Endothelial Progenitor Cell Dysfunction in Myelodysplastic Syndromes: Possible Contribution of a Defective Vascular Niche to Myelodysplasia

Luciana Teofili*,3, Maurizio Martini†1,3, Eugenia Rosa Nuzzolo*, Sara Capodimonti*, Maria Grazia Iachininoto*, Alessandra Cocomazzi†, Emiliano Fabiani*, Maria Teresa Voso* and Luigi M. Larocca†

*Istituto di Ematologia, Università Cattolica del Sacro Cuore, Rome, Italy; †Istituto di Anatomia Patologica, Università Cattolica del Sacro Cuore, Rome, Italy

Abstract
We set a model to replicate the vascular bone marrow niche by using endothelial colony forming cells (ECFCs), and we used it to explore the vascular niche function in patients with low-risk myelodysplastic syndromes (MDS). Overall, we investigated 56 patients and we observed higher levels of ECFCs in MDS than in healthy controls; moreover, MDS ECFCs were found variably hypermethylated for $p15\text{INK4b}$, $DAPK1$, $CDH1$, or $SOCS1$. MDS ECFCs exhibited a marked adhesive capacity to normal mononuclear cells. When normal CD34+ cells were co-cultured with MDS ECFCs, they generated significant lower amounts of CD11b+ and CD41+ cells than in co-culture with normal ECFCs. At gene expression profile, several genes involved in cell adhesion were upregulated in MDS ECFCs, while several members of the Wingless and int (Wnt) pathways were underexpressed. Furthermore, at miRNA expression profile, MDS ECFCs hypo-expressed various miRNAs involved in Wnt pathway regulation. The addition of Wnt3A reduced the expression of intercellular cell adhesion molecule-1 on MDS ECFCs and restored the defective expression of markers of differentiation. Overall, our data demonstrate that in low-risk MDS, ECFCs exhibit various primary abnormalities, including putative MDS signatures, and suggest the possible contribution of the vascular niche dysfunction to myelodysplasia.

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Introduction
Homeostasis of hematopoietic system requires that hematopoietic stem cells (HSCs) can either rest dormant or proliferate and differentiate in response to peripheral stimuli [1]. The bone marrow (BM) environment guarantees for these definite activities by keeping HSCs embedded within specialized niches [2]. The BM niches are composed by several kinds of cells, including osteoblasts and mesenchymal and endothelial cells [3–5]. The osteoblasts govern the quiescence and maintain the pool of HSCs through specific pathways including Jagged-Notch, Tie2/angiopoietin, and transforming growth factor β/SMAD signaling [2]. During the past decade, several evidences have been gathered, showing that endothelial cells also play a critical role in hematopoiesis government [5–7]. In this study, we investigated the crosstalk between endothelial microenvironment and HSCs in myelodysplastic syndromes (MDS). These clonal disorders are characterized by an increased BM cellularity, due to excessive apoptosis, coupled with aberrant differentiation [8]. Although MDS constitute a very heterogeneous group of diseases, all of them share variable degrees of ineffective hematopoiesis and susceptibility to leukemic transformation [8]. Importantly, aberrant morphology of myelodysplastic cells mirrors specific functional defects, which significantly affects the disease course also in patients at low risk for leukemic evolution [9]. Indeed, patients with MDS are prone to serious infections...
even when the neutrophil count is apparently preserved and they can have serious bleeding episodes despite reasonable platelet counts [9]. In general, MDS patients have a transient and inadequate response to chemotherapy, probably as a result of the “dormant” status of MDS HSC within protective BM niches [10]. Therefore, it is conceivable that BM niches might contribute to the biologic demeanor of MDS. Because endothelial progenitors have been proven to contribute to the BM microvasculature, we used circulating endothelial colony forming cells (ECFCs) as a model to replicate the BM vascular niche [11,12]. We isolated ECFCs from a homogeneous group of patients diagnosed with low-risk MDS and evaluated the influence of MDS endothelium on the differentiation of normal hematopoietic cells.

Material and Methods

Patients

The study included 56 patients (25 males and 31 females, median age 69 years, range 46–90) affected by low-risk MDS according to the International Prognostic Scoring System [13]. At the time of study, patients were not receiving antiproliferative or demethylating drugs. Twenty-eight healthy blood donors (14 males and 14 females, median age 61.3 years, range 45 to 77) were used as controls. All blood samples were obtained after informed consent. The study was approved by the Institutional Ethical Committee.

