

Normal and malignant megakaryopoiesis

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Megakaryopoiesis is the process by which bone marrow progenitor cells develop into mature megakaryocytes (MKs), which in turn produce platelets required for normal haemostasis. Over the past decade, molecular mechanisms that contribute to MK development and differentiation have begun to be elucidated. In this review, we provide an overview of megakaryopoiesis and summarise the latest developments in this field. Specially, we focus on polyploidisation, a unique form of the cell cycle that allows MKs to increase their DNA content, and the genes that regulate this process. In addition, because MKs have an important role in the pathogenesis of acute megakaryocytic leukaemia and a subset of myeloproliferative neoplasms, including essential thrombocythemia and primary myelofibrosis, we discuss the biology and genetics of these disorders. We anticipate that an increased understanding of normal MK differentiation will provide new insights into novel therapeutic approaches that will directly benefit patients.

Megakaryocytes (MKs), the precursors of platelets, are derived from pluripotent haematopoietic stem cells (HSCs). Every day approximately 1×10^{11} platelets are produced by the cytoplasmic fragmentation of MKs, a level of production that can increase 10- to 20-fold in times of demand and an additional 5- to 10-fold under the stimulation of thrombopoietin-mimetic drugs (Ref. 1). MKs are fairly rare: within the normal human marrow, only 1 in 10 000 nucleated cells is a MK. The hallmark of the MK is its large diameter (50–100 μm) and its single, multilobulated, polyploid nucleus. The external influences that impact megakaryopoiesis include a supportive bone marrow stroma consisting of endothelial and other cells, matrix glycosaminoglycans, and a

number of hormones and cytokines, such as thrombopoietin, stem cell factor and stromal-cell-derived factor-1.

In the canonical pathway of haematopoietic lineage development (Refs 2, 3, 4, 5, 6), the HSC gives rise to two major lineages: the common lymphoid progenitor (CLP) (Ref. 7) and the common myeloid progenitor (CMP) (Ref. 4) (Fig. 1). The CLP then generates lymphocytes (NK, T and B cells), whereas the CMP produces the granulocyte/macrophage progenitor and the megakaryocyte/erythroid progenitor (MEP) (Refs 8, 9). Other evidence suggests that the MEP can arise directly from the HSC to give rise to the erythroid and MK lineages without a CMP intermediate (Refs 10, 11). The existence of this alternate pathway is under debate. For example,

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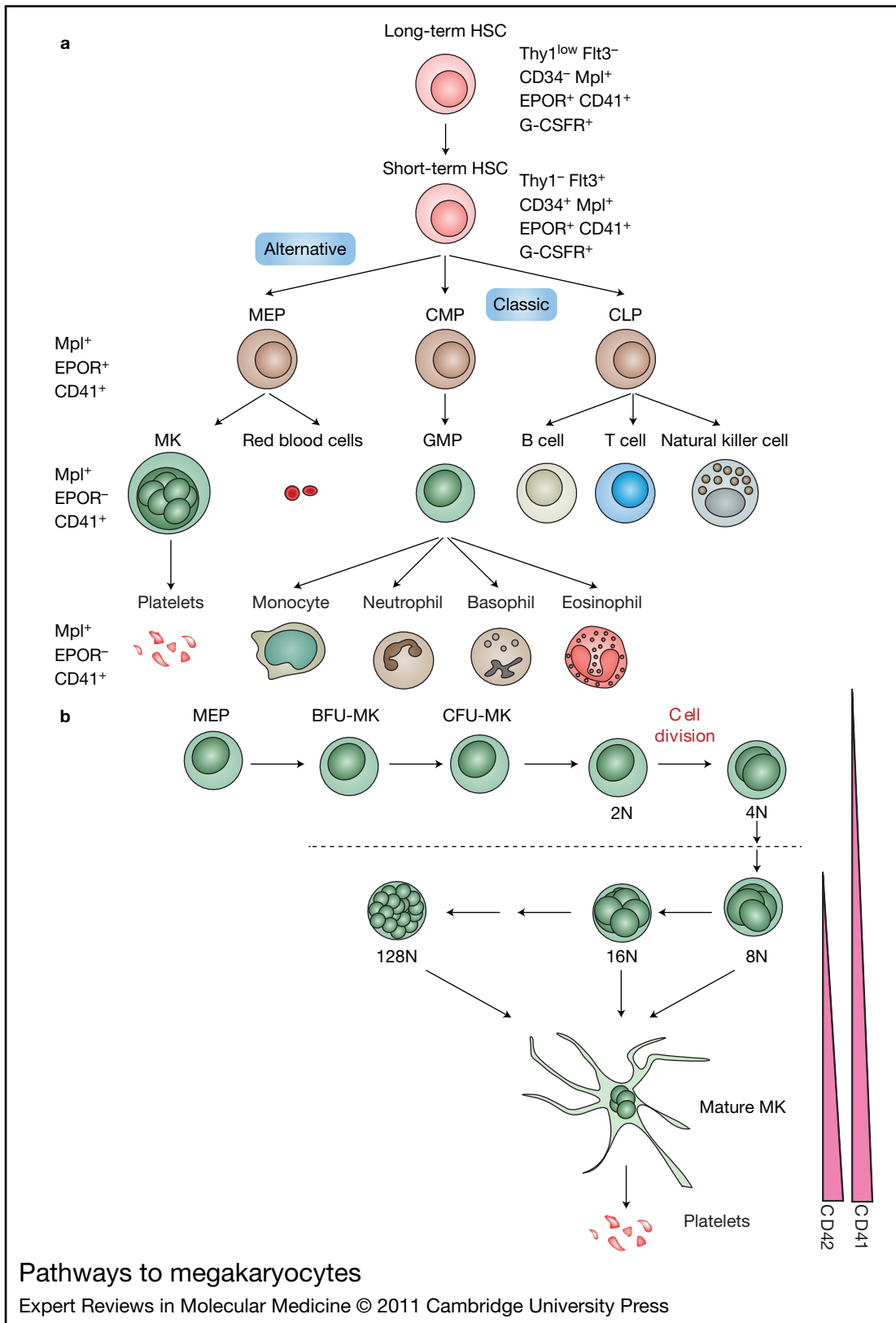


Figure 1. Pathways to megakaryocytes. (See next page for legend.)

