CALRETICULIN IN MYELOPROLIFERATIVE NEOPLASMS: THE OTHER SIDE OF THE ALICE MIRROR

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ABSTRACT

Calreticulin (CALR), a Ca^{2+} binding protein mostly localised in the endoplasmic reticulum, regulates Ca^{2+} homeostasis, chaperones, and other proteins to the nucleus and other cellular compartments. CALR has been implicated in several cellular processes including: signalling, regulation of gene expression, cell adhesion, apoptosis, autoimmunity, and, when expressed on the cell surface, induction of phagocytosis by macrophages. Reports indicating over-expression of CALR in cancer cells suggest that modulation of CALR expression may be exploited to increase their clearance by the immune system. In the hematopoietic system, CALR has been implicated in the activation of the stress pathway as an obligatory partner of the glucocorticoid receptor. More recently, somatic loss-of-function mutations in the CALR gene were discovered in a significant proportion of patients with Philadelphia-negative myeloproliferative neoplasms (MPN) who did not harbour gain-of-function mutations in Janus kinase 2 (JAK2), the first signalling element of cytokine receptors, and myeloproliferative leukaemia virus oncogene (MPL), the thrombopoietin receptor, usually associated with these diseases. This review will summarise current knowledge on the biological activity of CALR and MPL/JAK2 in hematopoiesis, delineate a unifying pathway for the pathogenesis of MPN, and discuss how this pathway may be exploited for therapy.

Keywords: Myeloproliferative neoplasms, calreticulin, JAK2, glucocorticoid receptor.

INTRODUCTION

The Philadelphia-negative myeloproliferative neoplasms (Ph-negative MPN) are a group of clonal stem cell disorders characterised by abnormal proliferation of myeloid cells. These disorders include polycythaemia vera (PV), essential thrombocythaemia (ET), and primary myelofibrosis (PMF).[1] These diseases are characterised by hyperproliferation of either erythroid cells, leading to erythrocytosis (PV), or of megakaryocytes (ET and PMF). Hyperproliferation of megakaryocytes may be associated with platelet overproduction (ET) or, when due to delayed maturation, with thrombocytopaenia (PMF). Since their discovery in 1951 by William Dameshek,[2] much progress has been made in our understanding of the pathobiology of myeloproliferative neoplasms (MPN), leading to the establishment of international consensus criteria for their diagnosis by the World Health Organization in 2008;[3] these were refined in 2011.[4]

The discovery in 2005[5-8] that the primary genetic lesion in these disorders was a gain-of-function mutation in the gene encoding a tyrosine kinase, Janus kinase 2 (JAK2), paved the way for development of targeted pharmaceutical inhibition. The positive clinical experience with imatinib, a tyrosine kinase inhibitor that targets the breakpoint cluster region protein/Abelson murine leukaemia viral oncogene mutation associated with Philadelphia-positive MPN (Ph-positive MPN), suggested that a similar approach could be successfully applied for the cure of PV, ET, and PMF as well. In as little as 9 years after the discovery of JAK2 mutations, JAK2 inhibitors have been designed, clinical trials with selected drugs conducted, and the drugs have been approved for clinical use by the FDA.[9] However, in contrast with

Ph-positive MPN that harbour one primary lesion, the genetic lesions found in Ph-negative MPN are heterogeneous, and so concerns were raised whether JAK2 mutations represent the primary transformation event in these diseases.[10]

This review will summarise the biology of the major mutations observed in MPN and discuss the possibility that different mutations may target a common pathway, suggesting that, in spite of their genetic heterogeneity, MPN patients may be treated with the same drugs.

MUTATIONS ASSOCIATED WITH PH-NEGATIVE MPN

The majority of MPN patients harbour acquired gain-of-function mutations in JAK2, the first signalling element common to several hematopoietic growth factor receptors. The most frequent of these mutations are in the region of the gene encoding for the tyrosine kinase domain of the protein (JAK2V617F[5-8] and JAK2 exon 12 mutations[11]). The JAK2V617F mutation is present in almost all PV, ~50% of ET, and ~65% of PMF.