Cell Cultures

ECFCs were obtained from peripheral blood of MDS patients or from healthy blood donors according to the method of Ingram et al., as previously described [14,15]. The endothelial nature of cells was confirmed by evaluating the expression of CD34, CD146, CD45, and vascular endothelial growth factor receptor by flow cytometry and reverse transcription–polymerase chain reaction (PCR) [15]. Confluent ECFCs at passages IV to VI were used for genotype profiling, hematopoietic cell co-cultures, and flow cytometry analysis. In selected experiments, Wnt3A or Wnt5A (R&D Systems, Space Import-Export srl, Milano, Italy) was added at 100 ng/ml. The influence of endothelial cells on normal hematopoietic differentiation was evaluated by co-culturing cord blood CD34+ cells over non-irradiated ECFC layers. CD34+ cells were isolated by immune-magnetic method with the purity always above 92%. Co-cultures were performed in 24 multiwell plates: 30 × 10^3 CD34+ cells per well were seeded on confluent ECFCs in Stem Span SFEM medium (Stem Cell Technology Inc., Vancouver, Canada). Different cocktails of cytokines were added to achieve the optimal commitment: 10 ng/ml each of stem cell factor (SCF), Granulocyte Macrophage - Colony Stimulating Factor, and Interleukin 3 for granulo-monocytic differentiation, 3 U/ml erythropoietin and 10 ng/ml SCF for erythroid differentiation, and 20 ng/ml Interleukin 6 and 10 ng/ml thrombopoietin for megakaryocytic differentiation (all purchased from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Every other day, 0.5 ml of supernatant was removed and replaced with fresh medium plus cytokines. CD34+ cultured without ECFCs served as controls.

Flow Cytometry

The differentiation toward different lineages was assessed by flow cytometry using a FACScanto flow cytometer (Becton-Dickinson, Franklin Lakes, NJ) equipped with a 488-nm excitation light source. The following monoclonal antibodies were used: phycoerytrin (PE)-mouse anti-human CD54, fluorescein isothiocyanate anti-human CD41a, PE anti-human CD11b, and PE anti-human CD71 (all purchased from Becton-Dickinson). Negative controls consisted of cells incubated with isotype-matched PE- or fluorescein isothiocyanate–conjugated irrelevant monoclonal antibodies. Results were expressed as percentage of positive cells. The cellular capacity of ECFCs was assessed by evaluating the percentage of carboxyfluorescein succinimidyl ester (CFSE)–labeled mononuclear cells remaining adherent to the layer of ECFC after 2-hour incubation [15]. The expression of intercellular cell adhesion molecule-1 (ICAM-1) in ECFCs was assessed with the PE anti-human CD54 (Becton-Dickinson).

Real-Time Analysis, Methylation Assay, and Gene Expression Array

Real-time analysis, methylation assay, and gene expression array are detailed in the Supplementary Materials. All used primers and product lengths are shown in Tables S1 and S2.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA) and MedCalc version 10.2.0.0 (MedCalc Software, Mariakerke, Belgium). Statistical comparison of continuous variables was performed with the Mann-Whitney U test or paired t test, as appropriate. Comparison of categorical variables was performed using chi-square statistic and the Fisher exact test. P values less than .05 were considered as statistically significant.

Results

MDS Patients Have a Higher Output of ECFCs than Controls

ECFCs were achieved in 29 of 56 MDS patients and in 17 of 28 controls (P = .491), with no differences in age (P = .356) and sex (P = .651) distribution between subjects producing or not producing ECFCs. In addition, we did not observe any clinical and biologic differences in MDS patients with ECFC isolation frequency similar to normal individuals (≤0.9/10^7 cells) and in MDS patients with ECFC isolation frequency higher than normal individuals (>0.9/10^7 cells). Among individuals with ECFCs, MDS patients had a significantly higher number of colonies than healthy controls (1.2 vs 0.2 ECFCs/10^7 cells, P < .0001, Figure 1). On the whole, the levels of ECFCs in our series of patients and the percentage of samples producing at least one ECFC are in good agreement with those reported by other authors using the same methodologies [16].