Figure 1. Pathways to megakaryocytes. (See previous page for figure.) (a) The HSC gives rise to all blood cell lineages. In the classical model, the first lineage commitment step of HSCs results in a separation of CMPs and CLPs. The MEP, the precursor of MKs and erythroid cells, is derived from a CMP. In the alternative model, the HSC directly gives rise to the MEP before restriction to myeloid or lymphoid lineages. (b) MK progenitors, including the MEP, BFU-MK and CFU-MK, proliferate and differentiate into platelet-producing mature MKs. During this process, MKs undergo endomitosis to increase their size and DNA content. In murine cells, the DNA content can increase up to 128N. Simultaneously, expression of the MK-specific markers CD41 and CD42 is upregulated. Abbreviations: BFU-MK, burst-forming unit megakaryocyte; CD41, glycoprotein IIb/IIIa or $\alpha\text{IIb}\beta\text{3}$ integrin receptor; CFU-MK, colony-forming unit megakaryocyte; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; EPOR, erythropoietin receptor; Flt3, FMS-like tyrosine kinase 3; G-CSFR, G-CSF receptor; GMC, granulocyte/macrophage progenitor; HSC, haematopoietic stem cell; MEP, megakaryocyte erythroid progenitor; MK, megakaryocyte; Thy1, thymus 1 (low, low surface antigen; –, none detectable).

Forsberg and colleagues showed that Flt3(+) early progenitors retain MK and erythroid potential *in vivo* at both the population and clonal levels (Ref. 12). In either case, the first cells fully committed to the MK lineage are BFU-MKs (burst-forming unit megakaryocytes), which give rise to a more differentiated MK progenitor, termed colony-forming unit megakaryocyte (CFU-MK). Committed MK progenitors can be identified by a distinct surface immunophenotype as a population of $\text{CD9}^+\text{CD41}^+\text{Fc}\gamma\text{R}^{\text{lo}}\text{c-kit}^+\text{Sca-1}^+\text{IL-7R}\alpha^-\text{Thy1.1}^- \text{Lin}^-$ cells in murine bone marrow. This population is restricted to producing MKs and platelets both *in vitro* and *in vivo* (Ref. 13).

Megakaryopoiesis is first observed in the embryonic yolk sac where it is closely associated with primitive and definitive erythropoiesis (Refs 14, 15, 16). Primitive MEPs are seen as early as E7.25, indicating that primitive haematopoiesis is bilineage in nature. The first glycoprotein-Ib-positive cells, corresponding to MKs, can be seen in the yolk sac at E9.5, whereas platelets can be detected in the embryonic bloodstream beginning at E10.5. At this time, the number of MK progenitors begins to decline in the yolk sac and expand in the fetal liver. Fetal platelets are extremely large, with a diameter 1.6 times larger than adult platelets, and contain a large amount of RNA. This feature is also observed in patients with immune thrombocytopenic purpura and highlights the similarity between embryonic megakaryopoiesis and adult stress megakaryopoiesis.

MKs and the endomitotic process

One of the most characteristic features of MK development is endomitosis, a modified form of mitosis in which the DNA is repeatedly replicated in the absence of cytoplasmic

division. During the endomitotic phase, each cycle of DNA synthesis produces an exact doubling of all the chromosomes, resulting in cells containing DNA contents ranging from 8N to 128N in a single, highly lobulated nucleus. Endomitosis is not simply the absence of mitosis, but is rather a series of recurrent cycles of aborted mitosis (Ref. 17). The cell cycle kinetics of endomitotic cells is also unusual, with a shortened G1 phase, a relatively normal S phase of DNA synthesis, a short G2 phase and a very short modified mitosis phase (Refs 18, 19). During the last phase, chromosomes condense, the nuclear membrane breaks down and mitotic spindles form upon which the replicated chromosomes assemble. However, individual chromosomes fail to complete their normal migration to opposite poles of the cell, the spindle dissociates, the nuclear membrane re-forms around the entire chromosomal complement and the cell again enters the G1 phase. Endomitosis departs from a normal mitotic cell cycle at cytokinesis, when furrow invagination aborts short of cell abscission (Refs 20, 21).

Because endomitosis is a modified form of the cell cycle, one might suspect that genes involved in normal cell cycle regulation would have an important role in endomitosis. Indeed, many of the genes that control the normal cell cycle are also required for the endomitotic cycle. For example, gain of function of cyclin D1 (*CCND1*) and cyclin D3 (*CCND3*) increases the polyploid state of MKs *in vivo* (Refs 22, 23, 24, 25), whereas loss of function of cyclin D1 in primary murine MKs induces low polyploidy (Ref. 26). Similarly, loss of cyclin E (*CCNE*) in mice leads to defective endoreplication of MKs and trophoblast giant cells (Ref. 27), whereas

increased expression of cyclin E enhances ploidy of MKs in transgenic animals (Ref. 22). In the same manner, it is likely that genes that normally restrict cell cycle progression also restrain polyploidisation of MKs. Evidence in support of this model includes observations that increased expression of p21, induced by loss of *SCL/tal1*, blocks endomitosis of murine primary MKs (Ref. 28) and knockdown of p19 in human $CD34^+$ cultures results in high polyploid MKs, whereas its overexpression causes a decrease in polyploidy (Ref. 29).

Because polyploid cells are genetically unstable and can progress to aneuploidy and promote tumours, organisms have developed ways of minimising this process. The prevailing model has been that cells maintain a checkpoint, which monitors the number of chromosomes and results in p53-dependent cell cycle arrest of tetraploid cells. However, a number of studies suggest that normal cells are instead protected from polyploidy by activation of a stress checkpoint, which stabilises p53 and leads to cell cycle arrest (Ref. 30). The absence of data to associate polyploidisation with cellular stress in MKs might provide an explanation as to why this lineage circumvents the cell cycle arrest that is seen in normal cells.