Less common mutations involving myeloproliferative leukaemia virus oncogene (MPL), LNK, TET2, ASXL1, EZH2, IDH1/2, CBL, IKZF1, have been observed in 3-20% of MPN patients.[12-14] These mutations may be found either alone or associated with JAK2 mutations. MPL and LNK mutations also affect the JAK2 signalling pathway.[12] MPL encodes the thrombopoietin receptor, and LNK encodes a chaperone protein that restrains MPL expression on the cell surface.

Other genes affected in MPN encode proteins involved in epigenomic DNA modification (TET2, ASXL1, and EZH2), cell metabolism (IDH1/2), protein stability (CBL), and gene transcription (IKZF1). TET2 encodes a methylcytosine dioxygenase that catalyses the conversion of methyl-cytosine to 5-hydroxy-methylcytosine; ASXL1 encodes a nuclear protein with a N-terminal helix-turn-helix domain associated with an unusual C-terminal domain that binds methylated lysines; EZH2 encodes the catalytic subunit of the polycomb repressive complex 2 (PRC2) responsible for methylation of histone H3 (H3K27) (methylation of H3K27 regulates stem cell renewal); IDH1/2 encodes isocitrate dehydrogenase 1 and 2, $NADP^+$ enzymes that catalyse the conversion of isocitrate to α-ketoglutarate; CBL encodes a protein of the Cbl family of E3-ubiquitin ligases that acts as a negative regulator of cell signalling by promoting ubiquitination, and decreasing stability of proteins involved in this process; IKZF1 encodes Ikaros, a transcription factor that regulates B and T cell development.

More recently, Klampfl et al.[15] and Nangalia et al.[16] used exome sequencing to identify novel mutations in exon 9 of calreticulin (CALR) in the majority of ET and PMF patients who did not harbour JAK2 or MPL mutations. CALR mutations were not found in healthy controls, lymphoid neoplasia, acute leukaemias, or solid tumours, indicating their specificity for ET and PMF. The mutations were either insertions or deletions, the commonest (80-90%) of which were a 52 bp deletion (CALRdel52) and a 5 bp insertion (CALRins5). All of them encode for a mutant protein with a novel C-terminus lacking the KDEL signal localisation domain (see the section of CALR functions). The mutations were detected in hematopoietic stem/progenitor cells and persisted with disease progression.[15] Re-sequencing samples from 1,107 MPN patients confirmed that CALR, JAK2, and MPL mutations were mutually exclusive.[15] Although the mutation rate in MPN is rather low, JAK2 and CALR mutations were found to be associated with other mutations such as loss of heterozygosity in p53 in those MPN patients at high risk of progression toward acute myeloid leukaemia.[17]

JAK2 AND PATHOBIOLOGICAL EFFECTS OF JAK2-MPL MUTATIONS

The mutation most frequently detected in MPN is JAK2V617F and several excellent reviews have been published on this subject.[18,19] Here we will summarise only information necessary to understand the relationship between the biological consequences of JAK2 and CALR mutations.

JAK2V617F constitutively activates the cytokine receptor signalling, including signal transducer and activator of transcription (STAT) proteins, and is largely responsible for the reported cytokine hypersensitivity and cytokine-independent growth *in vitro* of hematopoietic progenitor cells from MPN patients.[19] The addition in culture of JAK2 inhibitors reduces the erythropoietin-independent growth of erythroid colonies from PV patients, a property used for years as diagnostic criteria for this disease.[20,21] This observation provided the rationale for the development of JAK2 inhibitors,

such as ruxolitinib, currently used to treat patients with MPN.[22]

MPL - a gene first identified in a naturally occurring animal retrovirus - is located on chromosome 1p34 and, in addition to MPN, is present in Down's syndrome patients with acute lymphoblastic leukaemia (ALL).[12] The most frequent mutation is a point mutation in exon 10 that induces a tryptophan to leucine substitution in amino acid 515 (MPL^{W515L}). Additional MPL mutations found in a small number of JAK2 negative ET and PMF patients are MPL^{S505N}, MPL^{W515Ki}, and $MPL^{W515Kii}$.[23-27] All these mutations lead to ligand-independent activation of the receptor and of JAK2-STAT signalling, and to cytokine-independent cell growth, providing the rationale to use drugs that target JAK2 also for the treatment of patients carrying mutation in MPL.

