Third, what are the MNX1 target genes that mediate its leukemogenic potential? In a previous study, the same group identified binding to and regulation of the prostaglandin E receptor 2 (PTGER2) by MNX1 overexpressed in the human HL60 AML cell line.[24] However, critical targets might significantly differ in a context of fetal liver HSPC.

Finally, and most importantly, it needs to be shown whether high expression of MNX1 is critical to maintain a transformed phenotype. Knockdown or genome editing experiments in primary human AML cells (e.g. expanded in immune deficient mice) or conditional expression in transgenic mouse models may show the way. Further exploration of the MNX1 interacting proteome could provide some clues as how to develop strategies for targeted therapeutic intervention.

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References

1. Beverloo HB, Panagopoulos I, Isaksson M, et al. Fusion of the homeobox gene HLXB9 and the ETV6 gene in infant acute myeloid leukemias with the t(7;12)(q36;p13). Cancer Res. 2001;61(14):5374-5377.
2. Tosi S, Harbott J, Teigler-Schlegel A, et al. t(7;12)(q36;p13), a new recurrent translocation involving ETV6 in infant leukemia. Genes Chromosomes Cancer. 2000;29(4):325-332.
3. Slater RM, von Drunen E, Kroes WG, et al. t(7;12)(q36;p13) and t(7;12)(q32;p13)--translocations involving ETV6 in children 18 months of age or younger with myeloid disorders. Leukemia. 2001;15(6):915-920.
4. von Bergh AR, van Drunen E, van Wering ER, et al. High incidence of t(7;12)(q36;p13) in infant AML but not in infant ALL, with a dismal outcome and ectopic expression of HLXB9. Genes Chromosomes Cancer. 2006;45(8):731-739.
5. Ballabio E, Cantarella CD, Federico C, et al. Ectopic expression of the HLXB9 gene is associated with an altered nuclear position in t(7;12) leukaemias. Leukemia. 2009;23(6):1179-1182.
6. Espersen ADL, Noren-Nystrom U, Abrahamsson J, et al. Acute myeloid leukemia (AML) with t(7;12)(q36;p13) is associated with infancy and trisomy 19: Data from Nordic Society for Pediatric Hematology and Oncology (NOPHO-AML) and review of the literature. Genes Chromosomes Cancer. 2018;57(7):359-365.
7. Nagel S, Kaufmann M, Scherr M, Drexler HG, MacLeod RA. Activation of HLXB9 by juxtaposition with MYB via formation of t(6;7)(q23;q36) in an AML-M4 cell line (GDM-1). Genes Chromosomes Cancer. 2005;42(2):170-178.
8. Ross AJ, Ruiz-Perez V, Wang Y, et al. A homeobox gene, HLXB9, is the major locus for dominantly inherited sacral agenesis. Nat Genet. 1998;20(4):358-361.
9. Harrison KA, Thaler J, Pfaff SL, Gu H, Kehrl JH. Pancreas dorsal lobe agenesis and abnormal islets of Langerhans in Hlxb9-deficient mice. Nat Genet. 1999;23(1):71-75.
10. Li H, Arber S, Jessell TM, Edlund H. Selective agenesis of the dorsal pancreas in mice lacking homeobox gene Hlxb9. Nat Genet. 1999;23(1):67-70.
11. Thaler J, Harrison K, Sharma K, Lettieri K, Kehrl J, Pfaff SL. Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9. Neuron. 1999;23(4):675-687.
12. Flanagan SE, De Franco E, Lango Allen H, et al. Analysis of transcription factors key for mouse pancreatic development establishes NKX2-2 and MNX1 mutations as causes of neonatal diabetes in man. Cell Metab. 2014;19(1):146-154.
13. Desai SS, Modali SD, Parekh VI, Kebebew E, Agarwal SK. GSK-3beta protein phosphorylates and stabilizes HLXB9 protein in insulinoma cells to form a targetable mechanism of controlling insulinoma cell proliferation. J Biol Chem. 2014;289(9):5386-5398.
14. Zhang L, Wang J, Wang Y, et al. MNX1 Is Oncogenically Upregulated in African-American Prostate Cancer. Cancer Res. 2016;76(21):6290-6298.
15. Ingenhag D, Reister S, Auer F, et al. The homeobox transcription factor HB9 induces senescence and blocks differentiation in hematopoietic stem and progenitor cells. Haematologica 2019;104(1):35-46.
16. Prieur A, Peeper DS. Cellular senescence in vivo: a barrier to tumorigenesis. Curr Opin Cell Biol. 2008;20(2):150-155.
17. Viale A, De Franco F, Orleth A, et al. Cell-cycle restriction limits DNA damage and maintains self-renewal of leukaemia stem cells. Nature. 2009;457(7225):51-56.
18. Wildenhain S, Ruckert C, Rottgers S, et al. Expression of cell-cell interacting genes distinguishes HLXB9/TEL from MLL-positive childhood acute myeloid leukemia. Leukemia. 2010;24(9):1657-1660.
19. Bolouri H, Farrar JE, Triche T Jr, et al. The molecular landscape of pediatric acute myeloid leukemia reveals recurrent structural alterations and age-specific mutational interactions. Nat Med. 2018;24(1):103-112.
20. Johansson B, Billstrom R, Mauritzson N, Mitelman F. Trisomy 19 as the sole chromosomal anomaly in hematologic neoplasms. Cancer Genet Cytogenet. 1994;74(1):62-65.
21. Dastugue N, Lafage-Pochitaloff M, Pages MP, et al. Cytogenetic profile of childhood and adult megakaryoblastic leukemia (M7): a study of the Groupe Francais de Cytogenetique Hematologique (GFCH). Blood. 2002;100(2):618-626.
22. Dang J, Nance S, Ma J, et al. AMKL chimeric transcription factors are potent inducers of leukemia. Leukemia. 2017;31(10):2228-2234.
23. Lebert-Ghali CE, Neault M, Fournier M, et al. Generation of a novel mouse model recapitulating features of human acute megakaryoblastic leukemia. Exp Hematol. 2018;64:S79.
24. Wildenhain S, Ingenhag D, Ruckert C, et al. Homeobox protein HB9 binds to the prostaglandin E receptor 2 promoter and inhibits intracellular cAMP mobilization in leukemic cells. J Biol Chem. 2012;287(48):40703-40712.

