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REVIEW

BamHI-A rightward frame 1, an Epstein–Barr virus-encoded oncogene and immune modulator

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SUMMARY

Epstein–Barr virus (EBV) causes several benign and malignant disorders of lymphoid and epithelial origin. EBV-related tumors display distinct patterns of viral latent gene expression, of which the BamHI-A rightward frame 1 (BARF1) is selectively expressed in carcinomas, regulated by cellular differentiation factors including ΔNp63α. BARF1 functions as a viral oncogene, immortalizing and transforming epithelial cells of different origin by acting as a mitogenic growth factor, inducing cyclin-D expression, and up-regulating antiapoptotic Bcl-2, stimulating host cell growth and survival. In addition, secreted hexameric BARF1 has immune evasive properties, functionally corrupting macrophage colony stimulating factor, as supported by recent functional and structural data. Therefore, BARF1, an intracellular and secreted protein, not only has multiple pathogenic functions but also can function as a target for immune responses. Deciphering the role of BARF1 in EBV biology will contribute to novel diagnostic and treatment options for EBV-driven carcinomas. Herein, we discuss recent insights on the regulation of BARF1 expression and aspects of structure-function relating to its oncogenic and immune suppressive properties. © 2013 The Authors. Reviews in Medical Virology published by John Wiley & Sons, Ltd.

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INTRODUCTION

Epstein–Barr virus (EBV), a human gamma herpesvirus, infects over 90% of the world population and persists in its host for life, usually without complications. Primary infection frequently goes unnoticed early in life but may cause infectious mononucleosis if acquired during adolescence or adulthood. The virus initially infects submucosal B cells in the nasopharynx/oropharynx and transforms these into latently infected long lived memory cells, which are essential for virus persistence. EBV has dual tropism *in vivo*, infecting mainly B lymphocytes and epithelial cells [1–3]. This dual tropism is reflected by its association with several lymphomas and carcinomas, where distinct viral gene products support viral genome maintenance and contribute to the oncogenic process [4–7]. The immune system strongly responds to EBV-infected cells maintaining a lifelong well-balanced equilibrium, but the virus can escape into latency to evade elimination [7,8]. This latent state, which is regulated by a small subset of viral genes, enables the virus to persist lifelong in the human host [4].

Since its discovery and proven oncogenic role in African Burkitt lymphoma [9,10], EBV has been found to play a diverse and complex role in multiple chronic and malignant diseases occurring worldwide [11,12]. EBV causes most lymphoproliferative diseases that occur in immunocompromised individuals [13,14] and is causally linked to other lymphoid malignancies, i.c. 40–100% of classical Hodgkin's disease [14], 10–30% of non-Hodgkin lymphomas, and 100% of B-cell lymphomas in the elderly as well as extranodal T-cell/NK-cell

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Abbreviations used
BARF-1, BamH1-A rightward frame 1; BARTs, BamH1-A rightward transcripts; CTL, CD8+ T cells; DC, dendritic cell; DM, double-minute; FGFR, fibroblast growth factor receptor; GC, gastric cancer; HGFR, hepatocyte growth factor receptor; hTid, homolog of Drosophila tumorous imaginal disk 1; LMP1, latent membrane protein 1; M-CSF, macrophage colony stimulating factor; M-CSFR, macrophage colony stimulating factor receptor; NASBA, nucleic acid sequence-based amplification; NHL, non-Hodgkin lymphoma; NPC, nasopharyngeal carcinoma; PDGFR, platelet-derived growth factor receptor; rhLCV, rhesus lymphocryptovirus; sBARF1, soluble hexameric molecule BamH1-A rightward frame 1; TLR3, toll-like receptor 3; VEGFR, vascular endothelial growth factor receptor.

lymphomas in immunocompetent persons [4,15]. Moreover, EBV has a proven etiological role in certain epithelial malignancies, including 100% of undifferentiated nasopharyngeal carcinoma (NPC) [6,7,16] and approximately 10% of gastric cancer (GC) [4,17–19] worldwide. EBV-linked GC is considered a distinct disease entity compared with *Helicobacter pylori*-associated GC. EBV has been associated with breast cancer [20,21] and hepatocellular carcinoma [22,23], but supporting evidence is limited or contrary [24,25].

Besides two abundant small noncoding RNAs and about 44 distinct viral microRNAs that are widely expressed, the 172 000 basepair EBV genome contains >80 protein encoding reading frames that are largely silenced by epigenetic mechanisms [26]. Only a few latent genes are expressed in EBV-related malignancies in distinct expression patterns, called latency types [4,26,27]. Strikingly, EBV-positive epithelial malignancies show selective and abundant expression of a viral gene encoded in the BamHI-A rightward frame 1, encoding a polypeptide of Mr 29 000–33 000 called BARF1 [28–31]. In B cells and lymphomas, BARF1 expression is generally undetectable but can be induced during viral lytic replication [32,33].

In NPC, BARF1 may function as oncogene, parallel to the more widely studied latent membrane protein 1 (LMP1) [34]. In EBV-positive GC, BARF1 is expressed in the absence of LMP1, possibly functioning as the main EBV oncogene in this disease [31]. Since its first description, several distinct functions have been assigned to BARF1, varying from oncogenic to antiapoptotic and immune modulating. A schematic overview of BARF1 functions is given in Figure 1.

This review will discuss the role of BARF1 as a viral oncogene and immune modulator in EBV-driven carcinogenesis. It describes recent insights in BARF1 structure-function and its transcriptional regulation, as well as its potential use as a carcinoma-specific diagnostic factor.

BamHI-A rightward frame 1 protein structure

The first attempts to translate the BARF1 open reading frame (Figure 2A) *in vitro* revealed an intracellular monomeric polypeptide of Mr 26 000–33 000 [35,36], which was partially secreted in culture medium when linked to an immunoglobulin Fc-tail [37]. Low level BARF1 protein expression was observed in Burkitt lymphoma cells induced into the lytic phase but not in uninduced cells. [38]. Although initially a nuclear localization was reported [36], BARF1 protein was enriched in membrane fractions, and immunofluorescence analysis on fixed permeabilized cells showed a cytoplasmic Golgi localization [39–42]. Later reports confirmed that the monomeric form of BARF1 is around Mr 29 000 with a lower band at Mr 25 000 [40], and recent data show that BARF1 is distinctly glycosylated and