JAK2V617F constitutively activates the STAT5 pathways.[8,28] Intermediate and high levels of STAT5 activation favour, respectively, proliferation and maturation of human hematopoietic progenitor cells.[29,30] It may be argued therefore, that the JAK2V617F mutation (by inducing constitutive STAT5 activation) should decrease, and not increase, the intrinsic proliferative potential of erythroid cells. This apparent paradox was resolved by studies indicating that the glucocorticoid receptor (GR) plays a major role in fine-tuning the levels of STAT5 activation expressed by erythroid cells expanded from PV patients.[31]

GR is the stress nuclear receptor that mediates the increased erythroid output occurring under conditions of acute and chronic blood loss,[32,33] and may suppress megakaryocytic maturation.[34] Activation of GRα by its ligand induces its homodimerisation and heterodimerisation with signalling partners, such as phospho-STAT5. GRα/phospho-STAT5 complexes migrate to the nucleus to bind through the DNA binding domain (DBD) of GR specific consensus sequences activating/inhibiting the expression of target genes. However, STAT5 is also downstream to the erythropoietin receptor and, upon receptor stimulation, elicits the signal that allows erythroid cells to mature. Simultaneous activation of the glucocorticoid and erythropoietin receptor impairs the ability of both receptors to phosphorylate STAT5 and ultimately quenches the signal delivered by the erythropoietin receptor leaving the cells immature and capable of proliferation.[31]

In humans, alternative splicing of GR mRNA leads to synthesis of GRβ, a dominant-negative form of the receptor that retains GRα in the nucleus, preventing its interaction with STAT5.[31] A A3669G single nucleotide polymorphism (SNP) stabilises GRβ mRNA, increasing the cellular content of the GRβ protein.[35] The A3669G SNP is present at a frequency greater than normal in patients with PV31 and PMF, and in PMF patients it predicts poor survival.[36] We had determined that erythroid cells, expanded *in vitro* from PV patients, express high levels of GRβ. In addition, erythroid cells expanded from these patients, in spite of constitutive STAT5 phosphorylation, contain low levels of STAT5 in the nucleus (Figure 1) and do not express GR target genes, such as GILZ.[31] Transcriptosome profiling identified similarities in gene expression between erythroblasts expanded from normal donors in the presence of dexamethasone, a synthetic GR agonist, and those expanded from PV patients without this hormone, confirming that in both cases the transcriptional activity of STAT5 was greatly reduced.[37] Surprisingly, reduced nuclear localisation of STAT5 in association with nuclear retention of GRα was observed also in erythroblasts expanded *ex vivo* from JAK2-non mutated MPN patients who did not harbour the polymorphism (unpublished results). The reduced levels of nuclear STAT5 localisation in PV address the paradox described above suggesting that PV expression of GRβ, by sequestering GRα in the nucleus and preventing its interaction with phosphorylated STAT5 present in the cytoplasm, fine-tunes the nuclear levels of constitutively activated STAT5, assuring that these levels remain within the range that elicit a proliferation rather than a maturation response. They also reinforce the general impression that Ph-negative MPN results from genetic mutations that impair the stress response.

