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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2018

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Winkle, M. (2018). *The missing piece: Long noncoding RNAs in cancer cell biology*. [Thesis fully internal (DIV), University of Groningen]. Rijksuniversiteit Groningen.

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CHAPTER 2

Emerging roles for long noncoding RNAs in B-cell development and malignancy

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Critical Reviews in Oncology/Hematology December 2017
(doi: 10.1016/j.critrevonc.2017.08.011)

Abstract

Long noncoding (lnc)RNAs have emerged as essential mediators of cellular biology, differentiation and malignant transformation. LncRNAs have a broad range of possible functions at the transcriptional, posttranscriptional and protein level and their aberrant expression significantly contributes to the hallmarks of cancer cell biology. In addition, their high tissue- and cell-type specificity makes lncRNAs especially interesting as biomarkers, prognostic factors or specific therapeutic targets. Here, we review current knowledge on lncRNA expression changes during normal B-cell development, indicating essential functions in the differentiation process. In addition we address lncRNA deregulation in B-cell malignancies, the putative prognostic value of this as well as the molecular functions of multiple deregulated lncRNAs. Altogether, the discussed work indicates major roles for lncRNAs in normal and malignant B cells affecting oncogenic pathways as well as the response to common therapeutics.

1 Introduction

It is less than a decade ago that a genome wide long noncoding (lnc)RNA transcription expression profile has been reported for the first time[1]. Current annotations suggest the presence of 15,000 (GENCODE 26) to 60,000[2] lncRNA genes in the human genome. The majority of the lncRNAs (70%) are poorly conserved across species, i.e. they arose in the past 50 million years of evolution[3, 4]. Only a few lncRNAs show high sequence conservation across species, e.g. XIST, PVT1, MIAT, NEAT1, MALAT1 and OIP5-AS[5]. Globally, lncRNA genes show higher sequence conservation than randomly selected genomic regions, this includes positional conservation as well as conservation of promoter regions, splice sites or the act of transcription itself (reviewed in[5]). The biological significance of lncRNAs was proven in a murine knockout study in which developmental defects and lethality occurred upon deletion of several lncRNAs[6]. A recent large-scale lncRNA knockout screening in human cell lines identified for 50 out of 700 lncRNAs tested a significant effect on cancer cell growth[7]. Thus, although the functional significance of the bulk of lncRNAs is unclear, a significant proportion of them have major biological functions.

LncRNAs may be transcribed from intergenic (lincRNAs), genic regions (antisense, intronic, sense-overlapping) or (super-) enhancer regions (eRNAs) or they may share a promoter with the neighboring coding gene (bidirectional). Despite having a similar architecture as mRNAs including being subject to splicing, polyadenlylation and 5' capping, lncRNAs show characteristics that clearly distinguish them from mRNAs, such as a generally lower expression, a fewer number of exons and a much higher tissue specificity[2, 8]. The functional repertoire of lncRNAs appears to be broad, likely owing to their ability to interact with DNA, RNA and proteins or combinations thereof. Well described mechanisms include: (i) the modulation of three dimensional chromatin structure (e.g. Firre)[9], (ii) scaffolding functions for proteins (e.g. MALAT1, NEAT1)[10-12], (iii) transcriptional gene regulation via interaction with DNA and/or proteins including epigenetic regulators (e.g. HOTAIR)[13] and transcriptional (co)factors (e.g. lincRNA-p21, GAS5)[14-16], and (iv) posttranscriptional regulation affecting the stability of mRNAs or proteins (e.g. PVT1, GAS5)[17, 18] or competing with miRNA binding (competing endogenous RNAs, ceRNAs[19]; e.g. GAS5-miR-21)[20].

In solid cancers, lncRNAs have been shown to contribute to all known cancer hallmarks, i.e. viability, proliferation, growth suppression, motility, immortality and angiogenesis (reviewed in [21]). LncRNA BCAR4 represents an excellent example of a lncRNA regulating gene expression through direct and indirect effects. Mechanistically, BCAR4 directly interacts with the transcription factors SNIP1 and PNUTS[22]. The interaction of BCAR4 with SNIP1 releases its inhibitory effects on p300 histone acetylase, thereby causing increased H3K18 acetylation at migration-specific target gene loci. BCAR4 bound PNUTS then interacts with the acetylated histones to induce the transcriptional machinery via

activation of PP1 phosphatase. High BCAR4 expression thus results in increased cell motility, which is in line with the correlation of high BCAR4 expression with advanced, metastatic breast cancer[22]. Cancer cells may also become addicted to the expression of oncogenic lncRNAs. The melanoma-specific lncRNA SAMMSON is an example of a lncRNA exerting its function by binding to a protein and influencing its subcellular localization[23]. Mechanistically, SAMMSON increased mitochondrial metabolism by direct binding to p32, resulting in its mitochondrial localization[23]. Depletion of SAMMSON strongly decreased the viability of melanoma cells independently of mutational status[23].

