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RESPONSE TO LETTER TO THE EDITOR - “Critical assessment of putative endothelial progenitor phenotypes” by Gian Paolo Fadini, et al

Jamie Case, PhD, **Laura S. Haneline, MD**, **Mervin C. Yoder, MD**, and **David A. Ingram, MD**
Department of Pediatrics, Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, Indiana, USA

Dear Editor

We appreciate the comments by Fadini and colleagues concerning our recent manuscript (Exp Hematol. 2007;35:1109–18). It is important to re-emphasize that the major focus of our study was to phenotypically and functionally define the **clonal progeny** of $CD34^+CD133^+VEGFR\text{-}2^+$ cells since this had not been previously determined utilizing both hematopoietic and endothelial cell assays. This is a critical issue in endothelial progenitor cell (EPC) biology since this cell population has been shown to inversely correlate with cardiovascular disease risk in human patients [1–3]. However, the identity of this cell type remained unclear, which confounds the mechanism by which these cells may promote new vessel growth *in vivo*.

Fadini et al were concerned about the indirect staining method used for identification of the VEGFR-2 receptor (KDR) on the cells isolated from various human sources in our study. In our hands, we have shown that the indirect staining method is more accurate than the direct staining method given the specificity of the antibodies that are available to detect the KDR receptor. Given their concerns, however, we did confirm cell surface expression of KDR by RT-PCR analysis. Furthermore, a very stringent gating strategy was utilized for the identification of $CD34^+CD133^+VEGFR\text{-}2^+$ cells. Therefore, we believe that the increased levels of circulating $CD34^+CD133^+VEGFR\text{-}2^+$ cells in our study, compared to prior reports, were not due to “some aspecific signal” as pointed out by Fadini et al. In fact, when $VEGFR\text{-}2^+$ cells were back gated on and plotted on a forward and side scatter dotplot, the cells clearly fall within the original gated region of interest. Again, expression of the VEGFR-2 antigen on cells isolated within the stringently defined gates was confirmed by RT-PCR.

Fadini et al also raised concerns about “culturing rare cells sorted according to a restrictive phenotype” in the absence of other cells, which may be important in facilitating their clonal outgrowth. Though not shown in the published manuscript, we indeed performed multiple co-culture experiments with various mononuclear cell (MNC) populations and $CD34^+CD133^+VEGFR\text{-}2^+$ cells. However, $CD34^+CD133^+VEGFR\text{-}2^+$ cells consistently

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Corresponding Author: David A. Ingram, MD, Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, 1044 West Walnut Street, R4-470, Indianapolis, Indiana, 46202., dingram@iupui.edu.

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yielded hematopoietic but not endothelial cell colonies, which is consistent with their hematopoietic cell identity. We chose not to report this data since we provided other results to clearly show that $CD34^+CD133^+VEGFR\text{-}2^+$ cells are hematopoietic cells, which express the universal hematopoietic cell surface antigen CD45. Therefore, we were not surprised that co-culture of $CD34^+CD133^+VEGFR\text{-}2^+$ cells with MNCs did not yield endothelial cell colonies.

Concern by Fadini et al was also raised that $CD34^+VEGFR\text{-}2^+$ were not isolated and analyzed on our study. While the major focus of the manuscript was to analyze the clonal progeny of $CD34^+CD133^+VEGFR\text{-}2^+$ cells since this cell has been established by numerous groups as the most primitive EPC precursor [4–6], we did also plate $CD34^+VEGFR\text{-}2^+$ cells in hematopoietic and EPC assays for the reasons stated by Fadini et al. Not surprisingly, these cells also yielded hematopoietic cell colonies at nearly the same frequency as plated $CD34^+CD133^+VEGFR\text{-}2^+$ cells but did not form a single endothelial cell colony in 5 different independent experiments. Consistent with a hematopoietic cell phenotype, $CD34^+VEGFR\text{-}2^+$ cells uniformly expressed the CD45 antigen.

Fadini et al also state that “we don’t know how and why circulating cells develop an endothelial cell phenotype in CFU-EC and ECFC conditions.” Actually, this information is known. In a recent study, Rohde et al clearly show that outgrowth of CFU-ECs from MNCs requires a combination of purified T-cells and monocytes [7]. In addition, our group recently demonstrated that ECFCs are derived from a single cell under defined culture conditions. In fact, the ECFCs give rise to only endothelial cells when plated at the single cell level [8–9].

Furthermore, Fadini et al were also concerned that $CD34^+CD133^+VEGFR\text{-}2^+$ cells were not tested for their angiogenic potential utilizing *in vivo* assays. We previously demonstrated that the ability of putative EPCs to form capillary-like structures in Matrigel predicts their ability to from vessels *in vivo* in a vasculogenesis assay recently developed by our group and others [8, 10]. When the $CD34^+CD133^+VEGFR\text{-}2^+$ cells were plated in a Matrigel assay, no capillary-like formations were observed, which again is not surprising since these cells are hematopoeitic cells. Therefore, since these cells did not form capillary-like structures *in vivo*, they were not tested in the vasculogenesis assays. Of note, $CD34^+VEGFR\text{-}2^+$ cells also did not form capillary-like structures *in vitro,* which is consistent with their hematopoietic cell identity. However, our studies do not rule out the possibility that $CD34^+CD133^+VEGFR\text{-}2^+$ and/or $CD34^+VEGFR\text{-}2^+$ cells secrete angiogenic factors when injected *in vivo* to facilitate angiogenesis and arteriogenesis.

Finally, we agree with Fadini and colleagues that circulating levels of $CD34^+$ and $CD34^+VEGFR\text{-}2^+$ cell counts do correlate closely with a decrease risk of developing various cardiovascular diseases. However, in our opinion, there is no convincing data that $CD34^+CD133^+VEGFR\text{-}2^+$ or $CD34^+VEGFR\text{-}2^+$ cells differentiate into endothelial cells, either *in vitro* or *in vivo,* to form new blood vessels. Given our study, it is most likely that these cells are hematopoietic cells, which promote new blood vessel growth by paracrine effects. This is indeed a significant step forward since the original work of Asahara et al, which identified a cell type that promotes new blood vessel growth [4]. The determination of the hematopoietic identity of these cells clearly changes the interpretation of prior studies, which claim that these cells become endothelium in new blood vessels *in vivo*. Future studies should now be focused on how $CD34^+CD133^+VEGFR\text{-}2^+$ or $CD34^+VEGFR\text{-}2^+$ cells promote angiogenesis or arteriogenesis by paracrine affects. These studies would not only broaden our understanding of the molecular mechanism by which these cells correlate with cardiovascular disease risk, but also provide a better rationale for using these cells as therapies in human patients with cardiovascular disease.

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