Review of "The blunted vascular endothelial growth factor-A response to high-altitude hypoxia is associated with the genetic variants in the promoter region of VEGF A gene in Sherpa highlanders"

General: I commend the authors for undertaking this study as to understand the process of high-altitude adaptation, studies must go beyond simply measuring gene frequency differences or reporting on the existence of selected-for gene regions and actually measure relevant phenotypes. The authors have done this, which is good. But (1) their language still suffers from non-idiomatic and overly wordy expressions and (2) not sufficiently acknowledged the weakness of their present study design, and (3) over-interpreted their data findings.

1. Language. I was not among the reviewers of the prior version of this manuscript. From the authors' remarks, it appears they engaged a professional translation service and I commend them for doing so. However, perhaps because that service was not familiar with scientific writing, there are still numerous places where the writing should be improved. Hence, requiring quite a bit of my time to do so, I note the places below where changes could be made to remedy the manuscript's non-idiomatic and overly wordy expressions. (Line numbers below are from the printed versions of the files sent to me).
   a. Abstract:
      i. The 1st sentence is rough. Perhaps simplify as "Sherpa highlanders demonstrate extraordinary tolerance to hypoxia at high altitudes, which may be achieved by mechanisms promoting microcirculatory blood flow and capillary density."
      ii. Line 27: … "and angiogenesis which are stimulated
      iii. Line 33 & 35: "measured, ~~meanwhile~~ and, five single-nucleotide polymorphisms (SNPs, rs699947, rs833061, rs1570360, rs2010963, and rs3025039) in the *VEGFA* were genotyped. ~~The results~~ VEGF-A levels were …"
      iv. Lines 40-43: "high altitude was ~~on~~ the same ~~level~~ as that in the non-Sherpas at low altitude ~~without significant difference~~ (262.8 ± 17.9 pg/ml vs. 266.8 ± 21.8 pg/ml, P = 0.88). This result suggested that the plasma VEGF-A concentration in Sherpa highlanders was stable despite a ~~with the~~ high-altitude hypoxic stimulus ~~and did not respond to hypoxia~~, and that therefore the Sherpa exhibited a phenotype…"
      v. Lines 45-end: promoter region of the *VEGFA* ~~were significantly~~ different between the Sherpa highlanders and non-Sherpa lowlanders (corrected P values = 3.30×10^{-5}, 4.95×10^{-4}, and 1.19×10^{-7}, respectively). **Conclusions.** ~~The VEGF-A in~~ Sherpa highlanders exhibited a ~~feature of~~ blunted VEGF-A response to hypoxia at high altitudes, which we ~~was~~ speculated was ~~on an~~ associated with the distinctive genetic …"
   b. Introduction
      i. Line 54: ~~The history suggested~~ Historical sources indicate
      ii. Line 58: to hypoxia ~~at high altitudes, which is highly valuable during the~~ during expeditions
      iii. Line 61: chronic mountain sickness~~, resulting from the~~ due to various adaptations to hypoxia involving ~~through~~ hematology…"
      iv. Line 72: ~~because they play a role in~~ through their influences on endothelial …"

v. Lines 74-75: ~~members of~~ VEGF-A, VEGF-B, VEGF-C, VEGF-D (Holmes et al. 2005). ~~The~~ VEGF-A is the major member of the VEGF~~s~~ family, and ~~it~~ is an endothelial cell-specific ~~angiogenic inducer~~ protein that …"
vi. Lines 79-80: Thus, ~~the~~ we targeted VEGF-A and ~~the~~ its polymorphisms ~~in the gene~~ encoding VEGF-A (*VEGFA*) ~~were targeted~~ in the present …"
vii. Lines 82-86: Other members of the VEGF family were not included in the present study because there is little information available about the effects of hypoxia on these factors and further, the actions of VEGF-B, -C, and -D primarily involve other systems (i. e., ~~VEGF-B functions for the~~ embryonic angiogenesis, ~~VEGF-C activates~~ lymphatic vascular endothelium, and ~~VEGF-D is needed for the development of~~ lymphatic vasculature ~~surrounding lung bronchioles~~ respectively (Holmes et al. 2005). ~~There is little information available about the effects of hypoxia on these factors~~."
viii. Line 87: ~~The~~ VEGF-A is encoded…
ix. Line 90-92: … correlation of the genetic variants with VEGF-A production (Watson et al. 2000). Therefore, possibly VEGFA gene variants may alter ~~lead to differences in~~ *VEGFA* expression ~~in individuals~~ and ~~may~~ influence ~~a variety of~~ *VEGFA*-associated ~~phenotypes associated with~~ (Appenzeller et al. 2006; Tomar et al. 2015).