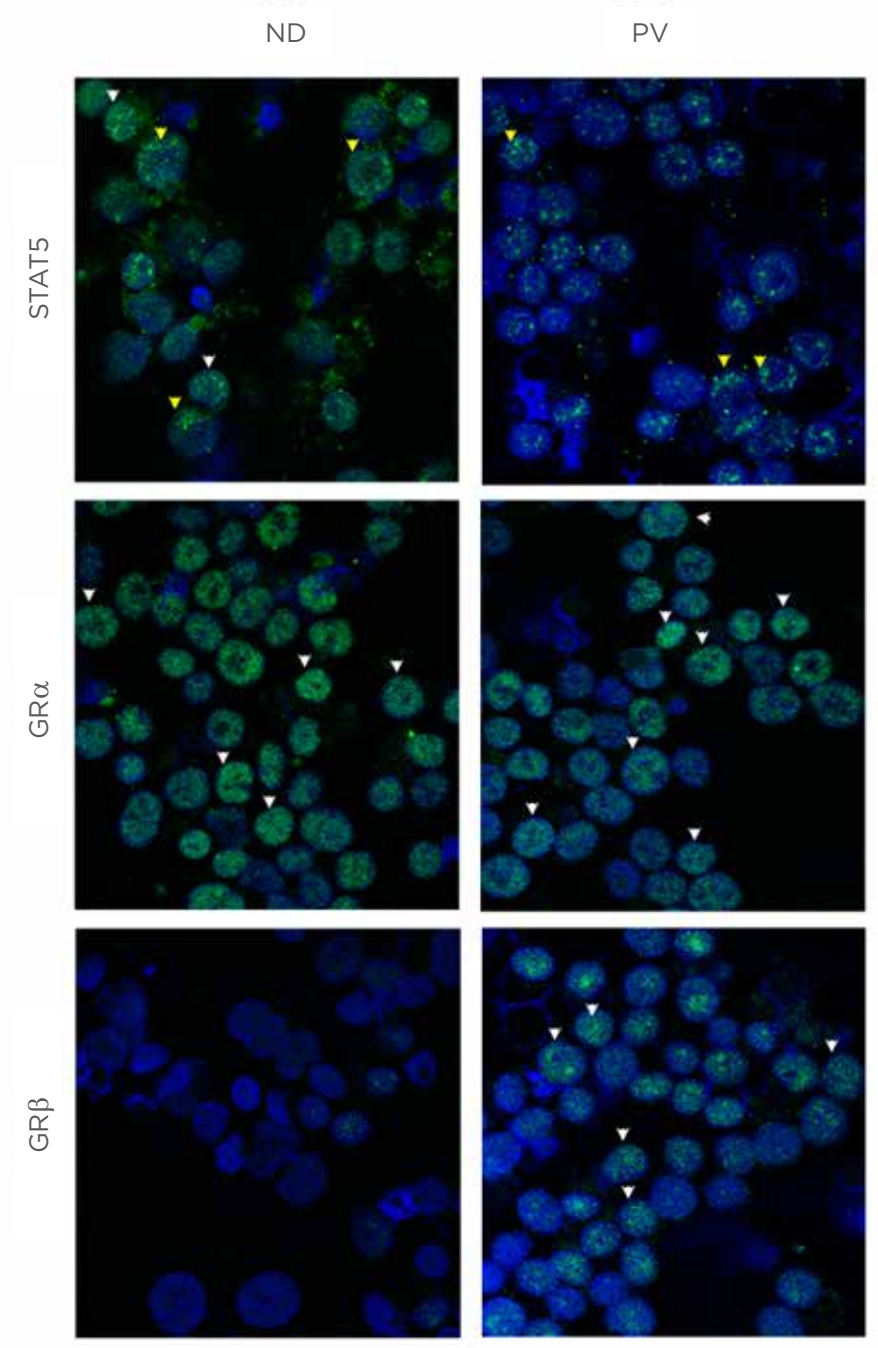

Figure 1: Confocal microscopy showing the cellular localisation of signal transducer and activator of transcription 5 (STAT5), glucocorticoid receptor alpha (GRα), and GRβ (in green) in erythroblasts expanded ex-vivo from Normal Donors (ND) with dexamethasone and from JAK2-mutated polycythaemia vera (PV) patients without dexamethasone.

Nuclei are evidentiated by DAPI staining. Erythroblasts from ND expanded with dexamethasone contain STAT5 both in the cytoplasm (yellow arrow) and in the nucleus (white arrows), GRα mainly in the nucleus, and do not express GRβ. Erythroblasts from PV patients contain STAT5 mainly in the cytoplasm, and GRα and GRβ mainly in the nucleus where the two subunits likely form a transcriptionally inactive complex. For reasons unknown at the time of this publication, a similar cytoplasmic-restricted STAT5 localisation and nuclear-restricted GRα localisation were also observed in erythroblasts expanded from JAK2-negative primary myelofibrosis patients that did not express GRβ (unpublished results).