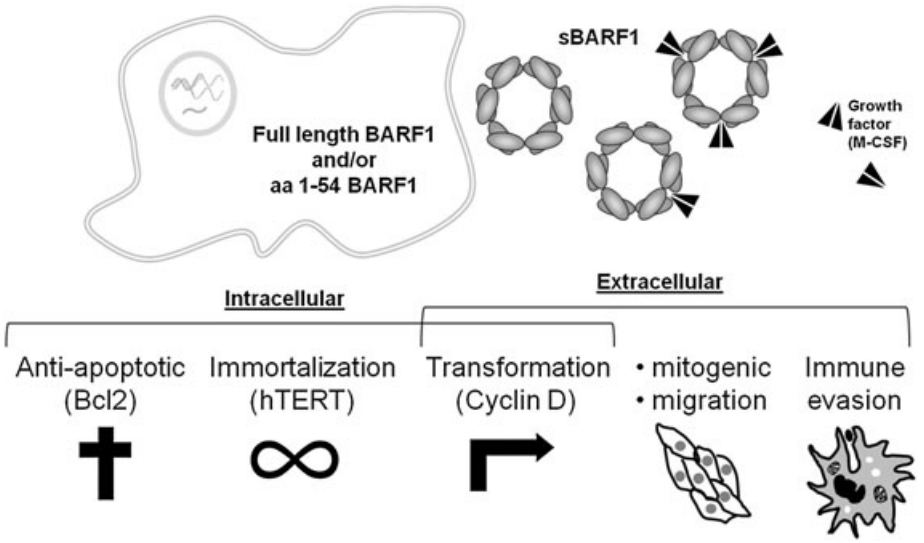

Figure 1. Putative functions of BamHI-A rightward frame 1. Schematic representation of functions ascribed to BamHI-A rightward frame 1

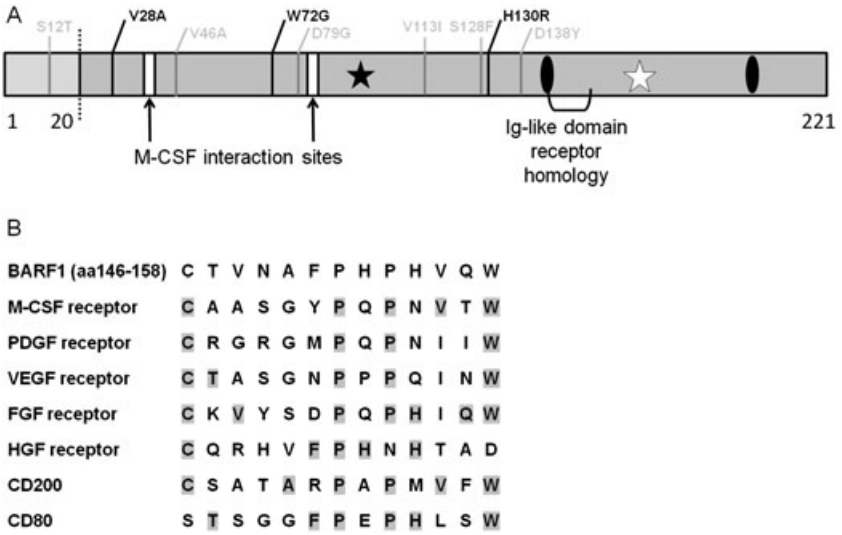

Figure 2. Mutations and homology domain of BamHI-A rightward frame 1(BARF1) protein. (A) Schematic representation of the BARF1 221 peptide. Left of the dotted line is the intracellular N-terminal part. Frequent amino acid mutations are depicted in black, rare mutations are depicted in gray. White bars; structural loops that interact with M-CSF, black asterisk; high mannose N-linked glycosylation at N95, white asterisk; predicted O-glycosylation site at T169, black ovals; C146 and C201 involved in folding and oligomerization. (B) BARF1 has sequence homology with a conserved domain found in several growth factor receptors. BARF1 aa146–158 is shown with homologous regions from the indicated receptors. Adapted from [37,48,53]

rapidly and completely secreted as a soluble hexameric molecule BamH1-A rightward frame 1 (sBARF1) when expressed in human epithelial cells [43–45]. A recent study suggests cellular uptake of secreted sBARF1 with subsequent nuclear localization [46], which remains to be confirmed.

The sBARF1 has high mannose N-linked glycosylation at Asn95, resulting in a sugar chain located at the inner side of the hexameric sBARF1 ring (Figure 3) [44,47–49]. Asn95 N-glycosylation is essential for folding and secretion [47]. An additional O-linked glycosylation site is present at Thr169, which carries a sialic end-group [44,48,49]. Secreted BARF1 can be phosphorylated on both serine and threonine residues [49], but this remains unconfirmed [44]. The cell-type expressing BARF1 may influence post-translational modifications, explaining small differences between publications. The human homolog of Drosophila tumorous imaginal disk 1 (hTid) was found to interact with BARF1, acting as a chaperone for proper folding and promoting its secretion as a monomeric protein [47]. However, other studies on BARF1 expressing cell lines indicate rapid secretion of hexameric sBARF1, despite the presence of innate hTid [44,49]. In human epithelial cells, most of the BARF1 translational product is rapidly and efficiently processed and cleaved from its putative aa1-20 leader sequence, yielding a secreted protein. Density gradient analysis revealed that sBARF1 is secreted as a hexameric complex of Mr 150 000–240 000[44,48]. In cells blocked for protein secretion by Golgi-modifiers monensin or brefeldin A, the BARF1 remains localized to perinuclear regions with reduced glycosylation, overlapping the endoplasmic reticulum. Upon release from blocking, BARF1 passes quickly through the Golgi system, paralleled by the addition of high mannose N-linked glycosylation, and can be detected in the cell membrane at later time points [44,47,49]. The post-cleavage fate of the intracellular aa1-20 leader sequence remains undefined, but some important functions are assigned to this fragment [50].

The BARF1 gene sequencing from NPC tumor samples revealed sequence variation, with 80.3% of samples having specific amino acid mutations compared with 33.3% in non-NPC isolates. The main substitutions found in Indonesian NPC samples are V29A, W72G, and H130R, but these conversions are not likely to change the protein tertiary structure or function [51]. In northern Chinese samples, the V29A mutation is the most prominent conversion and is found more frequent in NPC than in GC and healthy controls [52]. Four other amino acid changes (V46A, D79G, V113I, and D138Y) were detected in some samples (Figure 2A).