c. Materials & Methods

i. Line 118-120 UTR showed significant alteration of the *VEGFA* promoter activity: … reported to be ~~in a significant~~ associated with altered plasma VEGF-A concentrations (Renner et al. 2000). Individuals carrying ~~with~~ SNPs ~~of~~ rs833061 in the promoter and rs2010963 in the UTR have been reported to be related to ~~showed significant alteration of the~~ *VEGFA* promoter activity...
ii. Lines 135-129: Accordingly, ~~the~~ SNPs ~~of~~ rs699947, rs833061, rs1570360, rs2010963 in the promoter & 5'-UTR region and rs3025039 in the 3'- UTR region of the *VEGFA* were genotyped in the Sherpa highlanders and non-Sherpa lowlanders (Table 1). Among these ~~SNPs~~, the rs833061, rs2010963, and rs3025039 are ~~the~~ SNPs ~~referring to the expression of~~ are quantitative trait loci (eQTLs)~~, the genomic loci~~ and ~~contribute to~~ variations in mRNA the expression ~~levels of~~ (Table 1).
iii. YOU MUST DESCRIBE THE LOW-ALTITUDE GROUP IN GREATER DETAIL AS TO THEIR ANCESTRY, ALTITUDE EXPOSURE, HABITUAL EXERCISE LEVELS, HEALTH STATUS, INCLUSION CRITERIA. LIKEWISE, SUCH DETAIL IS REQUIRED FOR THE HIGH-ALTITUDE SUBJECTS.

d. Results

i. Get rid of the "The" (1st word) in each section. It's not needed. Throughout, eliminate the word "significant" as, per your methods, you are only reporting observations that meet your criteria for statistical significance.
ii. Lines 150-152: There were no significant differences in the gender ~~ratio and average~~ or age between the two groups (Table 2). None of the Sherpa highlanders complained of CMS symptoms at the high altitude, and thus all ~~were adaptive well~~ appeared adapted to high-altitude hypoxia. The SpO_2 was significantly lower in Sherpas at 3,440 m (93.7 ± 0.2%) than non-Sherpas at 1,300 m (96.7 ± 0.2%, $P = 6.43 \times 10^{-17}$, Table 2) ~~due to difference of altitude~~. The plasma VEGF-A concentration in the Sherpas at high altitude ~~exposed to hypoxia~~ was on the same ~~level without a significant difference~~ as that in the non-Sherpas at low altitude ~~without exposure to hypoxia~~ (262.8 ± 17.9 pg/ml vs. 266.8 ± 21.8 pg/ml, P = 0.88, Table 2).

iii. A comment here: stating the subjects did not complain of CMS symptoms is a very weak argument for concluding that they are "adapted". See PMID 28860167; "adaptation" in the context of naturally selected genes and evolutionary processes has very specific meanings.

iv. Lines 158-163: THIS ENTIRE SENTENCE, BEGINNING "ASSUING" SHOULD BE MOVED TO THE DISCUSSION AS YOU ARE DISCUSSING, NOT PRESENTING, RESULTS. this result suggested that the plasma VEGF-A concentration in Sherpa highlanders was stable ~~with the~~ despite a high-altitude hypoxic stimulus, and that Sherpa therefore ~~did not respond to hypoxia,~~ exhibited a phenotype of blunted response to hypoxic stress. In addition, the plasma VEGF-A concentration was slightly in a positive correlation with SpO_2 in Sherpas at high altitude ($r = 0.13$, Fig. 1A YOU MUST ADD THE P VALUE HERE AND ADD IT TO FIGURE 1A AS WELL), ~~whilst, it was in~~ whereas it was negatively correlated with SpO_2 in non-Sherpas at low altitude ($r = -0.27$, Fig. 1B ADD THE P VALUE HERE AND IN FIGURE 1A).