Modified from Varricchio L et al.[31]

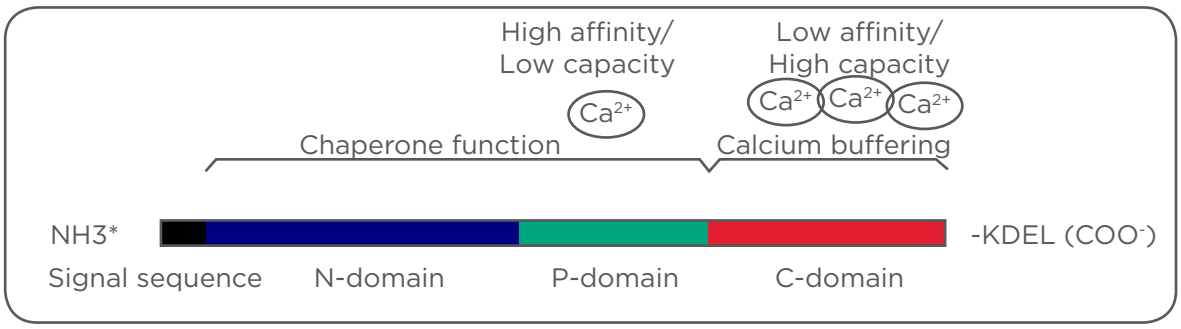

Figure 2: Structure of calreticulin (CALR) protein. Schematic representation of the structural domains of CALR.

The protein has at least three domains: N-domain in blue; the P-domain in green; and the C-domain in red. The protein contains an N-terminal amino acid signal sequence (black box) and a C-terminal KDEL endoplasmic reticulum retrieval signal.

Modified from Michalak et al.[41]

STRUCTURE AND BIOLOGICAL FUNCTION OF CALR

The CALR protein was first isolated by Ostwald and MacLennan in 1974[38] and its gene cloned by Smith and Koch[39] and Fliegel et al.[40] in 1989. The CALR gene is located on chromosome 19p13.2 and contains nine exons.

The CALR protein has three main functional domains (Figure 2). The first amino (N)-terminal domain (residues 1–180) contains a highly conserved amino acid sequence responsible for interactions with other proteins. This domain is homologous to that found in proteins with chaperone functions. The second domain is the central or P domain (residues 181–290) and is rich in the amino acid proline. It contains the region that binds calcium (Ca^{2+}) with high affinity and a lectin-like chaperone domain. The third carboxyl (C)-terminal domain (residues 291–400) is rich in acidic amino acids that confer to CALR its capacity to bind Ca^{2+} with low affinity. This domain regulates the Ca^{2+} levels in the endoplasmic reticulum (ER). This domain also contains the four amino acids KDEL (lysine, aspartate, glutamate, and leucine) motif that serves as a retention protein signal that, in the Golgi, switches the fate of CALR and its associated partners from extracellular secretion to ER localisation.[41]

Within the cell, CALR has multiple localisation sites and shuttles among different compartments (cytoplasm, ER, Golgi, cell membrane, nucleus, etc.) in response to its Ca^{2+} binding levels. The basic function of CALR is to bind Ca^{2+} in the ER (it binds >50% of the Ca^{2+} present in the ER). On the basis of these levels, it binds to newly synthesised proteins, assuring that they are properly folded, become proteasome-resistant, and reach their functional intracellular location. Therefore, CALR may be considered a sensor that, on the basis of the intracellular Ca^{2+} levels, fine-tunes the concentrations of other proteins in the various cellular compartments, allowing them to elicit the programmed cellular response. As such, CALR does not have a 'unique' biological function but cooperates with its multiple partners in the regulation of all cellular functions (proliferation, apoptosis, phagocytosis, gene transcription, etc.).

The role of CALR in the control of cell proliferation has been best described in the endothelial system. CALR expression is specifically upregulated in the heart during the middle stages of embryogenesis[42] and mice lacking CALR have an embryonically lethal phenotype with impaired heart development.[43] The survival functions of CALR are exerted through its partner p53. CALR has been shown to regulate p53 expression, localisation, and function. The observation that mouse CALR deficient embryonic fibroblasts (*calr*$^{-/-}$ cells) express significantly lower levels of p53 protein indicates possible p53 degradation in absence of CALR. These cells are also more resistant to apoptosis induced by ultraviolet light exposure.[44]

For their clinical relevance, the functions of CALR in phagocytosis have been extensively

