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Comments given by Reviewer 2618391

In current manuscript titled "New categorization of human vascular endothelial cells by pro- versus anti-proliferative phenotypes", the authors tried to solve the controversy in human vascular endothelial cells (VEC) to the proliferation of human vascular smooth muscle cells (VASMC) by characterize the human VECs from various sources in two groups as either pro-proliferative or antiproliferative.

Major concerns:

Comment 1

The authors use RGS5 expression level to categorize human VECs as RGS5-high (type I) and RGS5-low (type-II), but didn't indicate clearly that it is by RGS5 gene level or protein expression level. Furthermore, what is the reference number to separate the "low" and "high"? Is there any physiological meanings of this "low" and "high"?

Response 1

Although human VECs should be categorized into type-I and type-II by their effects on the proliferation of VSMCs as determined by co-culture experiments in principle, the level of RGS5 message expression (i.e. RGS5/GAPDH) is also useful in determining the phenotype of VECs. For example, the values on the vertical axis in Fig. 3A (i.e. Log RGS5/GAPDH) are as follows: 0.0079 ± 0.0010 (EPC1dEC[P6], type-II), 0.080 ± 0.0027 (EPC1dEC[P13], type-I), 0.087 ± 0.0017(EPC2dEC[P8], type-I), 1.090 ± 0.031 (HUVEC, type-I), 0.590 ± 0.023 (HAEC, type-I), 0.790 ± 0.030 (HMVEC, type-I) and

0.357 ± 0.014 (HCAEC, type-I). Additionally, we have data on Log RGS5/GAPDH values regarding two more lines of type-II VECs: 0.011 ± 0.0007 (SeV-iPS(BJ)dEC[p9]) and 0.0044 ± 0.0002 (SeV-hiPS(HUVEC)dEC[P10]) (*see* below). Therefore, human VECs whose Log RGS5/GAPDH values are "lower than 0.02" or "higher than 0.02" can be categorized into "type-II" or "type-I", respectively.
Alternatively, VECs that are evaluated as "negative" or "positive" by the immunostaining study using an anti-RGS5 antibody as performed under the condition described in Materials and Methods can be categorized into "type-II" or "type-I", respectively. (Figs. 3B, 4D and 5D).

Names of VECs	Log RGS5/GAPDH	Typing	Data
EPC1dEC[P6])	0.0079 ± 0.0010	Type-II	Fig. 3A
EPC1dEC[P13]	0.080 ± 0.0027	Type-I	Fig. 3A
EPC2dEC[P8]	0.087 ± 0.0017	Type-I	Fig. 3A
HUVEC	1.090 ± 0.031	Type-I	Fig. 3A
HAEC	0.590 ± 0.023	Type-I	Fig. 3A
HMVEC	0.790 ± 0.030	Type-I	Fig. 3A
HCAEC	0.357 ± 0.014	Type-I	Fig. 3A
SeV-iPS(BJ)dEC[p9]	0.011 ± 0.0007	Type-II	(Fig. 5C)
SeV-hiPS(HUVEC)dEC[P10]	0.0044 ± 0.0002	Type-II	(Fig. 5C)

Comment 2
For some statistical analysis, two way ANOVA should be used if there are more two groups of samples.
Response 2
ANOVA analysis should be applied to those cases where there is no specific information or particular meaning regarding the characteristics of each group. In other words, ANOVA analysis should be performed in the comparison among more than two groups of equal terms; for example, a comparison of body heights among French male population, American male population and Japanese male population. In our study, however, the comparison should be performed between "on gelatin", which is the *control condition*, and "on HUVECs", which is *the condition of interest*, but not among "on gelatin", "on HUVECs" and "Boyden" (in the case of Fig. 1D, for example). Although it was of no use to compare the results between "on HUVECs" and "Boyden",

we presented the data of “Boyden” to show that our experiments successfully reproduced the previously reported finding by Shinoda et al. (J Biol Chem 1999; 274:5379-5384; ref 2). Thus, it is valid to apply student-t test to evaluate the effect of VEC layers in comparison with that of gelatin layers in our case. Similarly, for an evaluation of the effect of RGS5 knockdown (Figs. 3E and 3F), the comparison should be performed between “CTL vector” and “Sh-RGS5”, but not among “Gelatin”, “CTL vector” and “Sh-RGS5”. In order to avoid the misunderstanding of readers, we depleted an arrow that was placed between “Gelatin” and “Sh-RGS5” from Fig. 3E in our revised manuscript.

Comment 3

In Figure 2 E and F, the ShRNA knockdown RGS5 experiment, the western blot should be used to show the expression level of RGS5 is also reduced.

Response 3

We suppose that the reviewer mentioned Figure 3E and 3F instead of Figure 2 E and F. To show the validity of our ShRNA knockdown experiments, we added the results of qRT-PCR and Western blotting in our revised manuscript (Figs. 3G and 3H) because RNA interference (RNAi) system can reduce gene/protein expressions *via* both an enhancement of message degradation and translational hindrance.

Minor Concerns:

There are some spelling and grammatical errors in the manuscript.