v. Line 164: ~~**The**~~ ***VEGFA*** **SNPs ~~of~~ in …**

vi. Lines 165-174: The genotype distributions and allele frequencies of the SNPs all met ~~the~~ HWE in both groups. The genotype distribution of the ~~Two~~ SNPs in the promoter region (rs699947 and rs833061) and one SNP in the 5′–UTR (rs2010963) differed ~~exhibited significant~~ between-groups ~~differences in terms of genotype distribution~~ ($Pc = 1.43 \times 10^{-3}$, 2.48×10^{-3}, and 1.04×10^{-7}, respectively) ~~and~~ as did their allele frequencies ($Pc = 3.30 \times 10^{-5}$, 4.95×10^{-4}, and 1.19×10^{-7}, respectively, Table 3). Particular attention was paid to the rs833061 and rs2010963 ~~that~~ as these are ~~the~~ eQTL loci in the promoter region that could influence ~~relating contribution to variations in the~~ mRNA expressions ~~of the *VEGFA* gene~~ and hence protein level. PLEASE BE AWARE THAT MANY FACTORS INFLUENCE PROTEIN LEVEL, NOT JUST MRNA EXPRESSION. On the other hand, ~~the~~ SNPs ~~of~~ rs1570360 and rs3025039 did not ~~significantly~~ differ between the two groups ~~concerning~~ nor did their genotype distributions ~~and~~ or allele frequencies (Table 3). In the Sherpa highlanders, the major alleles ~~of~~ for these SNPs were rs699947**C**, rs833061**T**, rs1570360**G**, rs2010963**C**, and rs3025039**C** and were PLEASE ADD THE MAJOR ALLELELS IN THE LOWLAND GROUP STUDIED.

vii. Lines 176-185: Pairwise LD analysis revealed that ~~the of~~ rs699947, rs833061, rs1570360, and rs2010963 SNPs in the promoter & 5-UTR region were strongly linked in both groups (Fig. 2). The haplotype comprising the Sherpa-major alleles (rs699947**C**, rs833061**T**, rs1570360**G**, and rs2010963**C**; **C-T-G-C** haplotype) exhibited a ~~significantly~~ higher frequency in the Sherpa highlanders (0.667) than the non-Sherpa lowlanders (0.280, $P = 3.1 \times 10^{-5}$, Fig. 2, Table 4). Note that the rs833061 and rs2010963 are the eQTL loci in the promotor region ~~presuming of~~ involved in the regulation of *VEGFA* mRNA expression ~~of the~~. Expectedly, among those ~~of~~ individuals carrying the **C-T-G-C** haplotype, there was no significant difference in the VEGF-A concentrations between the Sherpas at high altitude and non-Sherpas at low altitude (257.62 ± 29.76 pg/ml vs. 261.39 ± 17.13 pg/ml, $P > 0.05$).

e. Discussion. IT IS CURRENTLY 7.5 PAGES, WHICH IS TOO LONG AND THEREFORE SHOULD BE REDUCED TO ~5 PAGES. MY REMARKS BELOW ACCOMPLISH SOME OF THIS NEEDED REDUCTION BUT MORE COULD AND SHOULD BE DONE

i. Lines 189-191: thus suggested a ~~feature of~~ blunted response to hypoxia in Sherpa highlanders. Moreover, this blunted VEGF-A response to hypoxia was accompanied by differences in ~~speculated on association with~~ the genetic variants in the promoter …