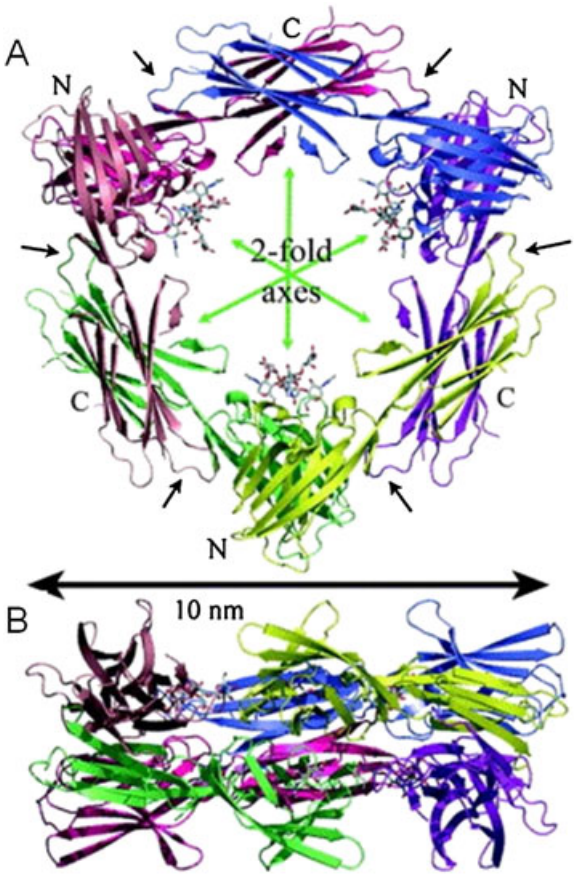

Figure 3. Cartoon of the soluble hexameric molecule BamH1-A rightward frame 1 (BARF1) hexameric structure. [48] (A) Top view of the BARF1 hexamer, the N-linked glycosylation is shown in a stick formation, (B) side view of the BARF1 hexamer

None of the mutations is located in functional relevant domains of BARF1 (see in the succeeding text).

Cysteïnes 146 and 201 are involved in folding and oligomerization [47]. Crystallographic analysis of sBARF1 (aa21–aa220) showed that sBARF1 forms hexameric rings in which three dimer molecules are interconnected head to tail, arranged in two layers (Figure 3). The C-terminal domains interact via hydrophobic contacts and hydrogen bonds, whereas the N-terminal domain also involves salt bridges representing a unique folding pattern [48]. Early studies on BARF1 protein function were carried out with an Fc-tagged protein, which might have affected the 3D-hexameric conformation. The two N-terminal domains of the sBARF1 dimer bind each other similar to the dimerization domain of CD80, a co-stimulatory factor for T cells, but the residues forming the interaction have no similarities. The BARF1 protein has defined regions of homology to the tyrosine kinase receptor family (Figure 2B) and immunoglobulin super-family members CD80 and CD200, both having immunoregulatory and cell activating properties upon binding to their receptor [37,48,53]. However, the relative orientations of the immunoglobulin-like domains differ between CD80 and BARF1 [48]. No study has yet addressed the latter homology at a functional level. The homology of BARF1 with the c-fms (the M-CSF receptor) will be discussed in detail later.

BamHI-A rightward frame 1 transcriptional regulation

The EBV gene expression is controlled by epigenetic modulation, and the majority of the EBV genome is methylated in latently infected cells [54]. The BARF1 promoter region was found to be highly methylated, both in carcinoma and B-cell lines [55], indicating that BARF1 transcription activator(s) must be able to overcome methylation-induced repression. In EBV-infected B cells, BARF1 is considered an early lytic gene, whose expression is triggered by immediate early proteins BZLF1 (Z, Zta, ZEBRA, and EB1) and BRLF1 (R and Rta). Z and R are transcription factors that stimulate their own expression, reciprocally activate each other, and cooperatively induce expression of other early lytic viral proteins, allowing the virus to replicate [56,57]. Z has enhanced ability to bind to methylated promoters [58]. The Z-responsive elements in the BARF1 promoter are reversely oriented [59], and Z was found to only moderately transactivate the BARF1 promoter [55]. R preferentially activates unmethylated lytic promoters; however, methylation does not inhibit DNA binding by R. Multiple potential R binding sites are present in the BARF1 promoter region [55], and Heilman *et al*. showed that R binds the bidirectional BALF2/BARF1 promoter [60]. R was defined as the viral factor directly responsible for BARF1 transactivation upon lytic induction, and BARF1 mRNA can be detected within 6 h after transfection with an R expression vector [55], confirming its classification as early gene [61]. A schematic view of BARF1 regulation is given in Figure 4.

Thus, during lytic replication, BARF1 expression is regulated by R, yet BARF1 is also expressed during latency. However, this occurs almost exclusively in undifferentiated epithelial tumors suggesting that

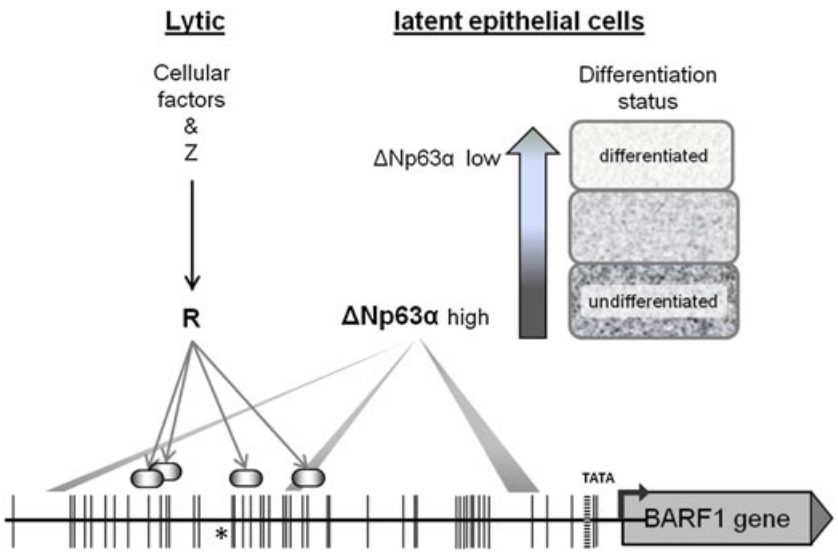

Figure 4. Schematic representation of BamHI-A rightward frame 1 (BARF1) transcriptional regulation. During lytic reactivation BARF1 is directly transactivated by the immediate early transcription factor R. Recent findings indicate that the differentiation marker ΔNp63α might be the cell type-specific transcription regulator enabling BARF1 transcription in latent in epithelial cells. The BARF1 promoter region is depicted up to −679 nucleotides relative to the BARF1 start-codon encoding methionine (ATG) start site and black vertical lines indicate methylation sites. Arrows point to the R binding sites in the BARF1 promoter [55], shaded bars indicate sites likely to be the most important for ΔNp63α BARF1 transactivation [62]

BARF1 promoter activation is cell type-specific and may involve epithelial cell-specific transcription factors. Therefore, we recently explored potential candidates involved in epithelial-specific BARF1 expression. The BARF1 gene control region harbors multiple p53-p63-p73 family binding sites. Indeed, from this family of transcription factors, we identified the epithelial differentiation marker ΔNp63α as the major activator of BARF1 transcription in the epithelial background, functioning independently of virus lytic activation [62]. ΔNp63α is a key regulator of epithelial cell development and differentiation [63], and overexpression of ΔNp63 is observed in nasopharyngeal carcinoma [64–66]. Both LMP1 and LMP2A are involved in activation of ΔNp63α transcription [67–70]. LMP1 down-regulates cellular microRNA 203, leading to higher levels of ΔNp63α [71]. Whereas p63 is expressed in lymphoid cells, the dominant forms of p63 detected in B cells and lymphoma tissues are TA-p63 isoforms and not the ΔNp63α isoform [72]. ΔNp63α is a stable factor during EBV latency in epithelial cells, and direct binding of ΔNp63α to the BARF1 control region enhances transcriptional activation in a reporter construct. However, overexpression of ΔNp63α was not sufficient to activate BARF1 expression in the context of the intact viral genome. Other cofactors might be required for full BARF1 upregulation by ΔNp63α in the epithelial cell background. The undifferentiated character of EBV-driven malignancies might be important to maintain expression of BARF1.