The spindle assembly checkpoint (SAC) is an evolutionarily conserved mechanism that ensures that cells with misaligned chromosomes do not exit mitosis. The ability of the checkpoint to monitor the status of chromosome alignment is achieved by assigning checkpoint proteins to the kinetochore, a macromolecular complex that resides at centromeres of chromosomes and establishes connections with spindle microtubules. The SAC prevents cell cycle progression by negatively regulating CDC20 and inhibiting the activation of the polyubiquitylation activities of anaphase-promoting complex (APC), which degrades proteins such as securin. Failure in the function of the checkpoint results in polyploidy (Ref. 31). Genes that are required for spindle checkpoint control include the kinases BUB1 and BUBR1, the WD40 repeat containing protein BUB3, which directs the localisation of the complex to kinetochores, and the APC inhibitors MAD2/CDC20. For example, loss of function of *BUB1*, *BUB3* and *MAD2* results in increased polyploidy (Refs 32, 33). Moreover, although homozygous loss of *Bubr1* leads to embryonic lethality, *Bubr1* heterozygous mutant mice

displayed splenomegaly and extramedullary megakaryopoiesis with a marked increase in polyploidisation (Ref. 34). It is important to note that haploinsufficiency of BUBR1 selectively induced polyploidy of MKs, underscoring the unique sensitivity of MKs to this process.

The chromosome passenger complex (CPC) includes Aurora-B (AURKB), inner centromere protein (INCENP), survivin (BIRC5) and borealin (CDCA8). The CPC localises at centromeres in metaphase and then translocates to the midzone late in mitosis (Ref. 35). The CPC is important for the recruitment and proper localisation of SAC proteins and is required for spindle checkpoint function. Based on this important function, one might expect that alterations in the CPC would affect MK development. Indeed, changes in survivin expression have been associated with aberrant MK polyploidisation: overexpression of survivin in normal murine bone marrow progenitors antagonised MK polyploidisation, growth and maturation (Ref. 36), whereas loss of survivin in primary bone marrow progenitors resulted in increased polyploidy of MKs (Ref. 37). These findings suggest that the tight regulation of survivin expression is important for MK development.

The function of Aurora-B in endomitosis has also been under intense study. Geddis and Kaushansky found that Aurora-B was expressed and properly localised during endomitosis of human MKs derived from $CD34^+$ cells (Ref. 38), whereas Zhang and colleagues reported that Aurora-B was absent or mislocalised in MKs derived from mouse bone marrow (Ref. 39). The discrepancy has been attributed to species-specific differences or to differential antibody sensitivity. Independently, Lordier and colleagues showed that inhibition of Aurora-B kinase activity induced apoptosis of low polyploid MKs and caused mis-segregation of chromosomes, mitotic failure and an increased proportion of high polyploidy human MKs (Ref. 21).

Precise activation of Rho-GTPase-ROCK signalling and its downstream targets, such as kinesin, myosin, tubulin and actin, are of critical importance in cytokinesis (Ref. 40). Unlike diploid MKs, RhoA and F-actin are partially concentrated at the site of furrowing in polyploid MKs and inhibition of the Rho-Rock pathway causes loss of F-actin from the midzone

and increases the degree of MK polyploidisation (Ref. 20). A direct role for regulating RhoA-ROCK signalling has recently been demonstrated for polo-like kinase 1 (PLK1) (Refs 41, 42, 43). PLK1 is important for precise localisation of ECT2 (Refs 41, 43), a GTP exchange factor for RhoA, to the central spindle. It has been reported that PLK1 expression is absent in polyploid MKs, although expression of other cell cycle proteins is similar in both populations (Ref. 44). Forced expression of *Plk1* during MK differentiation from murine bone marrow cells was associated with decreased polyploidisation, suggesting that PLK1 is indeed a regulator of this process in differentiating MKs (Ref. 44).

Transcriptional regulation of megakaryopoiesis

Lineage-specific transcription factors have essential roles in the development of MKs. Many human and murine leukaemias are associated with mutations, chromosomal translocations or viral insertions in these factors (Ref. 45). This finding suggests that the maintenance of a normal transcriptional programme has an important role in normal haematopoiesis. In this section, we will highlight several key MK transcription factors, such as GATA-1, FLI1 (and other E-twenty six (ETS) factors), Stem cell leukemia (SCL), Runt-related transcription factor-1 (RUNX1) and serum response factor (SRF).

GATA-1

GATA-1 is a zinc finger transcription factor that binds the consensus motif WGATAR. GATA-1 is required for normal development of MKs and platelets. Mice that lack *Gata1* expression in the MK lineage (*Gata1*^{low} or *Gata1*^{knockdown} mice) develop MKs, but they are abnormal in many respects (e.g. reduced polyploidisation and excessive proliferation) (Refs 26, 46, 47). Restoration of cyclin D1 expression, which is diminished in GATA-1-deficient cells, resulted in a dramatic increase in MK size and DNA content (Ref. 26). However, terminal differentiation was not rescued, suggesting that cyclin D1 is a mediator of polyploidisation but not of terminal differentiation. The finding also confirms the notion that polyploidisation and terminal maturation can be uncoupled. The GATA-1 protein consists of two zinc finger DNA-binding domains, the N- and C-fingers, and an N-