ECFCs Isolated from MDS Patients Show a Hypermethylated Phenotype

Although recent studies evidenced that in myeloproliferative neoplasms cells from the endothelial compartment harbor the disease-specific signature, i.e., the JAK2^V617F^ mutation [15,17], Della Porta et al. demonstrated that ECFCs isolated from MDS patients do not show the
chromosomal aberrations found in the neoplastic clone [16]. Because aberrant DNA methylation of CpG-rich promoters of a variety of genes is very frequent in MDS [17], we investigated in ECFCs an eventual deregulation of the epigenetic machinery. We examined in ECFC samples obtained from 20 patients the methylation status of four genes that frequently undergo hypermethylation in MDS hematopoietic cells: p15INK4b (p15), DAPK1, CDH1, and SOCS1. We found that all patients showed at least one hypermethylated gene, with 13 patients having multiple hypermethylated genes (Table 1 and Figure 2). All investigated genes are functional to various pathways of normal endothelial cells, thus excluding that hypermethylation might result from physiological gene silencing. In addition, none of 14 investigated healthy controls was found hypermethylated.

**ECFCs from MDS Patients Show Increased Adhesion to Normal Hematopoietic Cells.**

It has been recently reported that mesenchymal stromal cells (MSCs) from MDS patients have a defective expression of adhesive molecules and chemokines [18,19], and we evaluated the proficiency of ECFCs obtained from MDS patients to adhere to normal mononuclear cells in comparison with ECFCs isolated from healthy subjects. Normal mononuclear cells tracked with CFSE were incubated over ECFC layers obtained from healthy individuals and MDS patients: After 2 hours, non-adherent cells were discharged and adherent cells were recovered and counted by flow cytometry. Results obtained are shown in Figure 3. On the whole, 40 ± 4% of tracked cells adhered to MDS ECFCs, in comparison with 13 ± 1% remaining adherent to normal ECFCs (P = .005). Altogether, these observations suggest that endothelial cells from MDS patients show an “adhesion-proficient” phenotype.

**The Contact with MDS Endothelial Cells Perturbs the Expansion and Differentiation of Normal CD34+ Cells.**

Preliminarily, we observed that underlying ECFCs supported the expansion and differentiation of cord blood CD34+ cells. In fact, when CD34+ cells were seeded over normal ECFCs in erythroid, granulo-monocytic, and megakaryocytic differentiation media, we observed at day 7 a mean increase of 1.6 ± 0.1, 1.4 ± 0.1, and 2.0 ± 0.1 in total nucleated cell (TNC) number, respectively, compared to cultures performed under the same conditions but without endothelial layers. This was the result of an increase in the number of both differentiated (i.e., CD71+, CD11b+, and CD41+) and undifferentiated (i.e., CD34+) cells (Figure 4A). Accordingly, at the molecular level, we observed the harmonized up-regulation of genes related to the early phase of differentiation, including Friend of Gata (FOG), PU.1, and AML-1, and subsequently, the up-regulation of genes related to terminal differentiation, as Transferrin Receptor (TFR), Myeloperoxidase (MPO), and Glycoprotein Ib (GP Ib) (Figure 4B). Indeed, we investigated whether MDS ECFCs had the same supportive properties exerted by normal ECFCs. We found that ECFCs isolated from MDS patients were scarcely able or even at all incapable to support the expansion and differentiation of hematopoietic cells (Figure 4A). Although this defect was apparently more evident in granulocyte and megakaryocytic cultures than in erythroid cultures, the pattern of expression of lineage-related genes was deeply perturbed, clearly suggesting an imbalance between proliferation and differentiation (Figure 4B).