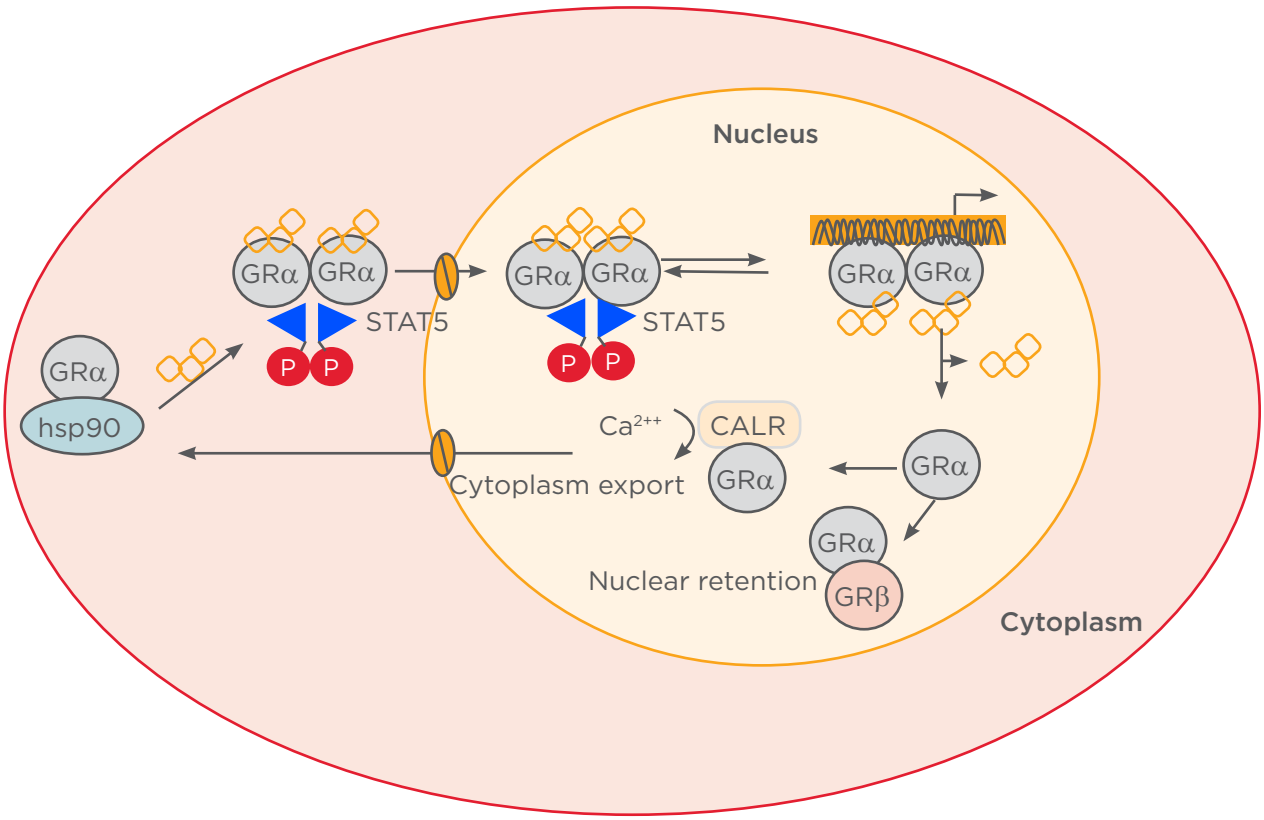

Figure 3: Calreticulin (CALR)-dependent trafficking of glucocorticoid receptor (GR) between the nucleus and the cytoplasm.