In recent years much progress has been made regarding the expression pattern of lncRNAs during different stages of B-cell development and in diverse B-cell derived malignancies. However, in contrast to solid cancers, a limited number of studies have addressed the consequences of deregulated lncRNA expression in B-cell malignancies. Here we provide an overview of the current knowledge on lncRNA expression changes in normal and malignant B cells, their potential clinical value as well as their molecular functions.

2 LncRNA expression in normal B cells

Multiple studies have analyzed lncRNA expression patterns in different stages of B-cell development in human[24-28] and mouse[29]. In general, marked lncRNA expression changes were observed during B-cell development and maturation. Consequently, B-cell subsets can readily be distinguished using their lncRNA expression patterns. Transcriptome sequencing of ten T-cell and three B-cell subsets[27] showed that expression of individual lncRNAs is more often restricted to a single subset (71% of 4,764 lncRNAs) than expression of protein coding genes (35% of 15,911 coding genes) or membrane proteins, which are commonly used for subset classification (40% of 1,051 membrane proteins). Of note, lncRNAs that showed the highest cell-type specificity appeared to be absent in whole lymphoid tissue or peripheral blood mononuclear cells (PBMCs) due to dilution of cell-type specific lncRNA transcripts in such heterogeneous cell samples[27]. This is also reflected by the fact that studies assessing lncRNA expression patterns in specific B-cell subsets[24, 25, 27, 29] led to the identification of a large number of previously unannotated transcripts. In contrast to mRNA expression patterns, lncRNA expression patterns can distinguish cells committed to the B and T cell lineage already at the progenitor stage in bone marrow[25], indicating their importance in lineage commitment. At later stages of B-cell development and maturation lncRNA expression profiles can be highly similar between functionally distinct B-cells such as follicular and marginal zone B cells in spleen[29] and naïve and memory cells in tonsils[28]. However, the strongly proliferative germinal center (GC) B cells showed lncRNA profiles that are very distinctive from other mature B-cell subsets[25, 28, 29].

To identify lncRNAs associated with cell fate-related transcription factors at different B-cell development stages a guilt-by-association analysis was done on 11 distinct B-cell subsets[26]. Early B-cell development specific genes, expressed in preBI, preBII and immature B cells, such as RAG2, VPREB1, DNTT, LEF1, SMAD1 and MYB, were associated with LEF1-AS1, SMAD1-AS1, MYB-AS1 antisense transcripts, as well as with the intergenic transcript CTC-436K13.6. Mitotic cell cycle related genes such as KIF23, PLK4 and CENPE were specific for the proliferative stages of B-cell development, i.e. preBI, preBII, centroblasts and centrocytes, and associated with the lncRNAs OIP5-AS, MME-AS1 and the bidirectional lncRNA CRNDE. CRNDE has previously been linked to cell cycle and proliferation[30-33]. Expression of AID and SERPINA9, two genes specifically expressed in GC centroblasts and centrocytes, was associated with PVT1 and multiple uncharacterized lincRNAs, e.g. LINC00487, LINC00877, RP11-203B7.2 and RP11-132N15.3. The latter one is located 240kb upstream of BCL6. RNA-seq of 11 murine B-cell subsets revealed 4,516 differentially expressed lncRNAs[29]. Assessment of the histone H3K4 mono/trimethylation ratio of these differentially expressed lncRNAs revealed 192 promoter (high H3K4me3) and 702 enhancer (high H3K4me1) associated lncRNAs (eRNAs). Comparison with previous human studies[24-26] identified 228 eRNAs with a potential human ortholog based on positional conservation and 185 based on sequence conservation. Of note, the above-mentioned GC-B cell-associated lncRNA RP11-132N15.3 located downstream of BCL6[26] has a murine ortholog showing both sequence and positional conservation. However, this lncRNA appears to be downregulated in murine GC B cells[29], while being upregulated in human GC B cells[26], indicating that despite strong similarities a functional conservation is unlikely.

Thus, although most current studies are limited by inclusion of a restricted number of B-cell subsets[24-26, 28] or the use of microarrays preventing the identification of novel transcripts[26, 28], they provide a valuable overview of B-cell subset-specific lncRNAs. Now, the field is open for studies that establish their roles in normal B-cell development.