In particular, in granulo-monocytic and megakaryocytic cultures, lineage-related gene expression was abnormally low in both early (PU.1 and AML-1, respectively) and late (MPO and GP Ib, respectively) phases of differentiation. In contrast, in cells pushed toward the erythroid lineage, FOG and TFR reached abnormally high expression values (Figure 4B). To discovery the alterations underlying the impaired capability of MDS ECFCs to sustain hematopoiesis in vitro, we evaluated through microarray analysis their gene expression profiles in comparison with normal ECFCs. We initially focused on genes involved in a wide range of endothelial cell pathways, including adhesion, angiogenesis, cell activation, cell survival, and apoptosis (the gene set is available in the Supplementary Materials). Overall, among six significant upregulated genes, four codified for cell adhesion molecules (ICAM-1, vascular cell adhesion molecule 1 (VCAM-1), L-selectin (SELL), and von Willebrand factor (vWF)). Two additional genes found overexpressed were nitric oxide synthase and tumor necrosis factor ligand superfamily, member 10 (Table 2, upper section). In contrast, matrix metalloprotease-1 (MMP1), BCL2-like 1, and CASP2 and RIPK1 domain-containing adaptor with death domain were significantly downregulated in MDS ECFCs compared to normal controls (Table 2, upper section). Some of these genes are implicated in the regulation of blood cell production. For example, nitric oxide delivery not only induces megakaryocyte apoptosis and platelet formation but also controls HSC production in embryonic and adult lives [20–22]. Likewise, MMP1 activates the hypoxia-inducible factor-1 pathway within niche cells, thereby inducing the transcription of hypoxia-inducible factor-responsive genes and terminal hematopoietic differentiation [23].

To explain our overall findings, we next examined additional pathways with putative effects on cell adhesion. Because the Wingless and int (Wnt) pathway is one of the main regulators of cell-to-cell interactions in hematopoietic tissues [24–26], we evaluated the expression profile of genes involved in the canonical and non-canonical Wnt pathways (gene sets, including ligands, receptors, and target of Wnt are available in the Supplementary Materials). We found that Wnt canonical signaling was significantly downregulated at multiple levels in MDS ECFCs (Table 2, middle section). Actually, MDS cells contained low RNA levels of several Wnt ligands (Wnt3A, Wnt5A, and Wnt5B), and several Wnt target genes were hence downregulated, including the transcriptional co-repressors amino-terminal enhancer of split and C-terminal binding protein 1, cyclin D1, and inhibitor of differentiation 2 [27]. On the contrary, the bone morphogenetic protein-4 (BMP4) and follistatin (FST), which are subjected to regulatory interactions with Wnt, were upregulated in MDS ECFCs in comparison with normal ECFCs (Table 2, middle section). Interestingly, both genes have specific activities in human hematopoiesis. In particular, BMP4 is a powerful inducer of erythroid differentiation in stress erythropoiesis and acts in concert with SCF and hypoxia to promote the proliferation and differentiation of erythroid progenitors in response to acute anemia [28]. The increased

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**Table 1. Incidence of Aberrant DNA Methylation of CpG-Rich Promoters of p15INK4b, CDH1, DAPK1, and SOCS1 genes in ECFCs Obtained from 20 Patients with MDS and 14 Controls**

| Gene          | Controls (n = 14) | MDS Patients (n = 20) | P   |
|---------------|-------------------|-----------------------|-----|
|               | Methylated | Unmethylated | Methylated | Unmethylated |
| p15INK4b      | 0          | 14         | 8          | 12          | .01  |
| CDH1          | 0          | 14         | 7          | 13          | .026 |
| DAPK1         | 0          | 14         | 3          | 17          | ns   |
| SOCS1         | 0          | 14         | 4          | 16          | ns   |
| One of the above | 0/14     | 20/20     | ns         | <.0001      |
expression of BMP4 can partly explain why MDS ECFCs forced the expression of FOG and TFR during erythroid differentiation of normal progenitors. In contrast, FST regulates the hematopoietic cell adhesiveness to fibronectin, favoring the expansion of immature progenitors [29]. We hypothesized that the extensive hypo-expression of several Wnt genes could depend on the aberrant hypermethylation of their promoters. Nevertheless, our search for methylation of the Wnt5A gene promoter region was unsuccessful (data not shown). We then investigated the expression profile of a set of 84 miRNAs with predictable effects on the Wnt pathway. Actually, we demonstrated that, in comparison with normal ECFCs, MDS ECFCs contained significantly lower amounts of miR-142-3p, miR-424-5p, and miR-196b-5p (Table 2, lower section). Whereas all these miRNAs are variously involved in hematopoiesis [30,31] or angiogenesis [32], the low levels of miR-142-3p were particularly interesting, because it has been demonstrated to modulate the Wnt pathway to balance proliferation and differentiation of mesenchymal cells [33]. Interestingly, miR-142-3p was predicted to inhibit the expression of BMP4 at both “Ingenuity” and “microRNA.org-Target and Expression” software.