ii. Lines 193-216: Hypoxia is an important regulator of blood vessel tone and structure, and a potent stimulus for vasculogenesis and angiogenesis (Liu et al. 1995). ~~The~~ VEGF-A production is mainly stimulated by hypoxia and promotes proliferations~~s~~ of vascular smooth muscle cells and endothelial cells under hypoxic conditions ~~with the outcome of~~ and thereby potentially vasculogenesis and angiogenesis (Moens et al. 2014; Namiki et al. 1995). Hypoxia-stimulated vasculogenesis and angiogenesis may increase microcirculatory blood flow and capillary density, thus restoring the oxygen supply in tissues exposed to hypoxia to compensate for oxygen insufficiency (Carmeliet 2000). ~~Studies on~~ High-altitude medicine studies have revealed ~~significantly~~ increased circulating VEGF-A concentrations ~~in individuals at high altitudes~~ which ~~. These elevations of VEGF-A concentrations~~ were negatively correlated with SpO_2 (Ge et al. 2011; Tissot van Patot et al. 2005; Walter et al. 2001; Zhang et al. 2018) ~~in individuals at high altitudes (Ge et al. 2011; Zhang et al. 2018).~~ Thus, we anticipated ~~assumed~~ that the VEGF-A concentration would increase in Sherpas at high altitudes compared with ~~that in the~~ non-Sherpa lowlanders, perhaps due to an ~~which might involve the~~ increase of capillary density in tissues exposed to high-altitude hypoxia ~~for the adequate oxygen supply~~. Indeed, compared to lowlanders, Sherpa highlanders exhibited a significantly higher number of capillaries per square millimeter ~~of~~ muscle cross-sectional area, which would be expected to improve ~~supply~~ efficient and effective diffusion of oxygen to muscles (Kayser et al. 1991). Moreover, Sherpas exhibited significantly higher sublingual capillary density and faster microcirculatory flow per time and volume of tissues compared to lowlanders at 5300 m (Gilbert-Kawai et al. 2017). All these studies suggested that the VEGF-A and the associated microcirculatory physiology could play~~ed~~ important roles in the process of adaptation to high-altitude hypoxia in Sherpa highlanders.

iii. Lines 217-239: ~~While on the other hand, concerning the role of~~ However, VEGF-A may also play a role in maladaptation to high altitudes as~~, the~~ excessive production of VEGF-A at high altitudes could contribute~~d~~ to ~~the~~ pathophysiological formation of abnormal ~~new~~ blood vessels, pulmonary vessel remodeling, vascular smooth muscle cell proliferation (Appenzeller et al. 2006; Ge et al. 2011) ~~in~~ and distal vasculogenesis ~~in skin and mucosa (Ma et al. 2015). Each of which is a~~ that are potential contributors to ~~the development of~~ chronic mountain sickness, ~~that is~~ a syndrome of maladaptation ~~representing a failure to~~ adapt to high altitudes ~~(~~Villafuerte & Corante 2016). ~~The~~ Plasma VEGF-A concentrations were negatively correlated ~~to~~ with SpO_2 at high altitudes and ~~significantly~~ elevated in ~~with~~ chronic mountain sickness patients (Ge et al. 2011). Thus, we propose that the blunted VEGF-A response to hypoxia may contribute to ~~properly inhibiting for the~~ preventing over-production of VEGF-A~~, at high altitudes to prevent~~ pathophysiological vasculogenesis and angiogenesis in Sherpas at high altitudes. A blunting of hypoxic response has also been seen ~~This model in adaptation to high altitudes was also observed and demonstrated~~ in ~~native~~ healthy Tibetans at high altitudes. For example, hemoglobin (Hb) ~~the~~ concentrations ~~of~~ were relatively low in high-altitude Tibetans ~~at~~ and ~~the Hb level was at~~ within the normal sea-level range compared ~~with the elevated Hb level typically exhibited in non-Tibetans at high altitude~~

(Beall et al. 1998) as were ~~the~~ pulmonary arterial pressures ~~were normal by sea-level standards in native healthy Tibetans at high altitudes~~ (Groves et al. 1993). ~~Both the~~ Hb and pulmonary artery pressure are sensitive to hypoxia stress and the hypoxia-induced polycythemia and pulmonary hypertension are typical phenotypes of maladaptation to high-altitude hypoxia (Appenzeller et al. 2006; Ge et al. 2011). ~~Such~~ Therefore, blunted responses to hypoxia ~~in~~ may serve to suppress~~ion on~~ the over-responses of Hb, ~~and~~ pulmonary vasoconstriction and vasculogenesis seen in maladaptive syndromes or non-adapted populations. ~~circulation to hypoxia at high-altitude contribute to the adaptation to high altitudes in Tibetans (Beall et al. 1998; Groves et al. 1993)~~.