3 Aberrant lncRNA expression in malignant B cells

LncRNA expression profiling studies in B-cell malignancies have been focused on classical Hodgkin lymphoma (cHL), diffuse large B cell lymphoma (DLBCL), Burkitt lymphoma (BL) and around the transcription factor MYC. Comparison of lncRNA expression patterns of cHL cell lines to sorted GC B cells from healthy individuals[28] revealed a total of 475 differentially expressed lncRNAs. The majority of them (74%) were downregulated, which may in part be a reflection of the lower degree of heterogeneity in cell lines compared to sorted B-cell subsets. In line with this, DLBCL cell lines also had a markedly lower number of expressed lncRNAs compared to sorted normal B cells and patient cases[34].

Re-analysis of RNAseq data of 116 DLBCL cases, 30 DLBCL cell lines, 4 naïve and 4 GC B-cell subsets[34] revealed 2,632 novel, multi-exonic lncRNAs. For approximately half of these, expression was restricted to DLBCL cases or to both DLBCL cases and cell lines. DLBCL cases display high heterogeneity in clinical behavior, mRNA expression and genomic aberrations[35] and this is also reflected in their lncRNA expression profiles, as most tumor-specific lncRNAs were found in only small subsets of cases[34]. Interestingly, among the lncRNAs with increased levels in a subset of the DLBCL cases, 33 were located in recurrently amplified genomic regions, supporting a potential role for these lncRNAs in the pathogenesis of DLBCL. To further establish a relation between the identified lncRNAs and DLBCL biology, their co-expression with the transcriptional repressors BCL6 and EZH2, a component of the polycomb repressive complex 2 (PRC2), was assessed. A significant negative correlation with BCL6 or EZH2 expression was found for 323 and 431 lncRNAs, respectively. A significant proportion of these (>40%) were also induced by EZH2 or BCL6 depletion, further verifying these lncRNAs as BLC6 or PRC2 target genes. Globally, the study showed that lncRNAs are an intrinsic part of tight regulatory networks in DLBCL; expression of 88% of the novel lncRNAs correlated significantly with at least one protein-coding gene[34]. Unfortunately, the study was restricted to newly identified lncRNAs and excluded >8,000 previously annotated lncRNAs. A total of 465 novel lncRNAs could readily distinguish ABC and GCB subtypes in unsupervised hierarchical clustering[34]. As expected these lncRNAs did not show any overlap with the 156 annotated ABC/GCB-specific lncRNAs identified in another study[36]. A subset of 17 of these 156 subtype-specific lncRNAs was sufficient to distinguish the ABC and GCB subtypes with a specificity and sensitivity of approximately 90% and accordingly predicted treatment outcome[36]. CRNDE and MME-AS1 both highly expressed in GC B cells[26] were among the lncRNAs with a significantly higher expression level in GCB subtype DLBCL[36]. Other studies analyzed expression differences in DLBCL for selected lncRNA candidates based on known functions or altered expression in other cancer types. These studies identified increased levels of HOTAIR[37], HULC[38] and LUNAR1[39] and decreased levels of lincRNA-p21[40] in DLBCL tissues. Lastly, MALAT1 levels were increased in eight DLBCL cell lines compared to Epstein-Barr virus (EBV)-transformed lymphoblastoid cells[41].

LncRNAs regulated by the well-known transcription factor Myc may have prevalent roles in the mediation of the oncogenic Myc-induced effects. The P493-6 B cell line carrying a tetracycline-repressible *MYC* allele was used to identify Myc-regulated lncRNAs. Microarray and RNAseq based studies identified >1,200[42] and 534[43] Myc-regulated lncRNAs. Reanalysis of these RNAseq data[43] by another group increased the number of Myc-regulated lncRNAs to 960[44]. In each study approximately half of the lncRNA loci were Myc-induced and the other half were Myc-repressed, similar to the pattern observed for protein coding mRNAs[42]. The comparison of Myc occupancy at Myc-responsive lncRNA and mRNA loci revealed an intriguing difference. Both, Myc-induced and -repressed lncRNAs were enriched for Myc binding sites, while only the Myc-induced but not the Myc-repressed mRNAs showed enrichment for Myc binding