New insights into the causes of thrombotic events in patients with myeloproliferative neoplasms raise the possibility of novel therapeutic approaches

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The Philadelphia chromosome-negative myeloproliferative neoplasms (MPN) include polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (MF). This group of clonal hematological malignancies is associated with a protracted clinical course frequently punctuated by thrombotic events. Such thrombotic events have been previously attributed to excessive numbers of functionally abnormal red cells, platelets and leukocytes. MPN patients are not only at a high risk of developing arterial and venous thromboses, but also throm-

boses at unusual sites including the hepatic, portal and splenic veins, the cerebral sinuses and the mesenteric arteries. The mechanism(s) underlying this pro-thrombotic tendency in MPN are incompletely understood and have been the subject of speculation for almost seven decades. Over the last 24 months, several reports have appeared which have shed new light on the mechanisms underlying this thrombotic tendency. They implicate a pro-inflammatory MPN milieu as well as interactions between excessive numbers of qualitatively abnormal blood cells and the vessel endothelium in the generation of these thrombotic events (Figure 1). In this issue of *Haematologica*, Guy *et al.*[1] demonstrate a role for integrins in the development of thromboses using endothelial cells (EC) engineered to overexpress the MPN driver mutation JAK2^{V617F} *in vivo* and *in vitro.*