terminal activation domain. The N-finger mediates the interaction between GATA-1 and its partner FOG-1, which is also essential for megakaryopoiesis. FOG-1-deficient mice do not generate any cells of the MK lineage (Ref. 48). Mice that express a *Gata1* mutant that fails to interact with FOG-1 show a phenotype that resembles that of *Gata1*-knockdown mice, suggesting that FOG-1 has GATA-1-independent functions, probably mediated through GATA-2 (Ref. 49). Furthermore, mutations in the N-terminal zinc finger that led to the dissociation of the GATA-1–FOG-1 interaction, or to a decreased ability of GATA-1 to bind DNA, are associated with a spectrum of benign haematologic diseases in humans, including dyserythropoietic anaemia and thrombocytopenia (Ref. 50), grey platelet syndrome (Ref. 51) and congenital erythropoietic porphyria (Ref. 52). NuRD interacts with the extreme N-terminus of FOG-1, and this interaction is important for GATA-1–FOG-1-mediated gene activation and repression (Ref. 53). Moreover, mice in which the FOG-1–NuRD interaction is disrupted produce MK progenitors that give rise to significantly fewer and less mature MKs *in vitro* while retaining multilineage capacity capable of generating mast cells and other myeloid lineage cells (Ref. 54). Further studies showed that NuRD could bind and repress expression of genes characteristic of mast cell lineage. These findings suggest that the interaction between NuRD and GATA-1–FOG-1 is required to maintain lineage fidelity in MK development. The mutations in the N-terminal domain of GATA-1 will be discussed in the acute megakaryocytic leukaemia (AMKL) section of this review.

RUNX1/AML1

The complex of RUNX1 and Core binding factor beta (CBF β) participates in programming haematopoietic ontogeny and represents the most common mutational target in human acute leukaemia (Ref. 55). RUNX1 and CBF β retain high expression during MK differentiation of MEP, but are downregulated during the early phases of erythroid differentiation (Refs 56, 57, 58). The absence of RUNX1 has profound effects on the polyploidisation and terminal maturation of MKs, leading to a significant reduction in polyploidisation and platelet formation *in vivo* (Refs 59, 60, 61). Expression of the proper amount of RUNX1 is also essential for normal platelet homeostasis, because *Runx1* heterozygous

animals display mild thrombocytopenia and a decrease in long-term repopulating HSCs (Ref. 62). Mutations of *RUNX1* are associated with familial platelet disorder with predisposition to acute myeloid leukaemia (AML) and sporadic cases of AML (Refs 63, 64, 65).

ETS factors

Several ETS factors, including FLI1, GABP α , ETS1, ETS2, ERG and TEL1, directly contribute to murine megakaryocytic development. The importance of TEL1 was highlighted by the finding that deletion of *Tel1* within erythroid and megakaryocytic cells in the GATA-1-CRE::*Tel1* floxed mouse strain causes a 50% drop in platelet counts with no effect on haemoglobin levels (Ref. 66). FLI1 is also necessary for MK development. Homozygous loss of *Fli1* in mice is embryonic lethal and results in severe dysmegakaryopoiesis (Refs 67, 68). Accumulated evidence indicates that FLI1 is a positive regulator of megakaryopoiesis (Refs 67, 68) and a negative regulator of erythroid differentiation (Refs 69, 70). *Fli1*^{-/-} mice show a marked decrease in the mature:immature MK ratio compared with wild-type littermates, consistent with a critical role in later stages of MK differentiation. Another ETS factor, GABP α , is a regulator of early MK-specific genes, including α IIb and c-mpl (Ref. 71). The ratio of GABP α /FLI1 decreases with MK maturation, consistent with a dependence of early genes on GABP α and late MK-specific genes on FLI1 for their expression. ERG is an ETS factor encoded on human chromosome 21 (Hsa21) and expressed within MKs. ERG facilitated the expansion of MKs from wild-type, *Gata1*-knockdown and *Gata1s*-knock-in progenitors, but could not overcome the differentiation block characteristic of the *Gata1*-knockdown MKs (Ref. 72). Overexpression of ERG immortalised *Gata1*-knockdown and *Gata1s*-knock-in, but not wild type, fetal liver progenitors. Moreover, high-level overexpression of ERG resulted in myeloid leukaemia in mice, with infiltration of CD41-positive cells in the spleen (Ref. 73). The latter findings highlight the important role of ERG in Down syndrome AMKL.

SCL/*tal-1*

It is well established that SCL is important for the development of HSCs. Mice with a conditional

knockout of SCL in haematopoietic cells display perturbed megakaryopoiesis and erythropoiesis with a loss of early progenitor cells in both lineages (Ref. 74). These mice consistently show a low platelet count and haematocrit compared with controls. The finding suggests that SCL is indispensable for the development of both megakaryocyte and erythroid lineage. SCL, along with LMO2, Ldb1 and E2A, forms a complex with GATA-1 at all activating GATA elements in MKs (Ref. 75). In striking contrast, at sites where GATA-1 functions as a repressor, the SCL complex is absent. Important MK targets of SCL include p21 (Ref. 28) and MEF2C (Ref. 76). Of note, *Mef2c*-deficient mice display similar MK defects as SCL-deficient animals.

Serum response factor

SRF is a MADS-box transcription factor that is important for muscle differentiation. Interests in the function of SRF in MK development was kindled when Mkl1, a cofactor of SRF, was discovered as part of the t(1;22) translocation in acute megakaryoblastic leukaemia (Ref. 77). The importance of SRF in MK maturation and platelet formation was revealed by two groups that used *Srf* floxed mice and PF4-Cre mice or MX-Cre mice (Refs 78, 79). *Srf*-deficient mice showed a reduction in platelet count and an increase of MK progenitors in the bone marrow (BM) by fluorescence activated cell sorting (FACS) analysis and CFU-MK. These mice also have defects in platelet function.

MKs and the myeloproliferative neoplasms (MPNs)

The MPNs include the 'classical MPNs' chronic myeloid leukaemia (CML), polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) (Ref. 80) as well as chronic eosinophilic leukaemia/hypereosinophilic syndrome, chronic myelomonocytic leukaemia, Ph-negative atypical CML, chronic neutrophilic leukaemia (CNL) and systemic mastocytosis. Many studies have demonstrated multilineage clonal haematopoiesis in the different MPNs (Refs 81, 82, 83), consistent with the notion that MPNs originate in the HSC compartment.

