In 1997, Asahara, Isner, and colleagues startled the vascular biology community by reporting that progenitor cells capable of differentiating into vascular endothelium are present in the circulation of adults and that these cells may contribute to the formation of new blood vessels at sites of injury. These findings challenged the then-established view that although endothelial progenitor cells (EPCs or angioblasts) contribute to de novo vessel formation (vasculogenesis) in the embryo, new blood vessels in the adult can only arise by outgrowth of mature endothelial cells from existing blood vessels (angiogenesis). In this new paradigm, it was proposed that EPCs develop and reside in the bone marrow until various forms of tissue injury or stress lead to their release into the circulation (where they could be isolated and enumerated) and then recruitment into sites of repair. A veritable cottage industry ensued correlating the frequency of circulating EPCs with the activity of different diseases. PubMed lists over 1800 publications describing circulating EPCs in the 15 years since the initial Asahara et al report and over 500 of these use flow cytometry (often referred to as FACS or fluorescence activated cell sorting even if sorting is not actually performed) to enumerate EPCs. In 2012, we are aware that there are several problems with such studies. First, many cells labeled as EPCs are actually bone marrow-derived leukocytes that can promote angiogenesis in vivo and can acquire endothelial cell markers when cultured with VEGF in vitro but cannot differentiate into true, blood vessel-forming endothelial cells. Second, it is controversial as to which markers, if any, can be used by flow cytometry to uniquely identify EPCs. So what have all (or at least most) of these groups actually been measuring? Ingram, Yoder, and colleagues previously defined a population of “late outgrowth cells” that form colonies of cells in vitro that express endothelial but not leukocyte markers. Significantly, these colonies can form human endothelial cell-lined blood vessels when implanted into immunodeficient mice, proving that the progenitor of these colonies is a true EPC, whereas other purported EPC populations uniformly fail in this assay.

Although the data are compelling that late outgrowth colonies are derived from true EPCs, this approach cannot directly detect the circulating EPC itself but rather relies on characterizing its progeny. In the current issue of this journal, Mund and coworkers present a primer in how to properly perform flow cytometry in 2012 (largely based on the work of Leonard Herzenberg and colleagues) and apply this methodology to the identification of circulating EPCs. There are several key points made by this study. First, one really must take advantage of multiple markers, something easily done with modern flow cytometers capable of detecting antibodies conjugated to 8 or more different fluoros. However, in order to do this properly, one must appropriately compensate for the staining seen with each individual fluorescently conjugated antibody in all of the other detection channels by analyzing samples in which all antibodies except the one being tested are used to stain the cell population (a process called “fluorescence minus one” gating). Few prior studies in vascular biology have performed proper compensation. Second, one must analyze low intensity staining regions using a linear scale, reserving log scales for brightly staining markers. Otherwise, important information is lost near the zero axis because low intensity signals are too compressed to be resolved. Again, this analytic technique is rarely used in vascular biology studies. Third, one must actually sort and collect the populations of interest to make sure they are what one thinks. Mund and coworkers show that some cell populations that contain EPCs by positive markers are contaminated by erythrocytes and monocytes as well as by un nucleated, subcellular microparticles derived from endothelial cells or platelets. One must actively gate out these false signals by positive staining for erythrocyte or monocyte markers instead of relying on light scattering as well as by using positive nDNA staining with DAPI, a feature lacking in microparticles. Fourth, one can improve the enumeration of rare cells like EPCs by efficient positive (with anti-CD31 or anti-CD146) and negative (with anti-CD45) selection of initial CD34 and AC133 (found on hematopoietic progenitor cells and often in the past used to [mis]identify EPCs). Disappointingly, this “final” population contains both the EPCs as well as mature circulating endothelial cells (CECs). Although these two cell types can be differentiated by the extensive (“clonogenic”) growth potential of EPCs in vitro, a characteristic that is lacking in CECs. for now, there is no convincing way to differentiate EPCs from CECs by flow cytometry alone. EPCs are rare, but interestingly, so are CECs once microparticles are gated out. Finally, what mes-

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Arterioscler Thromb Vase Biol is available at http://atvb.ahajournals.org
DOI: 10.1161/ATVBAHA.112.246280

837
sages does this technical tour de force bring to the vascular biology community? First, it will be necessary to re-examine past conclusions about EPC frequencies in the circulation in different patient populations as almost all of the literature in the past 15 years has misidentified the cells that have been enumerated. (The same may be true for CECs.) Second, even with optimal use of the modern flow cytometer, it still is not possible to accurately identify and enumerate circulating EPCs by flow cytometry alone; EPCs are currently indistinguishable from mature CECs except for their proliferative capacity. Both of these conclusions should be of significant interest to those who follow this field and should inform future research.

Acknowledgments
Jordan S. Pober is supported by NIH grants R01-HL36003 and R01-HL051014.

References

Key Words: imaging agents
Just the FACS or Stalking the Elusive Circulating Endothelial Progenitor Cell
Jordan S. Pober

doi: 10.1161/ATVBAHA.112.246280
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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