Our initial understanding of the MPN pro-thrombotic state was largely influenced by the seminal observations of Pearson and Wetherley-Mein.[2] They demonstrated that the incidence of thrombotic events in PV patients was directly related to the degree of hematocrit elevation. Red cells are the primary determinant of blood viscosity, which increases non-linearly with increasing hematocrit levels at both arterial and venous shear rates. Numerous studies have also suggested that increased red cell numbers increase the margination of platelets along the vessel walls. Recently, Walton *et al.*,[3] using a transfusion-based polycythemia model in healthy mice, showed that polycythemic mice had accelerated rates of arterial thrombus formation and shortened clotting times due to a platelet-dependent increase in thrombus formation. Their data collectively reflect the manner in which red cells independently promote the development of arterial but not venous thrombosis. Klatt *et al.*,[4] however, provided further data indicating that red cells trigger additional events beyond biophysical interactions that accelerate venous thrombosis. They showed that platelet/red cell interactions lead to increased platelet FAS ligand (FASL) exposure which then activates the death receptor (FASR) present on red cells. This ligand/receptor interaction ultimately results in further externalization of red cell phosphatidylserine which promotes the assembly of coagulation factor complexes leading to thrombin generation and the formation of occlusive thrombi. Klatt *et al.* reported that these events could occur on a collagen surface with low shear rates which resembles a venous system. The consequences of excessive numbers of red cells in MPN patients was validated by Marchioli *et al.*[5] who showed that sustained normalization of hematocrit levels (<45%) in high-risk PV patients was associated with reduced numbers of thrombotic events. Furthermore, Alvarez-Larran *et al.*[6] demonstrated that PV patients with higher phlebotomy requirements were at the highest risk of developing thrombotic events. However, several lines of evidence strongly suggest that additional mechanisms beyond hematocrit elevation are required to explain a number of observed clinical manifestations including: (i) the occurrence of thrombotic events in over a third of patients prior to the diagnosis of PV; (ii) the occurrence of splanchnic vein thromboses, frequently in patients with a JAK2^{V617F} mutation with normal blood counts; (iii) the increased incidence of thrombotic events in normal individuals found to have clonal hematopoiesis of indeterminate potential with a JAK2 mutation; (iv) the persistent rate of thrombosis following normalization of the hematocrit in PV patients; and (v) the increased rate of thrombosis in ET and MF patients without polycythemia. Intuitively, physicians have linked MPN-associated thrombocytosis to the high incidence of thrombotic events, however, the thrombotic risk in ET patients does not seem to be related to the degree of thrombocytosis[7] and those patients with extreme degrees of thrombocytosis (>1.5 million) are ironically at a higher risk of bleeding rather than clotting due to the development of a secondary form of von Willebrand disease.

The conclusion that additional factors beyond excessive numbers of blood cells contribute to the MPN pro-thrombotic tendency was bolstered by the more recent observation that patients with a JAK2^{V617F} mutation, particularly those individuals with a high variant allele burden, were at a greater risk of developing thrombotic events than those with calreticulin mutations.[8] Several groups have provided evidence that mutated JAK2 might affect not only hematopoietic cells but also EC, which raises the possibility that MPN might actually arise in some patients in a primitive cell that resembles the hemogenic endothelium.[9] In this issue of *Haematologica* Guy *et al.*[1] report the construction of several murine models which can be used to evaluate the contribution of EC to the MPN pro-thrombotic state. They demonstrate that mice that were genetically engineered to express JAK2^{V617F} in EC but not hematopoietic cells had a predilection to develop thrombotic events in spite of having normal blood counts and normal rates of thrombin generation. Importantly, this thrombotic tendency was accentuated by the creation of a pro-inflammatory milieu through the administration of low doses of tumor necrosis factor alpha. Using both *in vitro* and *in vivo* approaches they next showed that JAK2^{V617F+} human and murine EC were capable of promoting both leukocyte rolling and adhesion. Although the most common integrins associated with leukocyte adhesion to EC were not upregulated in these mutated EC, Guy *et al.* did demonstrate increased surface expression of P-selectin (CD62P) and von Willebrand factor (VWF), both of which are contained within Weibel-Palade bodies in EC. Importantly the pro-adhesive properties of the JAK2^{V617F+} EC were reversed by treatment with either a P-selectin blocking antibody or hydroxyurea, a drug that remains the standard of care for treating high-risk PV and ET patients. The authors concluded that hydroxyurea did not block the effects of P-selectin but rather decreased the release of P-selectin and VWF from Weibel-Palade bodies. The upregulation of P-selectin by mutated EC was attributed to increased STAT3 phosphorylation which is a downstream event of JAK/STAT signaling. Importantly, earlier this year, Guadall *et al.*[10] generated data that supported the findings of Guy *et al*. using a totally different experimental system. They developed wild-type and JAK2^{V617F+} EC from immortalized human pluripotent stem cells and showed that JAK2^{V617F+} EC promoted the adherence of leukocytes and were characterized by increase phosphorylation of STAT3 and overexpression of both VWF and P-selectin. The availability of large numbers of JAK2^{V617F+} human EC from immortalized human pluripotent stem cells allowed these investigators to document gene expression analyses, demonstrating increased expression of genes associated with inflammation and cell adhesion in JAK2^{V617F+} human EC. P-selectin has been previously implicated by the