Hyperproliferation of MKs and alterations in platelet counts are features of ET and PMF. ET is often discovered incidentally on blood counts in asymptomatic patients. The major causes of morbidity and mortality are bleeding or

thrombotic complications and rarely evolution to AML. Bone marrow pathology displays increased cellularity with marked megakaryocytic hyperplasia. There are frequently giant MKs with increased polyploidy, but the overall morphology of MKs is fairly normal (Fig. 2). By contrast, individuals with PMF present with splenomegaly, dysplastic megakaryopoiesis and marked bone marrow fibrosis. The peripheral blood often shows teardrop-shaped erythrocytes and large platelets. The bone marrow shows increased deposition of reticulin fibres and numerous morphologically abnormal MKs with multilobed nuclei. PMF might progress rapidly to AML, with nearly 20% of patients transforming within 10 years (Ref. 84).

Although MPNs were first described in 1951 by William Dameshek (Ref. 80), the genetic basis for the disease remained elusive until 2005 when a somatic point mutation in JAK2 tyrosine kinase (JAK2V617F) was identified in 50% of ET and PMF and 95% of PV patients (Refs 85, 86, 87, 88). JAK2 is a member of the Janus family of

cytoplasmic nonreceptor tyrosine kinases, which also includes JAK1, JAK3 and TYK2. The JAK kinases have seven homologous domains (JH1–JH7) and a catalytically inactive pseudokinase domain (JH2). The predominant JAK2 mutation is a guanine-to-thymidine substitution, which results in a substitution of valine for phenylalanine at codon 617 (JAK2V617F) within the JH2 domain. The V617F mutation abrogates the autoinhibitory activity of the JH2 domain (Ref. 89) and results in constitutive kinase activity (Refs 85, 86, 87, 88, 90). The mutation is present in myeloid cells, but not in germline DNA in patients with MPN (Refs 87, 88), demonstrating that JAK2V617F is a somatic mutation that is acquired in the haematopoietic compartment. When expressed in vitro, JAK2V617F, but not the wild-type protein, is constitutively phosphorylated (Ref. 88).

How does a single disease allele contribute to the pathogenesis of PV, ET and PMF, three distinct clinical disorders? Several lines of

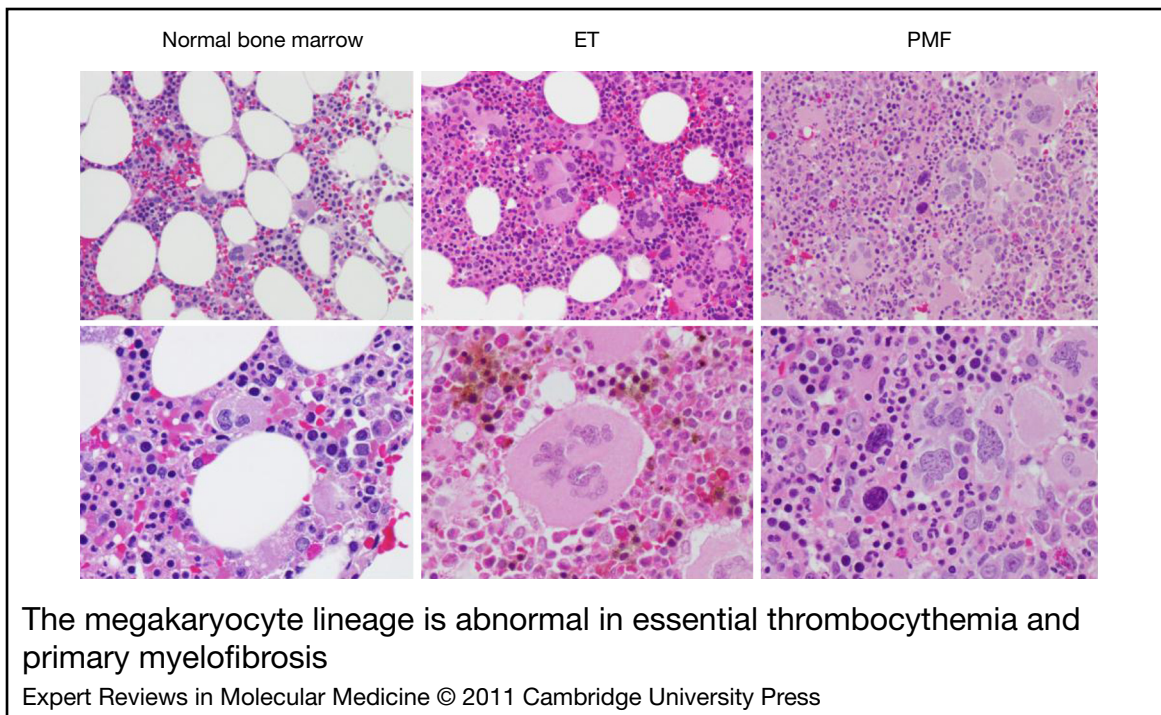


Figure 2. The megakaryocyte lineage is abnormal in essential thrombocythemia and primary myelofibrosis. Bone marrow sections from a healthy individual (left), and patients with essential thrombocythemia (ET; middle) or primary myelofibrosis (PMF; right). Top, 20 \times , bottom, 40 \times original magnification. Note that ET MKs are typically larger than normal, increased in number and contain chromatin that resembles leaves. By contrast, PMF MKs are small, dysplastic and contain chromatin that resembles clouds. Images courtesy of Dr Sandeep Gurbuxani (University of Chicago).

evidence suggest that a high dose of JAK2V617F, which results in a high level of JAK2 signalling, favours an erythroid phenotype and a low dose of JAK2V617F favours a megakaryocyte phenotype. First, homozygosity for JAK2V617F is much more common in PV than in ET (Refs 85, 86, 87, 88, 91). Second, mutations of JAK2 exon 12, which are associated with stronger downstream JAK2 signalling compared with JAK2V617F, are found in patients with PV, but not in those with ET (Ref. 92). Third, in vivo studies support the gene dosage hypothesis. For example, transplantation of JAK2V617F transduced mouse bone marrow cells with high-level expression resulted in a uniform PV phenotype without thrombocytosis (Ref. 93). Furthermore, transgenic mice that express a relatively low level of JAK2V617F, below that of wild-type allele, developed ET-like disease, whereas mice that express much higher levels caused a PV-like disease (Ref. 94). A similar induction of thrombocytosis was also seen in JAK2V617F transplant recipients with the lowest level of JAK2 expression (Ref. 95).