BamHI-A rightward frame 1 expression in Epstein–Barr virus-related malignancies

The BARF1 gene is consistently transcribed in EBV-linked carcinomas but virtually silent in EBV-driven lymphomas [29]. The abundant transcriptional activity within the BamH1-A region of the EBV genome in NPC tumors was first described in 1989 [73]. Subsequent work dissected these transcripts into a family of BamH1-A rightward transcripts (BARTs), later identified as precursors of EBV miRNA's [74,75], differently spliced transcripts encoding putative proteins with speculative function, such as RK-BARF0, RPMS1, and A73 [76–79], and a nonspliced transcript encoding the BARF1 protein.

The BARF1 transcription in NPC tissue was first demonstrated using reverse transcription polymerase chain reaction in a small cohort of North African NPC patients [80] and subsequently confirmed by nucleic acid sequence-based amplification (NASBA) (Figure 5), a technique allowing

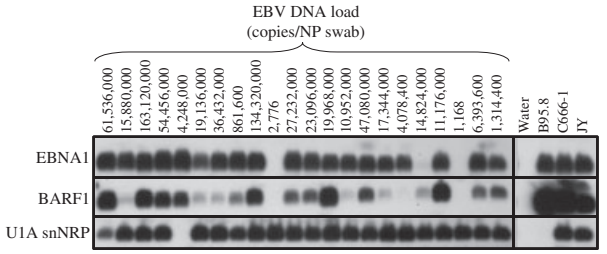

Figure 5. BamHI-A rightward frame 1 mRNA is abundantly detectable in nasopharyngeal (NP) brushings. [30] Epstein–Barr nuclear antigen 1 and BamHI-A rightward frame 1 RNA expression in NP brushings samples of NP carcinoma patients as determined by nucleic acid sequence-based amplification, in relation to Epstein–Barr virus DNA load. Shown is an autoradiogram of nucleic acid sequence-based amplification products hybridized with a radioactive-labeled internal oligonucleotide probe

specific nonspliced RNA analysis in a homologous DNA background [28,29]. BARF1 transcripts were also abundant in nasopharyngeal brushings of NPC patients, but not in healthy individuals, directly reflecting the presence of NPC cells *in situ* [30]. More recently, BARF1 transcripts were detected in microdissected NPC tumor biopsies [81], establishing BARF1 expression deeper in the tumor.

Approximately 10% of all gastric adenocarcinomas comprise monoclonal EBV-positive cells [5,19]. Zur Hausen *et al*. first demonstrated BARF1 expression in EBV-positive GC [31], later confirmed by Wang *et al*. [82]. Seto *et al*. [17] showed that BARF1 in GC cells is expressed as a true latent gene. GC has a unique EBV latency pattern without expression of LMP1 leaving BARF1 as the major EBV oncogene in these tumors [31]. In all carcinoma cells, BARF1 is expressed in absence of lytic cycle gene expression [17,83–85].

Nasal T-cell/NK-cell lymphoma is a rare and aggressive subtype of non-Hodgkin lymphoma (NHL), and particularly, the nasal type is closely associated with EBV [4,5,86–89]. Zhang *et al*. found BARF1 mRNA in EBV-positive T-cell/NK-cell lines [89]. Because these T-cell/NK-cell lines also expressed other early lytic genes, BARF1 expression may be induced by the immediate early gene R, which remains to be defined.

Several groups have investigated a role of EBV in breast cancer [20,21,90,91]. In two studies, BARF1 mRNA was detected in roughly half of the tumors analyzed [20,91]. However, EBV presence in breast cancer may be linked to sporadic lytic replication in ductal cells rather than associating with the proliferating tumor cells [24]. A similar situation may hold for the link between EBV and hepatocellular carcinoma, where conflicting data have been published [23,25].

Although expression of BARF1 mRNA in NPC and GC biopsies is clearly described, detection of BARF1 protein proved to be more challenging. BARF1 protein was detected in whole tissue lysates in only two of seven NPC tissue samples by immunoblot analysis [17], and only one study managed to detect BARF1 protein by immunohistochemistry [83], showing uniform cytoplasmic and membrane staining of NPC biopsy tissue. However, this may be due to false-positive staining (unpublished data) [19]. In our hands, despite having various monoclonal and polyclonal antibodies with excellent specificity to detect distinct linear and conformational epitopes of the BARF1 protein, suited for detection of recombinant BARF1 in formalin-fixed, paraffin-embedded insect cells, we were not able to detect BARF1 at the protein level in NPC or GC tissues, proven to have BARF1 mRNA expression [92]. BARF1 protein is probably rapidly and completely secreted from tumor cells, as shown for sBARF1 transfected cells [40]. In 2007, evidence for presence of sBARF1 in serum and saliva of NPC patients was provided [45], but this remains to be confirmed to date (see succeeding text).

ONCOGENIC PROPERTIES OF BamHI-A RIGHTWARD FRAME 1

The role of BamHI-A rightward frame 1 in host cell immortalization and transformation

Griffin *et al*. first suggested BARF1 to be involved in EBV-driven carcinogenesis 25 years ago [93]. A BamHI-A fragment of the EBV genome, named P31 and later demonstrated to encode the BARF1 protein, was found to immortalize monkey kidney epithelial cells, surviving more than 200 passages and gaining the ability to grow in low-serum conditions. Subsequent studies showed that human epithelial cells could be immortalized by P31 as well [94,95]. Moreover, mouse fibroblast lines NIH-3 T3 and Balb/c-3 T3 were immortalized upon transfection with a plasmid containing the putative BARF1 open reading frame, and importantly, BARF1 transfected cells induced tumor formation in newborn rodents [35]. The related tumor tissues were BARF1 mRNA positive, but the BARF1 transgene was lost upon further *in vitro* passage. BARF1-

immortalized monkey kidney epithelial cells were tumorigenic in severe combined immunodeficiency (SCID) mice but not in nude mice [96]

To define which part of the BARF1 protein was essential for malignant transformation, Sheng *et al*. created six deletion mutants of the BARF1 gene [50]. The first 54 amino acids at the N-terminus, including the membrane-spanning domain, were found to be responsible for the malignant transformation of BARF1 in rodent fibroblasts. In addition, this region was also responsible for the upregulation of the cellular antiapoptotic protein Bcl-2, mediating escape from senescence upon serum deprivation and mediating growth in soft agar. These results strongly suggest a role of BARF1 in immortalization and malignant transformation *in vitro*. Our unpublished data on the growth properties of stable BARF1-expressing human embryonic kidney epithelial (HEK-293) cells confirm the BARF1-driven increased growth in soft agar (Figure 6) and altered migration properties.