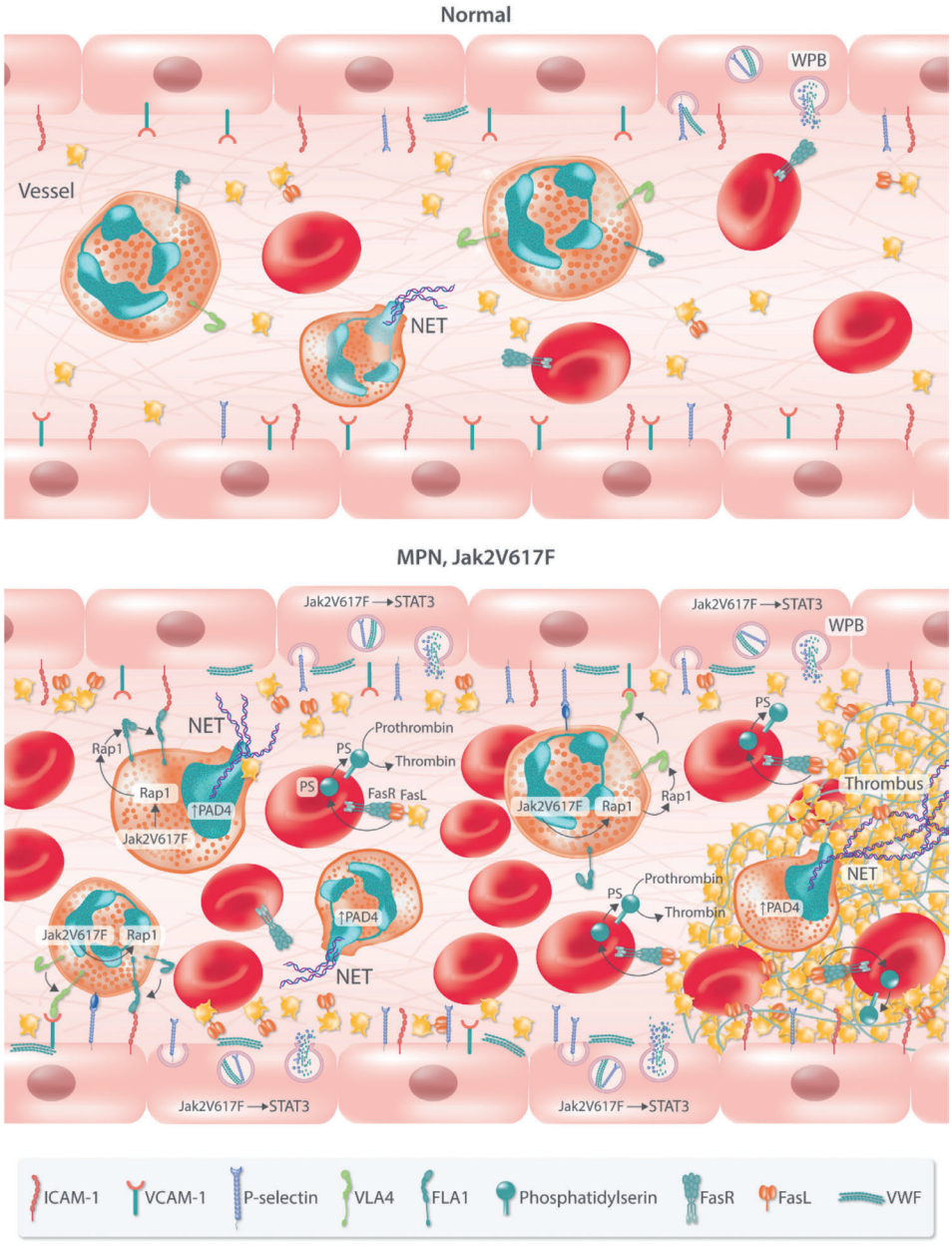

Figure 1. The mechanism of thrombus formation in myeloproliferative neoplasm. The Jak2V617F mutation causes an increase in endothelial cell (EC) Weibel-Palade body (WPB) degranulation of P-selectin and von Willebrand factor (VWF); translocation of Rap1 towards the cell membrane with activation of the integrins LFA1 and VLA4; and increased neutrophil extracellular trap (NET) formation. In addition, a red blood cell-platelet interaction through FasL/FasR causes externalization of phosphatidylserine (PS). All of these events play a role in thrombus formation. Rap1: Ras-related protein 1; LFA1: lymphocyte function-associated antigen 1; STAT: signal transducer and activator of transcription; PAD4: peptidyl arginine deimidase 4; ICAM-1: intracellular adhesion molecule 1; VCAM-1: vascular cell adhesion molecule 1; VLA4: very late antigen-4: FasL: Fas ligand.

