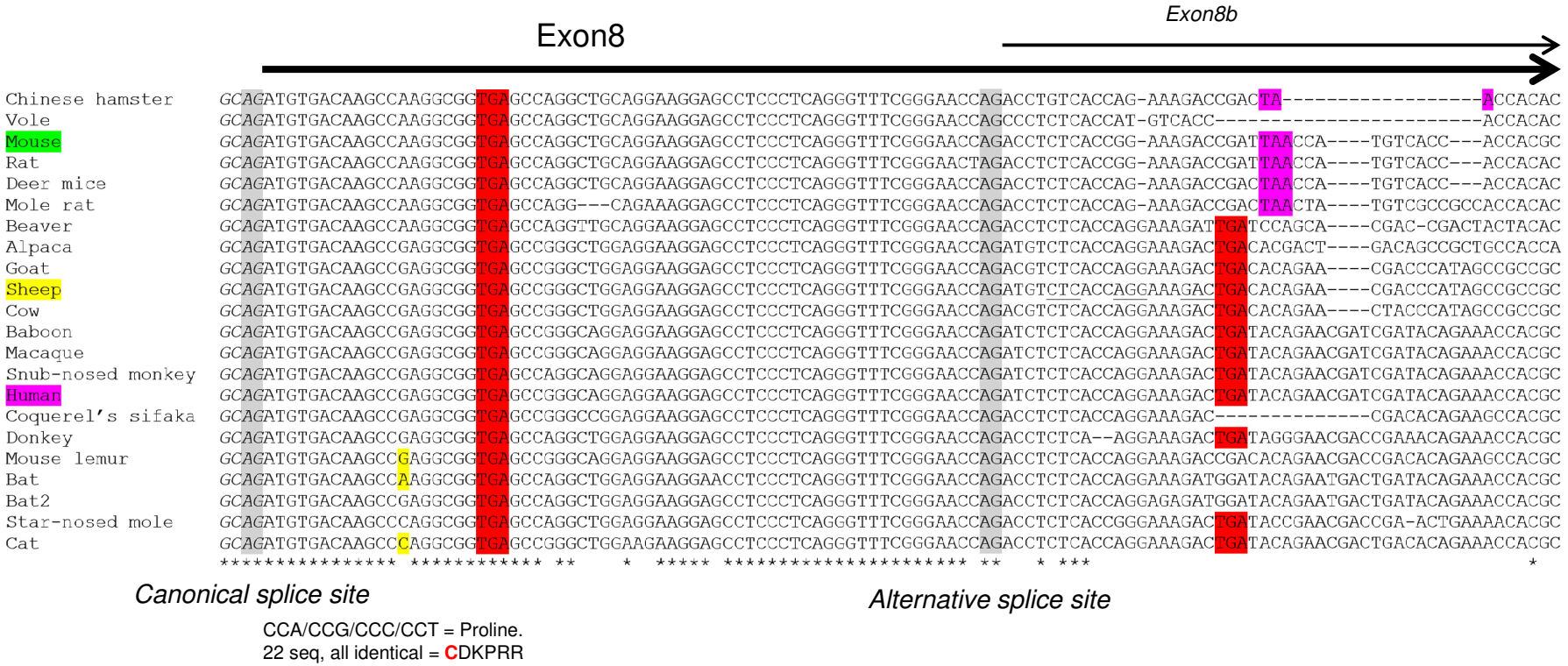
4 Li, X. *et al*. (2015) VEGF111b, a C-terminal splice variant of VEGF-A and induced by mitomycin C, inhibits ovarian cancer growth. *J Transl Med* 13, 164-015-0522-0