Primate epithelial cells infected with NPC-derived EBV virions became immortalized, expressing both Epstein–Barr nuclear antigen 1 and BARF1, in contrast to their non-infected counterparts [97]. Recently, Seto *et al*. [98] investigated the malignant phenotype of EBV-negative NPC cells, infected with a genetically modified EBV expressing BARF1 at increased levels from a SV40 promoter. In these cells, BARF1 expression was found to contribute to the tumorigenicity of EBV-infected NPC-cell lines *in vivo*. LMP1, the other known EBV oncogene, was not expressed in both epithelial models.

Although BARF1 expression can induce malignant transformation in several cell types, not all BARF1 immortalized primary epithelial cells were found to be tumorigenic [42,97,99]. Recent studies showed that h-RAS coexpressing epithelial cell lines (PATAS and human epithelial cell line N69) become tumorigenic upon introduction of BARF1 suggesting that h-RAS expression synergizes with BARF1. After immortalization and transformation of PATAS cells by BARF1, activation of telomerase was observed, as well as activation of other signal pathways, including c-myc [100]. These results suggest that the transforming abilities of BARF1 are partly mediated by activating pro-oncogenic cellular pathways [101]. In BARF1-transfected human cervical cancer cells (Hela), activation of telomerase was observed, but not in macaque fibroblast-like cells, suggesting species differences in BARF1-induced telomerase activation [33,102]. Intracellular pathways mediating BARF1-driven epithelial cell transformation and carcinogenesis should be further clarified to define the oncogenic role of BARF1.

Upregulation of the transcription factor and proto-oncogen c-myc was also described in the BARF1-immortalized EBV-negative human B-lymphocyte line Louckes [41]. Inoculation of BARF1 transformed Louckes cells induced tumors with diffuse lymphoid phenotype in newborn rats and immune compromised mice, though not as aggressive as observed with transformed rodent fibroblasts. This process proved to be BARF1-specific, since cells loosing BARF1 expression were no longer tumorgenic. Also EBV-negative subclones of Burkitts lymphoma and EBV-negative Akata cell lines underwent malignant transformation upon transfection with BARF1 and were tumorgenic in SCID mice, like their EBV-positive Akata counterpart [103]. Using a BARF1 knock-out virus, two studies described that BARF1 has a negligible role in the transformation

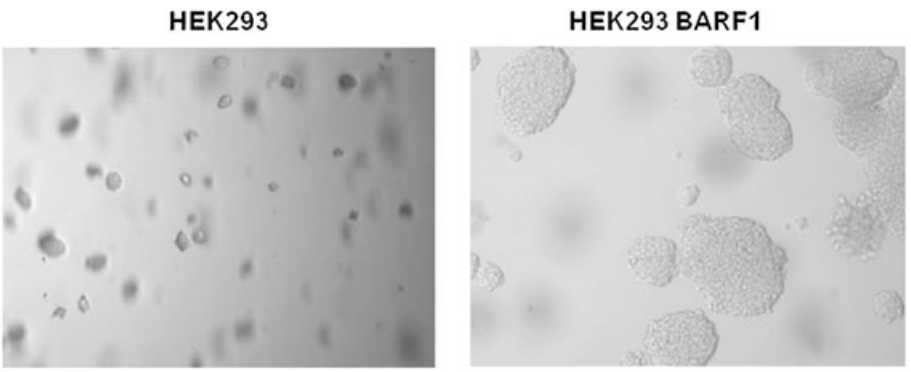

Figure 6. Anchorage-independent growth in soft agar. Unpublished results from our own group demonstrate contact independent growth of BamHI-A rightward frame 1 expressing 293HEK cells

or establishment of EBV latency in B cells. Cohen *et al*. [38] showed that addition of secreted BARF1 protein did not influence EBV transformation of B cells. Furthermore, an EBV virus with a BARF1-deleted genome was not impaired for B-cell transformation, and its related B-cell lines induced lymphoid tumors in SCID mice at the same rate as cells infected with wild-type (WT) EBV. In the closely related rhesus lymphocryptovirus (rhLCV) model, a minor role for BARF1 was found in establishing viral latency. rhLCV with truncated BARF1 was less efficient than WT virus for B-cell immortalization but only at low multiplicity of infection. Remarkably, this truncated BARF1 still contained the 54 N-terminal amino acids reported to be essential for malignant transformation of rodent fibroblasts [104]. These findings underline the different mechanisms of EBV-induced lymphomagenesis and carcinogenesis, the former preferentially involving LMP1 as oncogene and the latter involving BARF1, either with or without LMP1.

BamHI-A rightward frame 1 as mitogenic growth factor

A fundamental characteristic of tumors is their enhanced growth rate compared with normal tissues. BARF1-transfected mouse fibroblasts grow twofold to fourfold faster than control cells [35]. Similar results were found in human B cells [41] and primary monkey and human epithelial cells [50,94,95,100]. In some studies, the N-terminus seems to be essential for this growth-stimulating effect and considered responsible for the transforming properties. Cells transfected with BARF1 lacking the amino acids 1-54 stopped proliferating after 4 days and at low-serum growth conditions [50]. In other studies, autocrine cell growth was induced by secreted BARF1, which could be blocked by anti-BARF1 antibodies [105]. Moreover, sera of mice-carrying BARF1-positive human NPC tumor cells and semi-purified sBARF1 from NPC patient sera were described to have mitogenic activities, which could be specifically blocked by BARF1 antibodies [45]. Recently, uptake and cell cycle activation by sBARF1 was demonstrated in human keratinocytes [46].

These results indicate that BARF1 may exert its growth-stimulating effect both in cis and in trans. However, we and others could not confirm direct mitogenic effects of purified hexameric sBARF1 on either lymphoid or epithelial cells [106].

Transfection with BARF1 has been related to formation of double-minute (DM) extrachromosomal bodies, containing viral DNA [99], driving positive selection for DM-carrying cells. Furthermore, two recent microarray studies showed that BARF1 transfection into GC cell lines leads to deregulation of genes involved in cell proliferation, mitosis, and cell cycle regulation [107,108]. However, in one study, cell proliferation of non-transfected GC cells was not affected by BARF1 [107], whereas the other study found the BARF1-induced overexpression of Cyclin D1, a positive regulator of the cell cycle. *In vivo*, EBV-positive GC cells show upregulated Cyclin D1 protein expression [108]. These findings indicate a possible role for BARF1 in cell cycle deregulation.