Research has shown that STAT1 signalling promotes megakaryopoiesis and contributes to polyploidisation (Ref. 96). However, a connection between STAT1 and MPN was only recently discovered. An exciting study by Anthony Green and colleagues showed that the activation status of STAT1 could predict the phenotype of JAK2V617F disease (Ref. 97). By comparing clonally derived mutant and wild-type cells from individual patients, they showed that enhanced activation of STAT1 was associated with an ET phenotype, whereas downregulation of STAT1 activity was associated with a PV-like phenotype. It still remains to be determined whether the increased activation of STAT1 in ET is a result of change of JAK2V617F protein level.

Beyond its effects on the STAT signalling cascade, JAK2V617F was recently shown to promote changes in chromatin structure, resulting in differential gene expression (Ref. 98). The authors reported that JAK2 phosphorylates histone H3, leading to reduced binding of HP1 α in human haematopoietic cells. Furthermore, inhibition of JAK2 activity decreased the phosphorylation of H3Y41 at certain promoters, including that of the leukaemia oncogene *lmo2* (Ref. 98). More recently, it was found that compared with ES

cells that have wild-type JAK2, levels of chromatin-bound HP1 α were lower in JAK2V617F mutant cells, but these levels increased following inhibition of JAK2, coincident with a global reduction in histone H3Y41 phosphorylation (Ref. 99). JAK2 inhibition also reduced levels of the pluripotency regulator *Nanog*, with a reduction in H3Y41 phosphorylation and a concomitant increase in HP1 α levels at the *Nanog* promoter. These findings highlight the nuclear function of JAK2. Further genome-wide epigenetic and gene expression studies to probe JAK2 nuclear function will shed light on the pathogenesis of JAK2V617F MPNs.

This past year, four groups reported the creation of knock-in mouse models in which the mutant JAK2V617F allele was inserted into the germline in such a way that expression could be activated in a conditional fashion (Refs 100, 101, 102, 103). Three of these groups replaced the wild-type allele with the mutant murine *Jak2* gene. In all these cases, animals developed similar phenotypes that mimicked PV (Ref. 100, 102, 103). By contrast, the one animal strain created with the human *JAK2* gene developed an ET-like phenotype (Ref. 101). In this latter mouse, the human *JAK2* cDNA was incorporated into the murine locus, an event that itself may lead to changes in gene expression and phenotype. Thus, any direct comparisons of the four lines should be done with caution.

It is interesting to focus for a moment on one of the JAK2V617F knock-in models that develops a lethal MPN (Ref. 103). In this model, the mice were engineered so that the wild-type exon 14 is flanked by *loxP* sites, with a mutated version of the exon positioned downstream in an inverted fashion. On exposure to Cre recombinase, the wild-type exon was deleted and replaced by an inverted copy of the mutated exon, leading to expression of JAK2V617F protein. By breeding floxed heterozygous mice to the E2A-Cre strain, which leads to germline excision in early embryos, JAK2V617F-expressing animals were generated. All these animals developed a lethal MPN characterised by elevated haematocrit, splenomegaly and a median survival of 146 days. Flow cytometry and histology of the bone marrow and spleen confirmed that the mice developed marked erythroid and mild megakaryocytic hyperplasia. Together, the pathological findings revealed that the mice

develop an MPN that is closely reminiscent of human PV. Given that the MPN in these animals was transplantable to primary recipient mice, it was possible to identify the nature of the disease-initiating cell. JAK2 mutant stem cells exhibited a minor competitive advantage in recipients, the LSK population (and not more differentiated progenitor subsets) contained repopulating activity, and the presence of a minority of JAK2V617F LSK cells was sufficient to cause the PV phenotype. Perhaps the most fascinating aspect of the study, made possible by the ability of the LSK population to transplant disease, was the discovery that inhibition of JAK2 kinase did not eradicate the MPN-initiating population. Although treatment of primary diseased animals with the JAK inhibitor TG101348 led to significant reductions in spleen size and number of erythroid progenitors, the LSK population of TG101348-treated animals retained the ability to induce MPN in recipient mice. These studies demonstrate that JAK inhibition with this agent does not eliminate the MPN-initiating population in vivo and thus has profound implications for human therapy.

The common occurrence of *JAK2* mutations in patients with MPNs underscores the importance of JAK2 signalling in the pathogenesis of these diseases. However, nearly half of ET and PMF patients do not harbour a *JAK2* mutation. Shortly after the discovery of the *JAK2* mutations, activating mutations in tryptophan 515 of the thrombopoietin receptor, *MPL*, were identified in a subset of *JAK2* wild-type ET and PMF cases (Refs 104, 105, 106). These mutations occur in a subset of patients with *JAK2V617F*-negative ET and PMF, including 8.5% of *JAK2V617F*-negative ET patients (Ref. 107) and approximately 10% of *JAK2V617F*-negative PMF patients (Refs 104, 105, 106). Four patients have also recently been described with somatic *MPLS505N* mutations (Ref. 107), an allele that had previously been associated with inherited thrombocytosis (Ref. 108). *MPLW515*-positive ET patients have higher platelet counts and lower haemoglobin levels than *JAK2V617F*-positive ET patients (Refs 107, 109), and *MPLW515*-positive PMF patients present with more severe anaemia (Ref. 110). Moreover, endogenous MK colonies, but not endogenous erythroid colonies, can be grown from *MPLW515*-positive patient cells. These findings might be because *MPL* is not

expressed during terminal erythroid differentiation.