The Addition of Soluble Wnt3A Modifies the Adhesive Profile of MDS ECFCs

Overall, the analysis of gene expression profiles suggested that MDS endothelial progenitor cells show a defective expression of several Wnt pathway constituents. Previous studies demonstrated that canonical Wnt pathway signaling regulates hematopoietic homeostasis by shaping the niches supportive of hematopoietic stem/progenitor cells [25]. In particular, Wnt signaling modulates VCAM-1 expression by marrow stromal cells and controls the production of extracellular matrix components [34]. Therefore, our model suggests that the increased adhesiveness of MDS ECFCs may result from the ineffective Wnt signaling and that the aberrant attachment of hematopoietic cells to the endothelial environment could interfere with their normal differentiation programs. To test this hypothesis, we investigated whether the addition of 100 ng/ml of soluble Wnt3A and/or Wnt5A to ECFC cultures could influence the abnormally high RNA expression of ICAM-1, VCAM-1, vWF, and SEL. We observed that ICAM-1 RNA expression was the most significantly affected: either Wnt3A or Wnt5A was able to reduce ICAM-1 expression, but Wnt3A induced a more sustained and deep decrease (Figure 5A). These findings were further confirmed by flow cytometry. In particular, we found a significant reduction of ICAM-1 expression in MDS ECFC but not in normal ECFC after Wnt3A addition (Figure 5, B and C). In addition, we investigated

![Figure 2. Methylation-specific PCR of p15INK4b (p15), DAPK1, CDH1, and SOCS1 genes in ECFCs isolated from MDS patients: representative results of ECFCs isolated from four patients. UM indicates unmethylated gene; M indicates methylated gene; NC indicates negative control; PC indicates positive control for unmethylated and methylated DNA; MW indicates the molecular weight markers. Other abbreviations are detailed in the text.](image)

![Figure 3. CFSE-labeled mononuclear cell adhesion to normal or MDS ECFCs. (A) Percentages of mononuclear cells adherent to ECFCs obtained from six healthy controls and from six MDS patients. (B) Representative experiment showing on the left the cytofluorimetric analysis of unstained peripheral blood mononuclear cells (negative control) and on the right the number of CFSE-labeled mononuclear cells bound by ECFC in a healthy control (upper panels) and in an MDS patient (lower panels). Abbreviations are detailed in the text.](image)
the effect of Wnt3A addition on the capacity of MDS ECFCs to support hematopoietic differentiation. We found that the expression pattern of differentiation markers was positively affected by the Wnt3A addition, with a significant decrease in abnormally high TRF RNA values and a significant increase in both MPO and GPIb RNA expression (Figure 5D).

**Discussion**

This study demonstrates that endothelial progenitor cells isolated from patients with low-risk MDS have an increased adhesion capacity and fail to adequately sustain hematopoiesis, particularly in myeloid and megakaryocyte differentiation. Although the increased level of ECFCs in these patients has been previously reported [16], our findings suggest that the molecular defects underlying MDS endothelial progenitor dysfunctions are likely primary in their nature, since these cells differ from their normal counterparts in both genetic and epigenetic profiles. Since the transplant of allogeneic endothelial progenitor cells in myelosuppressed mice shortens the duration of aplasia and fosters blood cell recovery, it is conceivable that circulating ECFCs normally contribute to the BM
Gene Fold Regulation P

ICAM-1 14.06 .012
Constitutive nitric oxide synthase 3 13.17 .015
SELL 7.26 .041
Tumor necrosis factor ligand superfamily, member 10 11.08 .019
VCAM-1 5.46 .008
eWF 6.86 .001
BCL2-like 1 − 8.51 .006
CASP2 and RIPK1 domain containing adaptor with death domain − 4.92 .032
MMP1 − 4.78 .002
FST 5.38 .043
BMP4 4.85 .001
Amino-terminal enhancer of split − 4.48 .001
Cyclin D1 − 5.59 .004
C-terminal binding protein 1 − 4.32 .003
Inducible of differentiation 2 − 4.96 .024
Wnt3A − 4.54 .010
Wnt5A − 4.51 .021
Wnt5B − 83.51 .031
miR-142-3p − 6.99 .003
miR-424-5p − 4.33 .001
miR-196b-5p − 9.89 .033