The antiapoptotic role of BamHI-A rightward frame 1 in Epstein–Barr virus-related malignancies

The oncogenic role of BARF1 may involve inducing escape from apoptosis. Cells transfected with full length BARF1 escape from apoptosis induced by serum starvation, unlike cells transfected with a N-terminal 1-54 BARF1 deletion mutant, suggesting BARF1 to modulate the intrinsic apoptosis pathway. Gene expression profiling of BARF1-positive GC cells revealed down-regulation of defined caspases and upregulation of the antiapoptotic Bcl-2 protein [50,107]. By activating Bcl-2, the release of mitochondrial cytochrome-c is inhibited, which is necessary to recruit and activate effector caspases, the executioners of apoptosis [109]. Upregulation of Bcl-2 prevents cells from death induced by cytotoxic drugs such as Taxol, an activator of the intrinsic apoptotic pathway. BARF1-expressing GC cells were found to be resistant to Taxol-induced apoptosis [107]. An additional function of Taxol is to phosphorylate and inactivate Bcl-2 [110,111], which can be overcome by BARF1-expressing cells. *In vivo*, upregulation of Bcl-2 expression was observed in EBV-associated GC and NPC [108,112,113]. Because LMP1, also known to induce Bcl-2 expression, is not present in GC, this upregulation might directly relate to BARF1 expression [31,114].

Immune-modulating role of BamHI-A rightward frame 1

An immune-modulating role for BARF1 was first suggested by Strockbine *et al*. using basic local alignment search tool comparison of the BARF1

sequence to predict homologies to human proteins [37]. A small region at aa146-158 within BARF1 showed homology to the extracellular domain of the c-fms proto-oncogene, a member of the tyrosine kinase receptor family, which includes platelet-derived growth factor receptor (PDGFR), fibroblast (FGFR), hepatocyte (HGFR), vascular endothelial (VEGFR), and macrophage colony stimulating factor receptor (M-CSFR) (Figure 2B). BARF1 was shown to bind a ligand on the cell membrane of activated peripheral blood T cells, identified as the membrane-associated splice variant of human M-CSF.

Macrophage colony stimulating factor is a pleiotropic cytokine important not only for regulating viability, proliferation, and differentiation of mononuclear phagocytes including monocytes and macrophages but also for the differentiation of Langerhans and other dendritic cell (DC) subsets [115–118]. Macrophages are scavenger cells with pro-inflammatory and anti-inflammatory functions, which make them both ally and foe of tumor cells and a dangerous opponent for viruses.

Recombinant Fc-tagged monomeric BARF1 co-precipitated the α, β, and γ splice variants of M-CSF [37]. These results were recently confirmed with native hexameric sBARF1 [53]. Recombinant BARF1 inhibits proliferation of mouse bone marrow cells [37], and sBARF1 inhibits proliferation of the M-CSF-dependent human myeloid leukemia cell line MUTZ-3 in a dose dependent fashion, whereas granulocyte M-CSF-induced proliferation remains unaffected [53]. Tyrosine kinase analysis confirmed that sBARF1 inhibits M-CSFR downstream signaling [53]. In the rhLCV model, similar M-CSF-inhibiting and cell-immortalizing abilities of rhBARF1 were demonstrated [106], suggesting an evolutionary conserved function of sBARF1 as selective M-CSF scavenger, interfering with innate and adaptive immune processes. The sBARF1/M-CSF ratio needed to completely block M-CSF-dependent MUTZ-3 growth revealed that the complex should consist of one hexamer sBARF1 binding three M-CSF dimers [53]. This is in agreement with crystallography data of the sBARF1/M-CSF complex [48] as recently revealed by Shim *et al*. and Elegheert *et al*. [119,120].

The data imply that native sBARF1 interferes with human monocyte-macrophage differentiation [53]. Specific surface markers of normal macrophages were down-regulated, and cell viability was reduced when cultured in the presence of sBARF1 (Figure 7(A,B)). A feature of macrophages is the release of interferon alpha upon virus infection to stimulate different immune cells [121–124]. Recombinant BARF1 inhibited interferon alpha

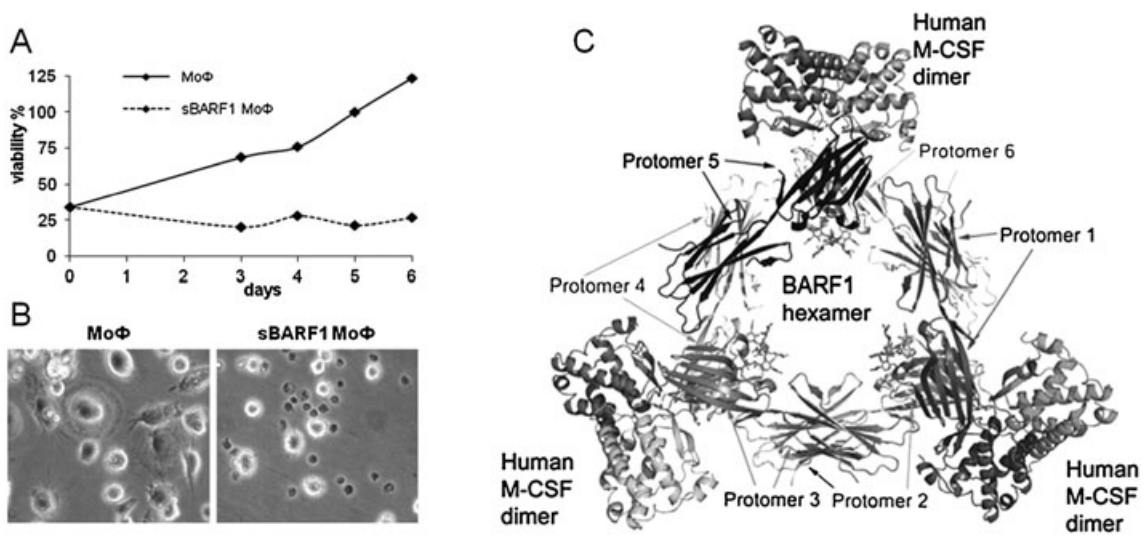

Figure 7. Soluble hexameric molecule BamH1-A rightward frame 1(sBARF1) is a macrophage colony stimulating factor (M-CSF) decoy receptor. (A) MTT proliferation test was used as readout for both increased cell numbers and level of differentiation. The inhibitory effect of sBARF1 on the monocyte to macrophage differentiation can be seen from day 3. The untreated cells showed increased viability, whereas the viability of sBARF1 treated macrophages remained the same in time. Adapted from [53]. (B) sBARF1 negatively influences monocyte to macrophage differentiation in response to M-CSF, here shown are the differences in morphology and viability between normal monocyte derived macrophages (MoΦ) and MoΦ differentiated in the presence of sBARF1 (sBARF1 MoΦ). Adapted from Hoebe *et al*. [53]. (C) Structure of the BARF1/M-CSF complex as by Shim *et al*. [120]. One sBARF1 hexamer can bind three human M-CSF dimers

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release from human mononuclear cells upon stimulation of toll-like receptor 3 (TLR3) with poly-IC, mimicking viral infection [38]. Down-regulation of interferon alpha release may counteract immune responses to EBV-positive cells. Because the amount of tumor infiltrating macrophages is positively correlated with NPC prognosis [125,126], blocking of M-CSF by sBARF1 might be a possible mechanism for evasion from macrophage-mediated innate antitumor responses.