Migliaccio Laboratory to play a critical role in the development not only of thrombosis but also progression to myelofibrosis in a GATA1^{low} mouse model.[11] In this animal model, abnormal localization of P-selectin in both megakaryocytes and platelets led to increased platelet/leukocyte interactions, an increased incidence of thrombotic events, and increased release of neutrophil proteases and transforming growth factor-beta which plays a critical role in the development of bone marrow fibrosis, osteosclerosis and disease progression in MPN. Importantly, the progression to MF as well as the increased frequency of thromboses in the GATA1^{low} mice was not observed in mice in which P-selectin was deleted by genetic approaches.[12,13] The role of integrins in MPN thrombosis has been further supported by the provocative work of Edelmann *et al.*[14] who showed that JAK2^{V617F+} granulocytes and monocytes were characterized by increased activation of VLA-4 and/or LFA1. These integrins are cell adhesion molecules which play an essential role in the attachment of leukocytes to EC by interacting with intracellular matrix proteins. The translocation of these two integrins to the granulocyte surface was due to the effects of mutated JAK2 on the inside–outside signaling molecule, Rap1. Most importantly these investigators demonstrated that the administration of integrin-blocking antibodies to JAK2^{V617F+} mice diminished the rate of thrombosis.

Additional evidence for the role of neutrophils in thrombosis in MPN was recently offered by Wolach *et al.*[15] with their demonstration that neutrophils from patients with JAK2^{V617F} MPN are primed to form neutrophil extracellular trap, implicated in the pathogenesis and promotion of thrombosis. Moreover, mice with conditional knock-in of

JAK2^{V617F} have an increased propensity to neutrophil extracellular trap formation and thrombosis. Inhibition of JAK-STAT signaling by ruxolitinib abrogated neutrophil extracellular trap formation and reduced thrombosis in this murine model.

The current report of Guy *et al.* as well as the other reports referred to in this Commentary each delineate the increasingly plausible role of various cell adhesion molecules (selectins and integrins) in MPN-associated thrombosis and in some cases evolution to MF. The question remains, how relevant are these observations in disease models to the pathophysiology of MPN in patients? This is especially relevant to the work dealing with JAK2^{V617F+} EC. Sozer *et al.*[16,17] previously documented that angiogenic monocytes as well as true EC were JAK2^{V617F+} in PV patients with splanchnic vein thromboses. Using laser capture microdissection they demonstrated that the EC within the hepatic veins of some PV patients with hepatic vein thrombosis were JAK2^{V617F+}. Furthermore, Rosti *et al.*[18] reported that splenic vein EC were JAK2^{V617F+} in 67% of patients with MF. To better understand the significance of these intriguing experimental findings, the frequency of JAK2^{V617F+} MPN patients with mutated EC, the extent of the distribution of these JAK2^{V617F+} EC within the vasculature of various tissues, and the relationship of these findings to the incidence of thrombosis in MPN require evaluation in larger numbers of patients. It will also be interesting to determine whether other driver mutations in MPN, such as calreticulin, share the same properties which might explain, in part, the different propensity to develop thrombosis relative to that in JAK2^{V617F}-mutated patients. The increased propensity to develop thrombosis in MPN patients is likely multifactorial in origin. An elevated hematocrit and a pro-inflammatory state, as well as a series of cellular interactions mediated by cell adhesion molecules that are expressed by red cells, platelets, leukocytes, monocytes and EC, may all play a role (Figure 1), and combinations of these events at any one time may further increase the risk of developing a thrombotic event. Most importantly, this recent round of studies provides a rationale for the evaluation of blocking antibodies to P-selectin, VLA-4 and LFA-1, which in part are already in clinical use for other conditions,[19-21] to further reduce the incidence of not only thrombotic events but also disease progression beyond that achieved with the presently available therapeutic options. The outcomes of such proposed clinical trials, which are at best presently in the planning stages, will be closely watched. Such studies will allow us to assess the importance of each of these membrane proteins in the development of life-threatening clinical events in MPN patients and are likely to increase the therapeutic options for such patients.