This function of CALR is Ca^{2+} dependent. Ligand binding to GRα leads to dissociation of a cytoplasmic complex interacting with GRα. Ligand-bound GRα rapidly translocates into the nucleus where it activates gene expression by directly binding to specific consensus sequences, either as a homodimer or a heterodimer with other transcription factors, such as STAT5. CALR inhibit the transcriptional activity of GR by masking its DNA binding domain and by facilitating its translocation from the nucleus to the cytoplasm. On the other hand, expression of GRβ leads to formation of a GRα/GRβ complex that is constitutively retained in the nucleus.

studied. In eukaryotes, CALR is the second signal - the membrane phospholipid phosphatidylserine being the first - present on apoptotic cells to be recognised by phagocytes. This function is highly conserved and is exerted in organisms as distant as *Drosophila*[45] and man.[46,47] A great variety of human cancer cells, including leukaemia blasts and progenitor cells from myelodysplastic syndrome, express increased levels of CALR; this increased expression exerts a pro-phagocytic signal that counter-balances the 'eat-me-not' signal provided by CD47.[46] In addition, Obeid et al.[47] have shown that when expressed on the cell surface, CALR may serve as an 'eat me' signal that allows dying cells to be recognised, ingested, and processed by dendritic cells. Based on these discoveries, molecules promoting apoptotic clearance of cancer cells, including leukaemic cells, through modulation of CALR/CD47 interaction have been devised.[48]

In contrast with JAK2, much less is known on the function of CALR in hematopoiesis. These functions may be inferred from studies with *calr*$^{-/-}$ cells indicating that CALR exerts an important function in the regulation of nuclear localisation and transcriptional activity of GR,[49,50] the nuclear receptor controlling the response of the hematopoietic system to stress. Both immunocytochemistry and sub-cellular fractionation studies have indicated that CALR may be localised in the nucleus[51,52] where it exerts the function to reset the cellular response of nuclear receptors, including that of GR (Figure 3). On one hand, CALR, through its N-terminal domain, binds to the DBD of GR, preventing its binding to glucocorticoid response elements (GRE) and activation of GR target genes.[49]

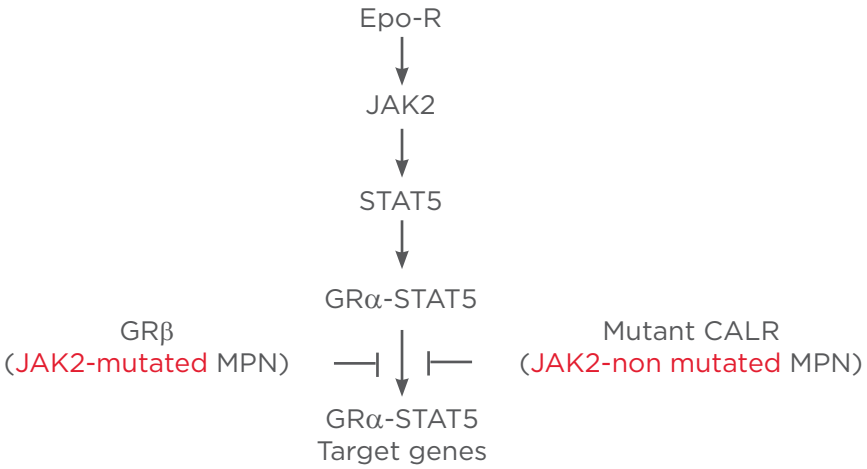

Figure 4: A unifying model for suppression of the expression of glucocorticoid receptor (GR)-target genes in JAK2-mutated (expression of the dominant negative GRβ isoform) and JAK2-non mutated (loss-of-function mutations) in myeloproliferative neoplasms patients.
In both cases, GRα is constitutively retained in the nucleus. In the case of JAK2-mutated patients, GRα is retained in the nucleus by the GRβ isoform. In the case of JAK2-non mutated patients, it is retained in the nucleus because the mutated CALR does not migrate to the nucleus and cannot exert its GR cytoplasm export function.

On the other hand, in response to changes in Ca^{2+} binding, CALR regulates the export of GR from the nucleus to the cytoplasm, enabling GR to respond to novel glucocorticoid stimulation[53] and to shuttle additional STAT5 to the nucleus. The function to export GR to the cytoplasm in response to Ca^{2+} is exerted by the KDEL in the C-terminal domain of the protein.

These data indicate that the role of CALR in the regulation of GRα activity (nuclear export) is antagonistic with that of GRβ (nuclear retention). It may be, therefore, hypothesised that while the function of GRβ in erythropoiesis is to suppress the cellular response to glucocorticoids, that of CALR is to prime erythroid cells to respond to glucocorticoids by increasing the amount of GRα present in the cytoplasm.