Response

We appreciate the comment. We have corrected spelling and grammatical errors in our revised manuscript

Comments given by Reviewer 2618027

There is controversy in the literature surrounding the contributions of experimental models of human vascular endothelial cells (VEC) to the proliferation of human vascular smooth muscle cells (VASMC). In this manuscript, the authors seek to characterize the phenotypes of current experimental models of human VECs from various sources as either pro-proliferative or anti-proliferative. The studies presented

herein implicate regulator of G-protein signaling 5 (RGS5) as a modulator of VEC phenotype, where VECs expressing high RGS5 are pro-proliferative and VECs expressing low RGS5 are anti-proliferative. Oxidative stress induces RGS5 expression and shifts VECs into a pro-proliferative phenotype. While the studies are novel and the manuscript is well written, there are a few concerns which should be addressed prior to publication:

Major Comments:

Comment 1

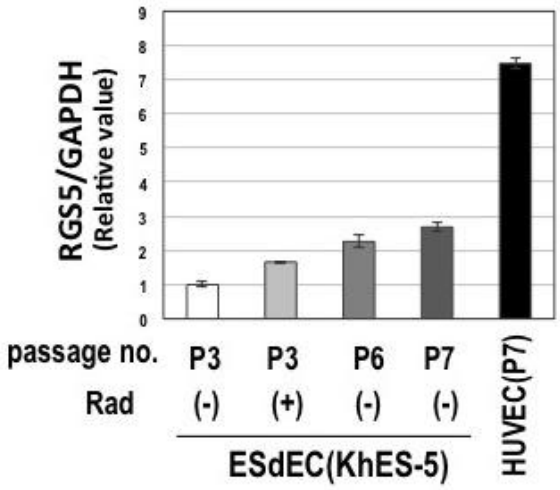
"…the proliferation of VECs had previously been arrested via a low-dose gamma ray irradiation." Did irradiation change the phenotype of these VECs? What experiments did the authors do to test that the phenotype of the irradiated VECs had not changed?

Response 1

To correctly evaluate the proliferation of VSMCs on the layer of VECs in VEC/VSMC co-cultured experiments, the proliferation of VECs should be arrested because the proliferation of VECs hinders that of VSMCs *via* the competition over nutrients and spaces. Therefore, it is impossible in principle to perform VEC/VSMC co-culture experiments without a *hitherto* gamma ray irradiation of VECs at a low dose rate (i.e. 5 Gy). In addition, VECs stay quiescent on the luminal surface of the artery *in vivo*, indicating that ordinary *in vitro* culture conditions without a low-dose-rate irradiation do not reproduce *in vivo* conditions.

Honestly, 5 Gy irradiation slightly up-regulated the level of RGS5 expression (*see* below), which is compatible with our finding that RGS5 is a stress-inducible gene. Nevertheless, 5 Gy irradiation did not affect the viability of VECs. Moreover, each experiment was performed under the same condition (i.e. we equally irradiated every kind of VECs before performing co-culture experiments), and thus, results of each kind of VECs can be validly compared. Moreover, the results of *in vitro* co-culture experiments were compatible with the results of *in vivo* transplantation experiments, which will be disclosed in our subsequent paper by Nishio et al., entitled "Pro- versus anti-stenotic capacities of type-I versus type-II human iPS-derived endothelial cells".

Collectively, our experimental procedures including a low-dose-rate irradiation of VECs, is valid and even indispensable for precise evaluation of the effect of VECs on the proliferation of VSMCs.



Comment 2

How confluent were the VECs prior to co-culture with VSMCs? If the VECs are too confluent, then they may produce pro-inflammatory mediators which may impact VASMC co-culture experiments.

Response 2

We performed co-culture experiments under conditions where VECs just covered the bottom surface of the gelatin-coated culture plate. In the case of HUVEC, for example, this stage was three days before full confluence or over-confluence, where the cells were highly packed, cell sizes were reduced by one third to one fourth of their original sizes and the localization of cadherin proteins at intercellular junctions became particularly dense. To avoid receiving non-specific signals from VEC-free gelatin-coated surfaces and also from pro-inflammatory mediators due to over-confluence, we performed the experiments under the condition described above.

Comment 3

The statistical tests performed are inappropriate. Student's t-test should only be used when comparing two sets of quantitative data. In studies where there are more than two groups, ANOVA followed by post-hoc tests of variance are required.

Response 3

The reason why we applied student-t test was described in the *Response 1* in the response to the comments by Reviewer 2618391.

Comment 4

The authors purport that "EPC2dEC might be in ageing states" due to "the gene function item "senescence" marked the highest value in the matching rate…" Since aging studies are not being performed in this manuscript, the conclusion that "the "type-II to type-I" phenotype conversion may well be considered as an ageing-associated degeneration" should be tempered. This inference is better described in the Discussion rather than in the Results section.

Response 4

According to the reviewer's kind suggestion, the description pointed above was moved to Discussion from Results in our revised manuscript.

Minor Comments:

Comment 1

There is a typographical error on page 5, where "fining" should be corrected to "finding".

Response 1

We appreciate the reviewer's comment. We corrected the spelling error.

Comment 2

There are two typographical inconsistencies, where "aging" (once on page 4) has been changed to "ageing" (twice on page 14).

Response 2

We appreciate the reviewer's comment. We corrected the spelling error.

Comment 3

There is a grammatical error on page 13, where “suspicious” may not be the intended word that was used.

Response 3

We appreciate the reviewer’s comment. We truly apologize for our terrible error. We replaced the word “suspicious” by “susceptible” in our revised manuscript.