Because both *JAK2V617F* and *MPLW515* activate JAK–STAT signalling, it is logical to surmise that loss of negative regulators of the pathway might also be associated with MPNs. Indeed, mutations in *LNK* [also known as Src homology 2 B3 (SH2B3)], a protein that downregulates JAK–STAT signalling after erythropoietin receptor (EPO-R) or *MPL* activation (Refs 111, 112, 113), have been identified in both ET and PMF patients with a frequency between 3% and 6% (Refs 114, 115, 116, 117). *LNK* has a number of protein–protein interaction domains: a proline-rich amino-terminus, a pleckstrin homology domain, an Src homology 2 (SH2) domain and many potential tyrosine phosphorylation motifs (Ref. 118). Human *LNK* mutations appear to cluster in the pleckstrin homology and SH2 domains, which physically interact with the cell membrane and EPO-R/*MPL*/*JAK2*, respectively. Loss-of-function mutations of *LNK* were also found in *JAK2* mutation negative ‘idiopathic’ erythrocytosis or PV (Ref. 114). Of note, mice with a complete loss of *LNK* display a number of features in common with human MPN, including an exaggerated response to cytokines, splenomegaly, thrombocytosis and extramedullary haematopoiesis (Refs 111, 113, 119). It should also be noted that *LNK* mutations and *JAK2V617* are not mutually exclusive and may co-occur in the same patient (Ref. 116). *LNK* mutations were recently shown to be acquired either early or late during the course of clonal evolution (Ref. 117). Other mutations identified in MPNs include *TET2* (Ref. 120), *ASXL1* (Ref. 121), *IDH1/IDH2* (Refs 122, 123), *CBL* (Ref. 124), *IKZF1* (Ref. 125) and *EZH2* (Ref. 126). The functional consequences of these mutations with respect to MPN initiation or progression are largely unknown, but are the subject of intense research.

Novel therapies for MPNs

The discovery of *JAK2V617F* and other JAK–STAT-activating mutations, including mutations in exon 12 of *JAK2*, *MPL* and *LNK*, has generated enormous interest in the development and therapeutic use of small-molecule JAK2-inhibitor-targeted therapy in these diseases. A handful of compounds are currently in clinical development for MPNs, including INCB018424,

TG101348, CEP-701, SB1518 and CYT387 (Ref. 127). These JAK inhibitors exhibit differential inhibitory activities against the JAK family members, whereas others are less selective. For example, INCB018424 inhibits predominantly JAK1, whereas CEP-701 and TG101348 inhibit FLT3 in addition to JAK2. Data from ongoing clinical studies show that JAK inhibitors provide symptomatic relief to patients, including spleen size reduction and improvement of constitutional symptoms such as fatigue, weight loss, night sweats and fever (Refs 128, 129). The improvement of constitutional symptoms by INCB018424 might be the result of a decrease in proinflammatory cytokines induced by inhibition of JAK1 (Refs 128, 129). Whether this is the main mechanism of action of JAK inhibitors in general remains to be determined. Indeed, longer-term studies are required to determine the full potential of JAK2 inhibitors and to show whether they will have an impact on survival in MPNs. In addition to JAK inhibitors, several other therapies are under study for use as single agents or in combination with JAK inhibitors. These include HSP90 inhibitors (e.g. PU-H71) (Ref. 130), histone deacetylase inhibitors (e.g. pabinostat) (Ref. 131) and PI3K–AKT pathway inhibitors (e.g. everolimus) (Ref. 132). Given these new drugs and other lines of clinical investigation, there are many reasons to be optimistic about the prospects for improved therapies for MPNs.

Acute megakaryocytic leukaemia

AMKL is a rare subtype of AML in which the leukaemic cells are derived from the MK lineage. The three major subgroups of AMKL include infants with Down syndrome (DS-AMKL), children without DS (non-DS paediatric AMKL) and adults (adult AMKL). The incidence in the paediatric population is 1:500 children with DS and 5–7% of paediatric AML cases overall in children without DS, whereas the incidence in adults is estimated to be approximately 1% of AML cases (Refs 133, 134, 135).

DS-AMKL, non-DS-AMKL and adult AMKL are distinct in that they have different genetic abnormalities and different outcomes. DS-AMKL often presents by age 2 with a myelodysplastic phase that includes thrombocytopenia. Bone marrow aspiration is

often difficult, with fibrosis detected on bone marrow biopsy (Refs 136, 137). The prognosis of DS-AMKL is favourable, with an approximately 80% cure rate (Refs 138, 139, 140). This outcome is based on the enhanced sensitivity of DS-AMKL blasts to chemotherapeutic drugs, especially cytarabine (ARA-C) (Ref. 141). On the other hand, children with non-DS-AMKL fare worse, with a 5-year event free survival (EFS) of 22–28% (Refs 135, 142). The prognosis of adult AMKL is even worse, with a median survival of approximately 10 months (Ref. 133).

Several genes and pathways have been found in the aetiology of the different forms of AMKL. In DS-AMKL, trisomy 21 and somatic mutations in the haematopoietic transcription factor gene *GATA1* are found in nearly every patient (Ref. 143). *GATA1* mutations, which include short deletions, insertions and point mutations clustered within exon 2, are a key step in the pathogenesis of DS-AMKL (Ref. 144). In every case, the mutations lead to a block in expression of the full-length protein, but allow for production of a shorter isoform, *GATA-1s*. Expression of *GATA-1s* alone is not sufficient to induce leukaemia, because humans with inherited mutant alleles fail to develop leukaemia (Ref. 145). However, in mice, expression of *GATA-1s* in place of *GATA-1* is sufficient to cause hyperproliferation of yolk sac and fetal liver MK progenitors (Ref. 146).