During viral lytic replication, sBARF1 inhibition of M-CSF might affect monocyte/macrophage particle opsonization and degradation, resulting in virus transmission rather than virus inactivation [53,127,128]. However, loss of BARF1 in the rhLCV model did not influence penetration of oral mucosa [104]. The same study indicated that the immune-modulating role of BARF1 is important for acute and persistent EBV infection [104].

Recent studies have characterized the BARF1/M-CSF interaction site, unexpectedly revealing that this site is opposite to the M-CSF receptor interaction site (Figure 7C). Two BARF1 N-terminal loops form structural "tweezers" that grab M-CSF at the dimer interface [119,120]. Mutations of the interface loops eliminated M-CSF-dependent MUTZ-3 growth [53]. sBARF1 binds M-CSF at the beta sheets and not at the alpha helices like the M-CSF receptor, locking the M-CSF dimer in an unfavorable global orientation for receptor binding. Conflicting results exist on whether BARF1-complexed M-CSF is still able to bind (but not signal) the M-CSF receptor, possibly because BARF1 from different host backgrounds was used [119,120]. A role for carbohydrate side-chains is suggested, indicating the need for using native glycosylated BARF1 molecules produced in a human cell background.

Next to the interaction with M-CSF and influence on the myeloid cell lineage, sBARF1 may affect other immune cells. BARF1 has 18% homology with CD80, also known as B7-1, suggesting that the BARF1 gene could have originated from CD80 during host-virus coevolution [48]. B7-1 is a costimulator for T-cell activation or inhibition depending upon interaction with CD28 or CD152 (CTLA-4), respectively. Importantly, blockade of CD80 costimulation may provide yet another way for BARF1-positive cells to evade cellular immune responses. The possible T cell-silencing effect of sBARF1 needs to be further investigated.

Furthermore, a homology with CD200 was noted (M. Ressing and C. Kesmir, personal communication). Upon ligand-receptor binding, the CD200 receptor transmits inhibitory signals to the myeloid lineage [129,130], but CD200R is expressed on some T-cell subtypes as well [129]. CD200 deficient mice are more readily immunologically activated compared with WT control mice [130], and lack of CD200 signaling is associated with an enhanced immune response to virus infection [131,132]. Virus-encoded CD200 orthologues can be found in the genomes of multiple viruses, including herpes viruses, suggesting the existence of yet another viral means to modulate immune reactivity [133]. Blockage of CD200 may also enhance tumor growth [134], but more research is needed to establish BARF1 effects on the CD200 receptor.

We conclude that sBARF1 may be important for carcinoma cells in mediating evasion from innate and adaptive immune responses. Next to its function as a M-CSF decoy receptor, BARF1 might have alternative immune-modulating functions that require further analysis. Interestingly, EBV-driven carcinomas are usually characterized by a significant infiltrate of activated T cells [135]. However, in NPC, the lymphocytes from the tumor environment are functionally impaired [136], and the amount of lymphocyte infiltrate does not affect prognosis [137]. In fact, the amount of tumor infiltrating activated granzyme-B expressing CD8 + (cytotoxic) T cells was shown to associate with worse prognosis, an effect linked to increased apoptosis-resistance of the tumor cells, possibly imposed by EBV gene products including either or both LMP1 and BARF1 [138,139]. The specificity of these T cells remains undetermined, but their presence illustrates the importance of immune evasion mechanisms in EBV-associated tumors.

BamHI-A rightward frame 1 and the human immune system

Although BARF1 has immune-modulating properties, its expression as nonself protein may trigger an immune response. Indirect immunofluorescence techniques have revealed antibody reactivity to BARF1-expressing cells in serum samples from NPC patients [39,41,42,140]. With defined immunodominant BARF1 epitopes, only weak IgG responses could be measured in 21% of the NPC patients compared with 5% in the regional healthy controls, relative to the immunodominant viral

capsid antigen and Epstein–Barr nuclear antigen 1 responses [140]. IgG reactivity to BARF1 was higher than IgA responses [44,140], and the C-terminal extracellular domain is most immunogenic [44]. Some healthy EBV carriers do have weak IgG responses to BARF1 possibly reflecting transcription of BARF1 during the lytic cycle during normal EBV persistence. Because of the low negative predictive value of the anti-BARF1 humoral response, this parameter has limited value for NPC diagnosis. It does however provide indirect proof that sBARF1 protein expression is elevated in NPC.

Orlova *et al*. [141] examined the immunogenic response to native hexameric sBARF1 and found that healthy EBV carriers and NPC patients have similar (possibly low level) IgG reactivity against sBARF1, in 66% and 70%, respectively. Unfortunately, BARF1 antibody responses were not correlated to those against viral capsid antigen and early antigen.

The BARF1 protein apparently has low immunogenicity *in vivo*, thus resembling LMP1 and LMP2 [142]. A prior study observed antibody-dependent cellular cytotoxicity against BARF1-transfected Raji cells using serum from NPC patients, but this study is poorly controlled for nonspecific interactions [143].

Martorelli *et al*. found that CD4+ and CD8+ T cells could be stimulated with BARF1 peptides, in particular when derived from the N-terminal 1-56 region and presented by professional antigen-presenting cells, like DCs [144]. NPC patients reacted more strongly than healthy EBV-positive individuals, whereas EBV-negative individuals showed no reaction. This implies expression and (cross-) presentation of BARF1 *in vivo* triggering T-cell immunity, which needs to be further unraveled. Moreover, cells expressing BARF1 peptides were efficiently lysed by *in vitro*-pulsed cytotoxic CD8+ T cells (CTL), showing that BARF1 is a natural immunogen for T cells and that tumor cells expressing BARF1 may be susceptible to CTL killing. These studies were performed in the context of human leukocyte antigen (HLA)-A2, the most frequent HLA-type in Caucasians. Importantly, this HLA allele is significantly underrepresented in Italian NPC patients [145], suggesting protection conveyed through this HLA allele by means of cytotoxic T-cell control *in vivo*.