iv. Lines 240-242: In this study, ~~the associations of the~~ blunted VEGF-A levels and *VEGFA* gene variants in Sherpa highlanders (Namche Bazaar) were compared to ~~with the *VEGFA* genetic variants were assessed by reference to the genetic information in the~~ non-Sherpa lowlanders ~~in~~ (Kathmandu) in Nepal.

v. Lines 243-251: THIS MATERIAL IS "HAND WAVING" – AN EXPRESSION THAT MEANS THAT YOU ARE BRINGING POINTS THAT ARE NOT VERY RELEVANT. THE GEOGRAPHIC DISTANCES BEING REFERRED TO BY THE VARIOUS STUDIES CITED ARE MUCH GREATER THAN 200 KM. YOU ALSO UNDERCUT YOUR OWN ARGUMENT BY NOTING THAT GENE FLOW HAS OCCURRED BETWEEN SHERPA AND PERSONS OF SOUTH ASIAN ANCESTRY. RATHER THEN THAN BRINGING IN EXTRANEOUS, AND RATHER WEAK ARGUMENTS FOR WHY YOU DIDN'T STUDY SHERPA AT LOW (AS WELL AS AT HIGH) ALTITUDE, AND NON-SHERPA AT HIGH (AS WELL AS AT LOW) ALTITUDE, MOVE THE MATERIAL MENTIONED IN LINES 320-333 TO HERE AND BEGIN THIS SECTION WITH A LINE SUCH AS "Our study suffered from several weaknesses…" . IF YOU DO THAT, THE FOLLOWING EDITS OF THE MATERIAL PRESENTLY ON LINES 320-333 MAY BE USEFUL.

One of the limitations of this study is that there ~~is~~ were no data ~~of~~ for plasma VEGF-A concentrations in Sherpas at low altitudes nor were studies conducted in non-Sherpa at high altitude. ~~It would indeed be superior to monitor the changes of VEGF-A concentrations in the Sherpa highlanders with altitude ascending. However, it was unfeasible~~ Such studies are needed but it was not possible for the present study to carry out such ~~a large population~~ group movements ~~from low-altitudes to high-altitudes in the huge and mountainous Himalayan region, which was beyond the scale of the present study~~ due to logistical and other constraints ~~the issues of and reasons of subjects themselves~~. Nonetheless, several studies indicate ~~By the evidence~~ that ~~the~~ VEGF -A levels are ~~was~~ increased by hypoxia stress (Ge et al. 2011; Tissot van Patot et al. 2005; Walter et al. 2001; Zhang et al. 2018). ~~we assumed that the VEGF-A concentration would be higher in Sherpas at high altitudes than the non-Sherpas at low altitudes and thus compared the VEGF-A concentration between the two populations.~~ Another limitation is that it lacked ~~phenotypes~~ measurements of angiogenesis and vasculogenesis in Sherpas at ~~the~~ high altitude due to the lack of requisite analytic equipment and the limitations ~~unavailable device capable~~ of performing ~~non~~invasive studies in ~~pathological study at~~ Namche village. However, the strength of our study is that ~~Nevertheless,~~ it was the first study to measure ~~the~~ plasma VEGF-A concentrations

in combination with *VEGFA* gene ~~and investigate the~~ variants of *VEGFA* in Sherpas at a high altitude.