The microenvironment participation in determining clinical and biologic features of MDS has been extensively investigated. Tennant et al. first showed that the adherent layers obtained from BM of MDS patients were significantly defective in supporting hematopoiesis in long-term Dexter-type cultures [41]. Tauro et al. observed heterogeneous abnormalities of MDS stromal cells including altered matrix molecule expression and changes in superoxide production, possibly contributing to the abnormal survival and development of hematopoietic cells [42]. Moreover, BM stromal cells of MDS patients exhibit abnormal production of several cytokines contributing to the impaired immune function observed in these patients [43]. More recently, Ferrer at al. showed that primary MSCs obtained from MDS patients express low levels of adhesion and cell surface molecules and, when co-cultured with CD34+ cells from healthy donors, produce lower numbers of cobblestone area–forming cells and fewer colony-forming units [18]. Similarly, Geyh et al. found that MSCs from patients with all MDS subtypes exhibit impaired growth and osteogenic differentiation, associated with specific methylation patterns and altered expression of several molecules involved in the interaction with HSCs, including osteopontin, Jagged1, Kit-ligand, and angiopoietin as well as several chemokines [19]. Finally, Pavlaki et al. observed that MSCs obtained from low- to intermediate-risk patients displayed downregulated canonical Wnt pathway due to up-regulation of inhibitors [44]. When pharmacologically activated, Wnt signaling led to an increased cell proliferation and restored their osteogenic differentiation ability [44]. The findings obtained in our model suggest for the first time that specific primitive perturbations could occur also at the level of the MDS vascular niche. Primitive abnormalities of endothelial cells or endothelial cell progenitors have been previously demonstrated in chronic myeloproliferative neoplasms. In these conditions, the “diseased vascular niche” facilitates clonal stem cell proliferation, favors extramedullary hematopoiesis through mobilization and homing of neoplastic HSCs in new niches in the spleen, and contributes to vascular complications [15,45,46]. In contrast, in MDS patients, endothelial niche dysfunctions could sustain BM hypercellularity embedded in dense microvasculature scaffolds. In addition, the pathologic involvement of the circulating endothelial compartment might be implicated in the association between MDS with systemic vasculitis [47] or in thrombotic proficieny of MDS patients subjected to specific therapies despite concomitant thrombocytopenia [48].

The canonical and non-canonical Wnt pathways consist of a complex network of interrelated and reciprocally connected signals [24–26]. Previous studies carried out in MDS hematopoietic cells reported hypermethylation of several Wnt antagonists, contributing to the activation of the Wnt pathway and the expression of β-catenin [49,50]. In these patients, a correlation between risk of leukemic transformation and nuclear β-catenin expression or Wnt antagonist hypermethylation was noticed, confirming the linkage between Wnt pathway activation and leukemogenesis [49,50]. Our observations, in contrast, suggest that the defective function of the Wnt pathway in vascular niches could mainly affect the physiological differentiation processes of hematopoietic cells, resulting in the myelodysplasia observed also in patients at low risk for leukemic transformation. Accordingly, harvest of immature progenitors (i.e., CD34+ cells) in cultures supported by MDS endothelial cells was not so different from that obtained in the presence of normal endothelial cells. Furthermore, we found that supplementation of exogenous Wnt3A to cultures

### Table 2. Genes Differentially Expressed in ECFCs Isolated from MDS Patients and Healthy Controls.

The Upper Section Includes Genes Functional to Adhesion, Angiogenesis, Activation, Survival, and Apoptosis of Endothelial Cells; the Middle Section Includes Genes Belonging to the Wnt Pathway; and the Lower Section Includes miRNAs.
partly reverted the impaired hematopoietic differentiation of hematopoietic cells.

**Conclusion**

Overall, our observations add a new tile to the complex mosaic of the MDS pathogenesis and offer an innovative perspective on these diseases, pointing to the primary dysfunctions of the vascular niche as important drivers for myelodysplasia.

**Appendix A. Supplementary Materials**

Supplementary data to this article can be found online at [http://dx.doi.org/10.1016/j.neo.2015.04.001](http://dx.doi.org/10.1016/j.neo.2015.04.001).
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