BamHI-A rightward frame 1 as target for diagnosis

Because BARF1 RNA is present in a high percentage of NPC and GC cells, it is considered a good diagnostic marker for detection of EBV-positive carcinoma cells *in vivo*. Despite having elevated levels of circulating EBV DNA, NPC patients have no detectable BARF1 mRNA in the circulation, excluding the presence of circulating viable NPC tumor cells [146]. This is in line with the observation that circulating EBV DNA in NPC patients is highly fragmented and probably derives from apoptotic tumor cell fragments [147]. The presence of BARF1 mRNA *in situ* in a high percentage of NPC patients is valuable for early-stage diagnosis and post-therapy monitoring of NPC. Stevens *et al*. demonstrated the diagnostic value of detecting BARF1 transcripts in noninvasive nasopharyngeal brushings reflecting the *in situ* presence of EBV-positive carcinoma cells [30]. EBV DNA quantification in NP brushings combined with quantitative BARF1 mRNA detection may have promising diagnostic and prognostic utility and reduce the number of invasive NP biopsies.

The BARF1 protein may escape detection *in situ* because it is probably rapidly secreted from the tumor cells. Houali *et al*. developed a sandwich Enzyme ImmunoAssay (EIA) to measure BARF1 protein levels in sera of NPC patients. In young NPC patients, the BARF1 concentrations reached as much as 5000 ng/ml and in sera from adult patients at 500 ng/ml ($n = 30$) were detected [45]. In addition, BARF1 was detected in saliva of NPC patients. However, the values reported are extremely high for a secreted factor. Serum levels of M-CSF in normal adults range from 1.7 to 8.4 ng/ml. The consequence of microgram levels of circulating sBARF1 would be major disruption of patient monocyte-macrophage pool. There are no indications that NPC patients have disturbed blood monocyte values. Importantly, no report has yet conformed the results of Houali *et al*., and our own efforts to detect sBARF1 using a variety of mouse and rabbit anti-BARF1 antibody combinations directed to conformational or linear epitopes on the BARF1 hexameric molecule have yielded mostly negative results in NPC sera, whereas the detection limit of the assay was around 5–10 ng/ml sBARF1 spiked in human serum (unpublished findings).

By mass spectrometry (MS)/MS ,we have defined a peptide that could reliably be detected in sBARF1-spiked human serum, yet no such peptide could be detected in NPC sera (unpublished findings). Our results showed that by using trypsin digestion, a BARF1 peptide LGPGEEQVLIGR (aa51–62) is the

main MS-identifier, and a quantitative targeted proteomics approach should be feasible to identify BARF1 values below 100 ng/ml in serum. No detailed data are available for BARF1 protein detection in GC blood samples, but it is unlikely to be much different from NPC.

Further evaluation and quantification of sBARF1 in serum as a carcinoma-specific diagnostic marker is needed. The addition of BARF1 mRNA or protein detection to current diagnostic screening efforts using EBV-IgA serology [148] or EBV DNA load testing may provide a new option for early tumor diagnosis, which is highly needed in both NPC and GC.

Concluding remarks

The BARF1 is a viral oncoprotein with pleiotropic functions, contributing to cell growth and survival as well as to immune modulation, allowing virus-producing cells and latently infected carcinoma cells to escape elimination. BARF1 mRNA is detected frequently in latently infected carcinomas and rarely observed in lymphomas except for a few cells induced into the viral lytic cycle [28,29,80]. BARF1 stimulates cell growth and survival when transfected in epithelial cells [94,95], up-regulating the transcription factor and proto-oncogen c-myc [100], the chromosome stabilizer hTERT [100], the cell cycle regulator cyclin-D [108], and the antiapoptotic Bcl-2 protein [50,107], as well as other genes [108], indicating a transforming and stimulating role of BARF1 in the cell cycle. In combination with the putative role of BARF1 in preventing apoptosis and immune evasion, BARF1 is a potent multifunctional player in the carcinogenesis of NPC and GC. To further define sBARF1 as suggested, mitogenic growth factor [105] would require identification of a putative BARF1 receptor as well as a signaling pathway underlying this mitogenic effect. It also remains undefined to which extend structural, conformational, and post-translational modifications in BARF1, such as glycosylation and phosphorylation play a role in BARF1 function(s) [44,47–49]. Insight in the exact mechanisms of BARF1-supported tumor cell growth can open the way for therapeutic intervention with specific antibodies or small molecule inhibitors.

Epstein–Barr virus-positive cells are in danger of being eliminated by the immune system. sBARF1 can function as a decoy receptor to functionally inhibit M-CSF, thereby acting as an immune modulator, interfering with antigen processing and innate danger-signaling functions of myeloid cells [37,38,53]. Because BARF1 has homology with several secreted growth factors, further characterization of BARF1 binding partners may clarify the role of BARF1 as an immune modulator. Recent *in vivo* studies using a BARF1-truncated EBV-homologue rhLCV demonstrate that BARF1 is important for acute and persistent viral infection [104,106], which could be a suitable model to further explore BARF1 functions *in vivo*.

During lytic replication, BARF1 expression is directly regulated by the immediate early gene R and is expressed as an early gene [55,60]. Without BARF1, B-cell immortalization is less effective, and viral loads during the acute phase of infection are lower [104,106]. Interestingly, the first 54 amino acids described to be sufficient for transformation and not affecting the apoptotic pathway were not deleted in this truncated rhLCV virus, suggesting that BARF1 immune-modulating function is important for the initial EBV infection. Blocking formation of BARF1/M-CSF complexes may reduce immune evasion of EBV-positive cells, and the recent structural and functional definition of the BARF1/M-CSF interaction domain [53,119,120] might be the first step toward a novel therapeutic approach, either to minimize EBV viral amplification during acute infection or to treat patients with BARF1-expressing carcinomas.

The clear influence of tissue type on the expression of BARF1 in EBV-associated malignancies may reflect differences in BARF1 promoter regulation by host factors. The epithelial differentiation marker ΔNp63α may be responsible for BARF1 transactivation in latent epithelial cell types [62], but more research is needed to confirm these findings. The BARF1 promoter is highly methylated in B-cell and epithelial cell lines, which could influence transcription of the BARF1 gene [55]. Thorough analysis of additional epigenetic events and/or transcription factors modifying BARF1 expression could identify the specific regulators in epithelial cells and carcinomas driving BARF1 expression.

Detection of BARF1 mRNA and protein in nasopharyngeal brushings and/or plasma may provide new options for early and specific diagnosis of NPC [30,45]. Both diagnostic options need to be further explored for gastric carcinoma as well. It is clear that understanding the full range of activities of BARF1 in EBV persistence, immune modulation

and oncogenesis is important for developing new treatment strategies for EBV-driven carcinomas.

CONFLICT OF INTEREST

The authors have no competing interest.

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