BIOLOGICAL CONSEQUENCES OF CALR MUTATIONS IN MPN

The limited number of studies on the function of CALR in hematopoiesis prevents a comprehensive analysis of the pathobiological consequences of CALR mutations in MPN. Some insights on these consequences may be inferred by the biological data included in the recent papers by Klampfl et al.[15] and Nangalia et al.[16] The CALR mutations found in MPN encode a protein lacking the C-terminal regions including the KDEL signal and the low affinity Ca^{2+} binding sites suggesting that this protein is mislocated and responds poorly to Ca^{2+}. In agreement with this hypothesis, myeloid cells from MPN patients carrying CALR mutations, and cells expressing ectopic levels of the mutant genes, express the mutated protein at normal levels in the Golgi and on the cell surface but at levels greater than normal in the ER, where they may acquire novel functions, and lower than normal in the nucleus, where they may instead lose function.[15,16] These reports also showed that CALR mutations increase STAT5 phosphorylation conferring cytokine hypersensitivity, suggesting that in the cytoplasm the mutated CALR acquires the novel function to constitutively activate the hemopoietic cytokine signalling pathway. Further studies are necessary to decipher the mechanisms that result in cytokine-independent signalling activation in hematopoietic cells harbouring CALR mutations.

The extensive data indicating that CALR resets GR signalling by favouring its export from the nucleus suggest that the nuclear function lost by the mutated protein may be the regulation of GR activity. This interpretation is consistent with our unpublished observations that STAT5 and GRα were similarly mislocated in erythroid cells from JAK2-positive PV patients that expressed GRβ and in those from JAK2-negative MPN patients

that did not express GRβ but probably expressed the mutated form of CALR (Figure 1). This interpretation provides a unifying mechanism for the hyperproliferation observed in JAK2-mutated and in CALR-mutated MPN patients, according to which in both cases the hyperproliferative response is elicited by constitutive STAT5 activation quenched within levels consistent with a proliferative response by a mechanism, expression of GRβ or CALR mutation, that restrains the localisation of GRα in the cytoplasm (Figure 4). This hypothesis must be tested by direct experiments.

FUTURE THERAPEUTIC STRATEGIES FOR MPN

The different forms of Ph-negative MPN have a heterogeneous prognosis; this ranges from the benign course of PV, usually well controlled by minimal interventions (phlebotomy), to the more challenging PMF, presently cured mainly by bone marrow transplantation. This variability likely reflects the interaction between the variegation of molecular lesions underscoring the insurgency of the disease and genetic factors, such as GR polymorphism and possibly other gene modifiers.[10,13,36] These considerations suggest that MPN patients may represent suitable candidates for the development of personalised therapies. This concept is exemplified by recent retrospective analyses of European and USA patients that have identified the presence of mutations in EZH2, ASXL1, SRSF2, and IDH1/2 as possible biomarkers to predict overall lower survival and increased risk of leukaemic transformation in PMF.[13]

Unexpectedly, the results of a large clinical trial have recently identified that the JAK2 inhibitor ruxolitinib is effective in reducing spleen size and disease manifestation both in JAK2-mutated and JAK2-non-mutated patients,[54] and the JAK2 inhibitor fedratinib reduced splenomegaly in two CALR-mutated patients.[55] As discussed in Cazzola and Kralovics' paper,[56] these results confirm the concept discussed here that mutations leading to development of MPN occur along a unifying pathogenetic pathway including JAK2 and CALR. Since the clinical response to JAK2 inhibitors is far from optimal, the discovery of CALR, as the other side of the Alice mirror suggests, highlights that drugs that will improve recognition and clearance of the MPN clone by the immune system (and/or target additional mutations [IDH1/2] or the genetic background of these patients) should be considered as candidates in combination with JAK2 inhibitors for personalised therapy in MPN.

CONCLUSION

In conclusion, loss-of-function CALR mutations, and gain-of-function JAK2/MPL mutations identified in MPN both target STAT5, suggesting that CALR and MPL/JAK2 represent the negative and positive effectors of a unique mechanism that controls proliferation of hematopoietic cells in response to stress, the alteration of which results in MPN.

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