vi. Lines 252-266: We genotyped the rs699947, rs833061, rs1570360, rs2010963, and rs3025039 SNPs for ~~of~~ *VEGFA* in the Sherpa highlanders and non-Sherpa lowlanders. The results revealed ~~significant~~ genetic divergences in the frequencies of the ~~of~~ rs699947, rs833061, rs2010963 SNPs between the two populations (Table 3) and the ~~.~~ ~~In addition, the~~ haplotypes composing the major alleles ~~of~~ (rs699947**C**, rs833061**T**, rs1570360**G**, and rs2010963**C** (**C-T-G-C**) ~~was significantly enriched in Sherpa highlanders~~ (Table 4). ~~By the evidence~~ Given that ~~the~~ VEGF-A levels were increased in patients with chronic mountain sickness ~~(mal-adaptation to high altitudes) and based on the present result that the VEGF-A concentrations were~~ but blunted ~~to increase~~ in Sherpas ~~exposed to hypoxia in the individuals carrying~~ with the **C-T-G-C** haplotype (Table 4), ~~it might suggest that this significant prevalence of~~ the **C-T-G-C** haplotype may have contributed ~~was related~~ to the blunted VEGF-A response ~~to hypoxia~~ in Sherpas. Further we speculate~~d~~ that the rs833061 and rs2010963 eQTL loci ~~of~~ in the haplotype ~~might involve the~~ may down regulate ~~of the~~ mRNA expression and hence VEGF-A levels ~~of the *VEGFA* gene~~. Further study is required to determine whether such effects occur and can help explain the Sherpas' ~~on the function of this haplotype will surely discover the mechanisms of this haplotype with this issue. The Sherpas carrying this haplotype exhibited a phenotype of~~ blunted VEGF-A response to hypoxia ~~in high altitudes~~.

vii. Lines 267-280: THIS PARAGRAPH CAN BE REDUCED TO A COUPLE OF SENTENCES SAYING THAT SHERPA ARE CLOSELY RELATED TO TIBETANS AND THAT THEIR VEGFA ALLELE FREQUENCIES ARE SIMILAR.

viii. Lines 281-291: THIS PARAGARPH CAN ALSO BE REDUCED TO 1-2 SENTENCES SAYING THAT VEGFA ALLELE FREQUENCIES IN SHERPAS (AND TIBETANS) DIFFER FROM THOSE OF THE VARIOUS LOW-ALTITUDE POPULATIONS THROUGHOUT THE WORLD. YOUR CONCLUDING SENTENCE SHOULD BE TONED DOWN ALONG THE LINES OF: Thus, together these observations ~~Taking all these together into consideration, it is highly~~ suggest~~ed~~ that ~~the~~ ~~significant~~ differences in the distribution~~s~~ of ~~the~~ *VEGFA* gene~~tic~~ variants ~~of~~ in Himalayan highlanders ~~from other lowlander populations probably~~ likely result~~ed~~ from a process of natural selection ~~through thousands of generations during their permanent settlements at high altitudes in~~ resulting in genetic adaptation to ~~environmental hypoxia stress in~~ high altitude ~~populations (Beall, 2007)~~.

ix. Lines 292-319: LIKEWISE, THIS PARAGRAPH CAN BE REDUCED TO ~HALF A PAGE.

f. <u>Conclusions.</u> AGAIN, THIS SECTION IS OVERLY WORDY AND SPECULATIVE. I SUGGEST REWORDING ALONG THE LINES OF THE FOLLOWING:

In summary, ~~the~~ VEGF-A levels in Sherpa highlanders exhibited a ~~feature of~~ blunted response to hypoxia at high altitudes, which was accompanied by differences in *VEGFA* SNPs ~~speculated on an association with the distinctive genetic variations of the SNPs and haplotype~~ in the promoter region ~~of *VEGFA* in Sherpa highlanders~~. Natural selection by ~~the~~ high-altitude hypoxia ~~would~~ may have favored a ~~specific~~ *VEGFA* haplotype conferring an ~~to~~ adaptive advantage to ~~for~~ Sherpa ~~people dwelling at~~ high-altitude populations. Further study~~ies~~ , with adequate controls for ancestry and altitude of

residence, are ~~is~~ necessary to identify the mechanisms by which such SNPs may be acting to affect protein levels and phenotypic correlates, as well as to identify possible ~~figure out the~~ interactions between ~~of the~~ *VEGFA* with other hypoxia-associated, naturally selected genes. ~~(for example, *EPAS1* and *EGLN1* genes) on the regulations of VEGFA gene mRNA expressions of these genes in Sherpa highlanders~~