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



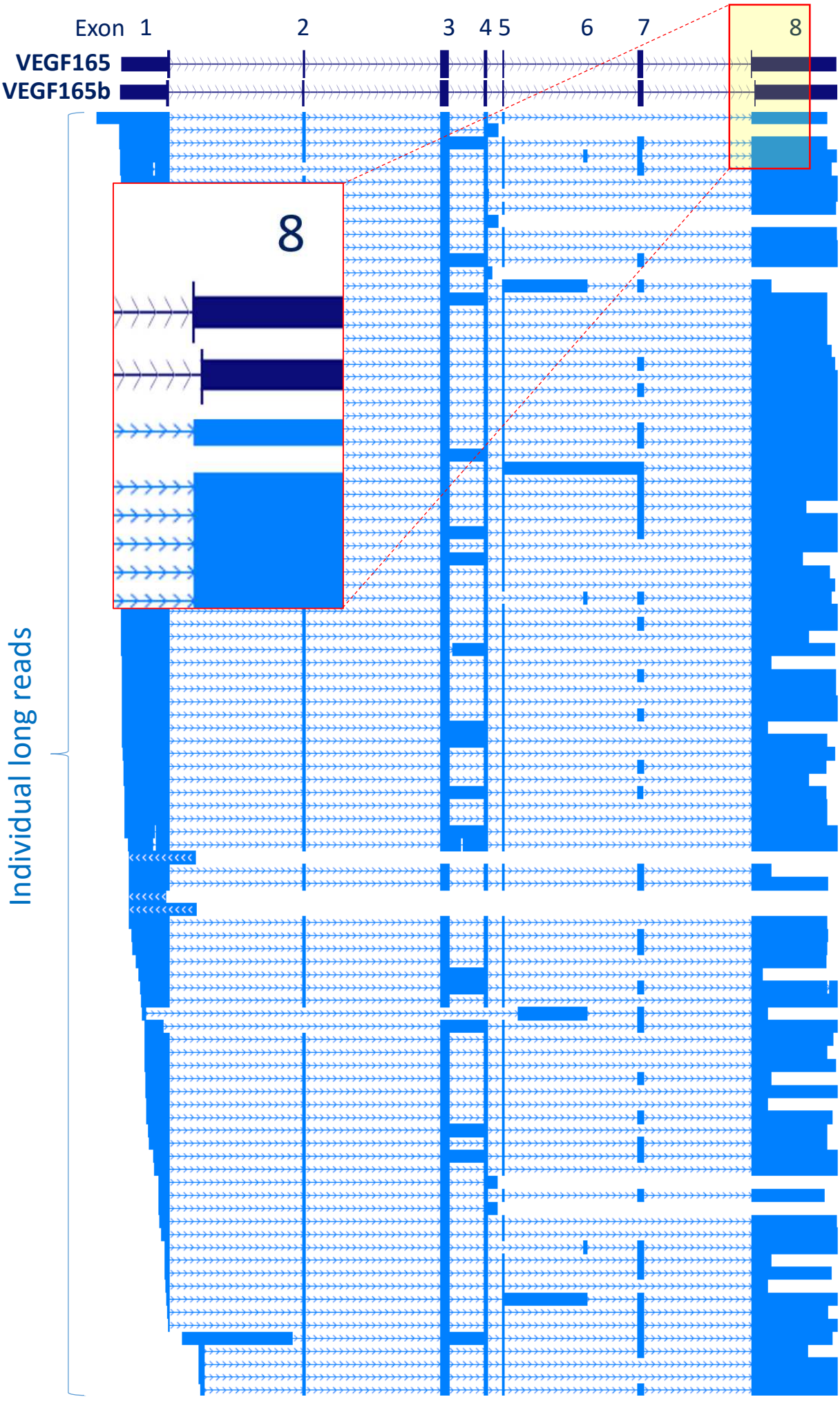
CCA/CCG/CCC/CCT = Proline.
22 seq, all identical = **C**DKPRR

Putative VEGFAxxxb sequences

Chinese hamster	PVTRKTD
Deer mice/Mole rat	PLTRKTD
Mouse/Rat	PLTGKTD
Star-nosed mole	PLTGKD
Sheep/Alpaca	CLTRKD
Goat/Cow	RLTRKD
Human/Babbon/Macaque	SLTRKD
Beaver/Cat	PLTRKD
Vole	PLTMSPPHRHRHHRQNSP
Bat	PLTRKDGYRMTDTETTPLPPHHHH
Bat2	PLTRRDGYRMTDTETTPLPPHHHHHHRQNNP
Mouse	PLTRKDRHRTTDTEATPLPPHHRHHRQNRP
Donkey	PLKERLIGNDRNRNHAAAATTSPSTEQSLIQKPEMKEEETAQSTLGPEGETPAEAFPGG
Coquerel's sifaka	PLTRKDRHRSHAAATTPPSPSTEQSLDQKPEMKEEETLRRALWVRRARLRQKHSRAGDQARSLLELDSPFRFSCC

:

Figure S1


Exon 1
2
3
4 5
6
7
8
VEGF165
VEGF165b
8
Individual long reads

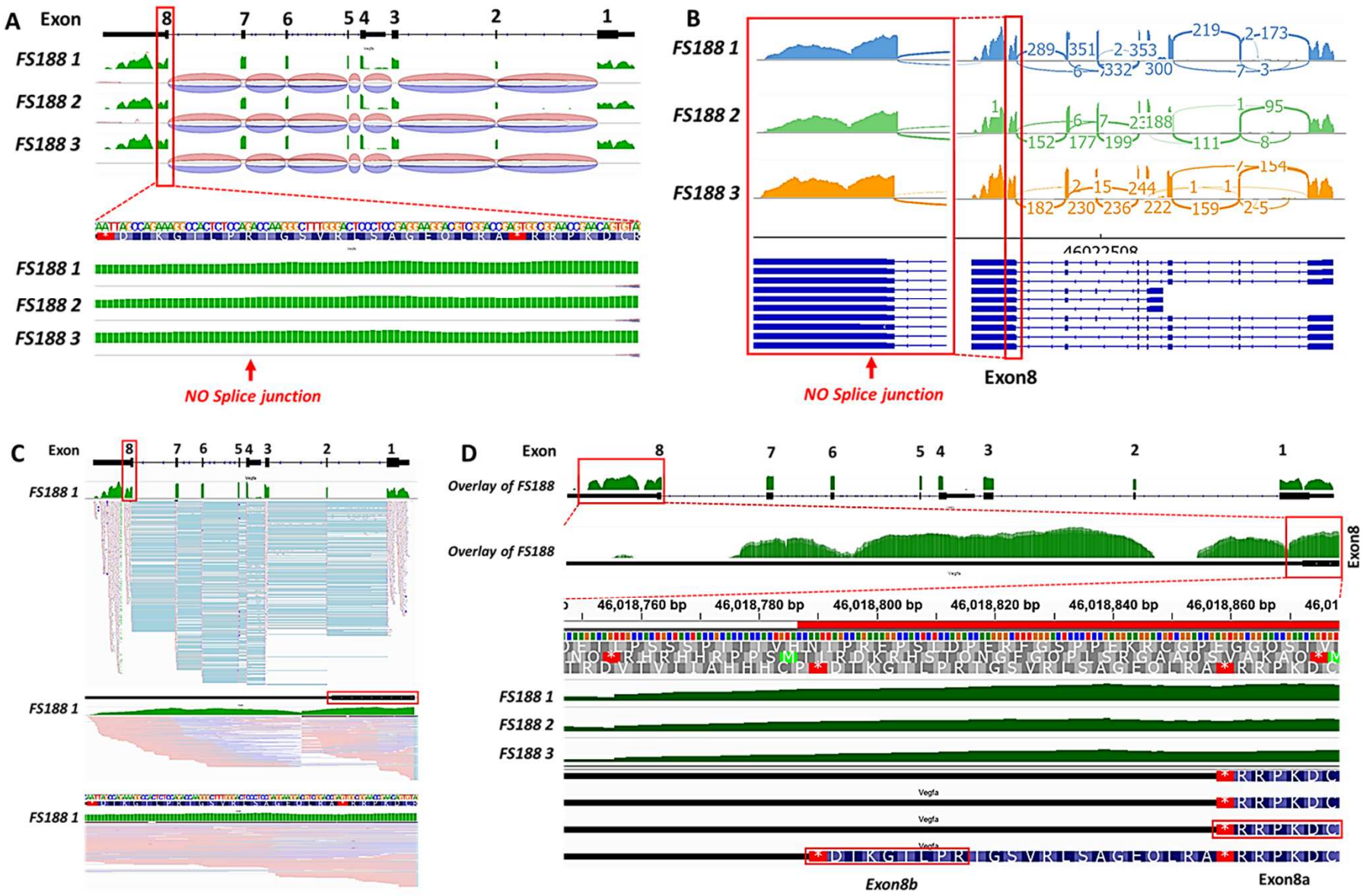

A
Exon
8
7
6
5 4
3
2
1
FS188 1
FS188 2
FS188 3
NO Splice junction
B
FS188 1
FS188 2
FS188 3
289
351
2-353
219
2-173
6
332
300
7
3
1
95
6
7
2188
152
177
199
111
8
154
2
15
244
1
1
182
230
236
222
159
2-5
Exon8
NO Splice junction
C
Exon
8
7
6
5 4
3
2
1
FS188 1
FS188 1
FS188 1
D
Exon
8
7
6
5 4
3
2
1
Overlay of FS188
Overlay of FS188
Exon8
46,018,760 bp
46,018,780 bp
46,018,800 bp
46,018,820 bp
46,018,840 bp
46,018,860 bp
FS188 1
FS188 2
FS188 3
Vegfa
Vegfa
Vegfa
Exon8b
Exon8a

Figure S3