References

1. Guy A, Gourdou-Latyszenok V, Le Lay N, et al. Vascular endothelial cell expression of JAK2V617F is sufficient to promote a pro-thrombotic state due to increased P-selectin expression. Haematologica. 2019;104(1):70-81.
2. Pearson TC, Wetherley-Mein G. Vascular occlusive episodes and venous haematocrit in primary proliferative polycythaemia. Lancet. 1978;2(8102):1219-1222.
3. Walton BL, Lehmann M, Skorczewski T, et al. Elevated hematocrit enhances platelet accumulation following vascular injury. Blood. 2017;129(18):2537-2546.
4. Klatt C, Kruger I, Zey S, et al. Platelet-RBC interaction mediated by FasL/FasR induces procoagulant activity important for thrombosis. J Clin Invest. 2018;128(9):3906-3925.
5. Marchioli R, Finazzi G, Specchia G, et al. Cardiovascular events and intensity of treatment in polycythemia vera. N Engl J Med. 2013;368(1):22-33.
6. Alvarez-Larran A, Perez-Encinas M, Ferrer-Marin F, et al. Risk of thrombosis according to need of phlebotomies in patients with polycythemia vera treated with hydroxyurea. Haematologica. 2017;102(1):103-109.
7. Vannucchi AM, Barbui T. Thrombocytosis and thrombosis. Hematology Am Soc Hematol Educ Program. 2007:363-370.
8. Falchi L, Kantarjian HM, Verstovsek S. Assessing the thrombotic risk of patients with essential thrombocythemia in the genomic era. Leukemia. 2017;31(9):1845-1854.
9. Pereira CF, Chang B, Gomes A, et al. Hematopoietic reprogramming in vitro informs in vivo identification of hemogenic precursors to definitive hematopoietic stem cells. Dev Cell. 2016;36(5):525-539.
10. Guadall A, Lesteven E, Letort G, et al. Endothelial cells harbouring the JAK2V617F mutation display pro-adherent and pro-thrombotic features. Thromb Haemost. 2018;118(9):1586-1599.
11. Zetterberg E, Verrucci M, Martelli F, et al. Abnormal P-selectin localization during megakaryocyte development determines thrombosis in the gata1low model of myelofibrosis. Platelets. 2014;25(7):539-547.
12. Centurione L, Di Baldassarre A, Zingariello M, et al. Increased and pathologic emperipolesis of neutrophils within megakaryocytes associated with marrow fibrosis in GATA-1(low) mice. Blood. 2004;104(12):3573-3580.
13. Spangrude GJ, Lewandowski D, Martelli F, et al. P-selectin sustains extramedullary hematopoiesis in the Gata1 low model of myelofibrosis. Stem Cells. 2016;34(1):67-82.
14. Edelmann B, Gupta N, Schnoeder TM, et al. JAK2-V617F promotes venous thrombosis through beta1/beta2 integrin activation. J Clin Invest. 2018;128(10):4359-4371.
15. Wolach O, Sellar RS, Martinod K, et al. Increased neutrophil extracellular trap formation promotes thrombosis in myeloproliferative neoplasms. Sci Transl Med. 2018;10(436).
16. Sozer S, Fiel MI, Schiano T, Xu M, Mascarenhas J, Hoffman R. The presence of JAK2V617F mutation in the liver endothelial cells of patients with Budd-Chiari syndrome. Blood. 2009;113(21):5246-5249.
17. Sozer S, Ishii T, Fiel MI, et al. Human CD34+ cells are capable of generating normal and JAK2V617F positive endothelial like cells in vivo. Blood Cells Mol Dis. 2009;43(3):304-312.
18. Rosti V, Villani L, Riboni R, et al. Spleen endothelial cells from patients with myelofibrosis harbor the JAK2V617F mutation. Blood. 2013;121(2):360-368.
19. Ataga KI, Kutlar A, Kanter J, et al. Crizanlizumab for the prevention of pain crises in sickle cell disease. N Engl J Med. 2017;376(5):429-439.
20. Schwab N, Schneider-Hohendorf T, Wiendl H. Therapeutic uses of anti-alpha4-integrin (anti-VLA-4) antibodies in multiple sclerosis. Int Immunol. 2015;27(1):47-53.
21. Vincenti F, Mendez R, Pescovitz M, et al. A phase I/II randomized open-label multicenter trial of efalizumab, a humanized anti-CD11a, anti-LFA-1 in renal transplantation. Am J Transplant. 2007;7(7):1770-1777.