Distinct cytogenetic abnormalities, including t(1;22)(p13;q13), which leads to expression of the OTT-MAL fusion protein, t(9;11), t(10;11) and +8, are present in cases of non-DS paediatric AMKL (Ref. 143). As in the case of MPNs, alterations in tyrosine kinase signalling are probably necessary for AMKL. *JAK3* mutations were originally identified in the DS-AMKL cell line, CMK, as well as in two DS-AMKL specimens, and were later detected in other DS-AMKL cases (Refs 147, 148, 149, 150, 151, 152). Apart from other rare mutations in *c-MPL*, *c-KIT*, *JAK2* and *FLT3*, no other specific chromosomal rearrangements or genetic mutations have been identified in adult AMKL (Refs 143, 147). Recent studies have shown that paediatric AMKL shows many more copy number alterations than other forms of AML (Ref. 153). However, how these changes influence the disease remains unknown. Rare instances of *FLT3* mutations in AMKL do not influence prognosis (Ref. 154). Recent studies

have implicated dysregulation of miR-125b-2, which is overexpressed in AMKL relative to normal MKs, in DS-AMKL (Ref. 155).

Two microarray studies comparing primary DS-AMKL versus non-DS AMKL specimens found 76 genes that discriminate between DS and non-DS AMKL. Genes encoding erythroid markers, such as glycophorin A and CD36, appear to be significantly overexpressed in DS-AMKL (Refs 156, 157). Further analysis of the gene expression data revealed that there is an overall increase in expression of chromosome 21 genes in DS-AMK than in non-DS AMKL (Ref. 157). Forty-seven chromosome 21 genes were found to contribute to the enrichment by gene set enrichment analysis. However, the distinction between the two types of AMKL was not solely driven by differences in expression of chromosome 21 genes. The second study found that only 7 of 551 differentially expressed genes were on chromosome 21 (Ref. 156).

Non-DS paediatric AMKL is characterised by an expansion of megakaryoblasts within the bone marrow and is frequently accompanied by hepatosplenomegaly, myelofibrosis and pancytopenia (Ref. 158, 159, 160). The majority of cases are associated with the t(1;22) translocation, which results in fusion of the RNA-binding motif protein 15 (RBM15; aka OTT) and MKL1 (aka MAL) genes (Refs 77, 161). The localisation and function of MAL, a coactivator of SRF with strong transcriptional activation properties, is deregulated by fusion with OTT (Refs 162, 163). OTT is related to the SHARP transcription factor that acts in transcriptional repression complexes in canonical Notch signalling pathways (Refs 164, 165). OTT might also inhibit myeloid differentiation (Ref. 166). A conditional knock-in of the OTT-MAL fusion protein at its endogenous promoter resulted in deregulated transcriptional activity of the canonical Notch signalling pathway and abnormal fetal megakaryopoiesis. Cooperation between OTT-MAL and an activating mutation of MPL, but not the OTT-MAL knock-in alone, efficiently induced a short-latency highly penetrant AMKL that recapitulates the human phenotype (Ref. 167). Other cytogenetic abnormalities have been observed, including t(10;11), which leads to CALM-AF10 fusion (Ref. 168), t(9;11), +8 or +21 (Ref. 135).

Adult AMKL is a rare malignancy that comprises nearly 1% of adult AML cases (Refs 133, 134).

Similar to the other subtypes of AMKL, adult AMKL is characterised by excessive production of immature megakaryoblasts within the bone marrow and extensive myelofibrosis. Peripheral blood features frequently include anaemia and thrombocytopenia. Adult AMKL frequently arises in individuals with an antecedent blood disorder or myelodysplastic syndrome (Ref. 169). Although some patients achieve complete remission, the long-term outcome is significantly worse for adult AMKL than for other forms of adult AML, with a median survival of 40 weeks or less (Refs 133, 134, 169).

To date, no specific chromosomal rearrangements or genetic mutations have been found in adult AMKL. In one study, it was noted that nearly 50% of patients had one or more cytogenetic abnormalities, including -5, -7, +8 or 11q involvement (Ref. 169). These findings partially explain the poor outcome of this subtype of AMKL, although the poor prognosis is not fully dependent on cytogenetic abnormalities. Several studies have now reported that the tyrosine kinases JAK2 or JAK3 are mutated in a subset of AMKL patients, suggesting that perhaps JAK inhibitors should be considered. Given the poor prognosis for all patients with AMKL without DS, new, targeted therapies are desperately needed.

Summary and perspective

MKs have been recognised as rare marrow cells for nearly two centuries, but it was the elegant studies of Howell in 1890 and his coining of the term 'MK' that led to their broader appreciation as distinct entities. The field has entered a new age in which modern genetic and molecular biological tools are being applied to these unique cells both in situ and in vitro to tease out novel insights into their growth and differentiation. With this increasing knowledge base, it is likely that we will soon know the complete genetic defects related to abnormal megakaryopoiesis and will be in a strong position to develop new therapeutics for both excessive and inefficient megakaryopoiesis.

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Further reading, resources and contacts

The following recent papers may be of interest to those who want to delve deeper into recent reports of normal and malignant megakaryopoiesis:

Tefferi, A. and Vainchenker, W. (2011) Myeloproliferative neoplasms: molecular pathophysiology, essential clinical understanding, and treatment strategies. *Journal of Clinical Oncology* 29, 573-582

Provides a state of the art review of MPNs.

Verstovsek, S. (2010) Therapeutic potential of Janus-activated kinase-2 inhibitors for the management of myelofibrosis. *Clinical Cancer Research* 16, 1988-1996

Highlights the clinical data for INCYTE JAK inhibitor.

Pardanani, A. et al. (2011) JAK inhibitor therapy for myelofibrosis: critical assessment of value and limitations. *Leukemia* 25, 218-225

Highlights the clinical data for the TargeGen JAK inhibitor.

Chen, E. et al. (2010) Distinct clinical phenotypes associated with JAK2V617F reflect differential STAT1 signaling. *Cancer Cell* 18, 524-535

Reveals that STAT1 activation differentiates PV from ET.

Chagraoui, H. et al. (2011) SCL-mediated regulation of the cell-cycle regulator p21 is critical for murine megakaryopoiesis. *Blood* 118, 723-735.

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State-of-the-art review of megakaryocyte lineage commitment and transcription factors.

Features associated with this article

Figures

Figure 1. Pathways to megakaryocytes.

Figure 2. The megakaryocyte lineage is abnormal in essential thrombocythemia and primary myelofibrosis.

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