sites[42]. For mRNAs, this is in line with the hypothesis that Myc tends to further upregulate transcripts already active in a specific cell type[45, 46]. However, downregulated lncRNAs also seem to be actively repressed by Myc[42]. In a comparison of primary lymphoma cases with low (CLL) and high Myc levels (BL) the expected Myc-regulated pattern was observed for 54% of the differentially expressed lncRNAs[42]. Of the lncRNAs significantly up- or downregulated in BL cases compared to normal GC B cells (514 and 367 lncRNAs respectively), 27% were identified as Myc-regulated lncRNAs[44]. Further overlap of these lncRNAs with lncRNAs regulated in the hT-RPE-MycER model (retinal pigment epithelial cell line with *MYC-ER* fusion gene) defined a limited set of 13 common Myc-regulated lncRNAs. This small overlap is likely due to the difference in cell types, in line with previous reports describing a limited set of cell type common 'core' Myc target genes in the coding genome[47]. One of these lncRNAs, termed MINCR (Myc-induced noncoding RNA), showed a significant correlation with Myc expression in BL cases as well as in *MYC* translocation positive DLBCL and FL cases[44]. No clear correlation with Myc expression was observed in normal tissues or BL cell lines[48]. MINCR was also identified as a Myc-regulated lncRNA in P493-6 cells[42, 43] and shown to have Myc binding sites[42] Other annotated lncRNAs defined as Myc-induced based on multiple datasets[42, 43] include e.g. GAS5, DANCR, KTN1-AS1, MCM3AP-AS1 and OIP5-AS. As the specificity of Myc-regulation has been under debate[45, 46, 49], disabling the nearby Myc binding sites using CRISPR-Cas9 technology may provide a more definitive answer on whether these and other Myc-induced and -repressed lncRNAs are directly regulated by Myc.

Thus although the number of profiling studies is limited, they clearly show that lncRNA expression patterns are deregulated in lymphoma. The candidate lncRNAs derived from these studies provide an excellent starting point for further studies aiming at identifying their function or assessing their potential clinical value.

4 LncRNAs with clinical value in B-cell malignancies

Reanalysis of microarray data of >1,000 DLBCL patients revealed a 6 lncRNA signature[50] correlating with overall survival in DLBCL patients in two independent cohorts. High expression of MME-AS1, CSMD2-AS1, RP11-360F5.1, RP11-25K19.1, CTC-467M3.1 was associated with a favorable outcome, while high expression of SACS1-AS was associated with poor outcome. The six-lncRNA score remained a significant predictor of overall survival in multivariate analysis, together with age at diagnosis, lactate dehydrogenese (LDH) levels and ECOG performance status[50]. In line with the better prognosis of GCB DLBCL, the expression levels of MME-AS1, CSMD2-AS1 and CTC-467M3.1 was enhanced in GCB DLBCL, while expression of the risk associated lncRNA SACS1-AS was higher in ABC DLBCL[36]. These studies thus show that lncRNA expression patterns are potentially valuable biomarkers for prognosis.

High levels of MALAT1 in a group of 40 MCL patients predicted worse overall survival[51]. In contrast, a meta-analysis in multiple myeloma (MM) (n = 144) and DLBCL (n = 200 and 414) patients indicated a good prognostic potential of high MALAT1 expression levels[52]. Moreover, pre-treatment MALAT1 plasma levels were decreased in MM patients compared to healthy controls and the decrease was associated with advanced clinical stage[53]. However, reduced MALAT1 levels were associated with a prolonged progression free survival in post-treatment bone marrow samples of MM patients[54]. Further adding to the controversy, high expression levels of MALAT1 in mesenchymal stem cells present in the tumor microenvironment of MM patients was shown to be detrimental to disease progression[55]. In summary, the prognostic potential of MALAT1 remains inconclusive and requires further study in large patient cohorts using plasma, tumor cell and tumor microenvironment derived samples.

Low expression levels of lincRNA-p21 were an independent predictor of poor 5-year OS and PFS in a cohort of 105 DLBCL patients[40]. In CLL patients, lincRNA-p21 plasma levels were decreased compared to healthy controls and this decrease was associated with advanced clinical stage[53]. High HULC[38] and LUNAR1[39] expression levels predicted worse OS and PFS in DLBCL cohorts of 147 and 87 patients, respectively. Furthermore, LUNAR1 is activated by NOTCH1[56] and the presence of activating NOTCH1 mutations correlated with worse prognosis in CLL[57, 58]. High HOTAIR expression was shown to be a predictor of worse OS in 50 DLBCL patients also as an independent predictor next to high international prognostic index (IPI) scores[37]. However, in a DLBCL cohort of 164 cases, high HOTAIR expression levels correlated with better outcome, possibly due to the negative correlation between HOTAIR and Myc expression[59]. MIAT expression was studied in 67 CLL cases[60] and high expression was observed more often in aggressive types of CLL. Lastly, plasma levels of the lncRNA TUG1 were significantly decreased in MM and its downregulation correlated with advanced disease state[53].

Thus, lncRNAs are building up potential as valuable tools in the clinic, e.g. as prognostic indicators and biomarkers for disease.

5 Molecular functions of lymphoma-associated lncRNAs

For multiple lncRNAs deregulated in lymphoma, functional studies have been performed to shed light on their precise involvement in cancer cell biology. In this section, we discuss knowledge on the oncogenic or tumor suppressive functions of these lncRNAs revolving around apoptosis and cell growth.

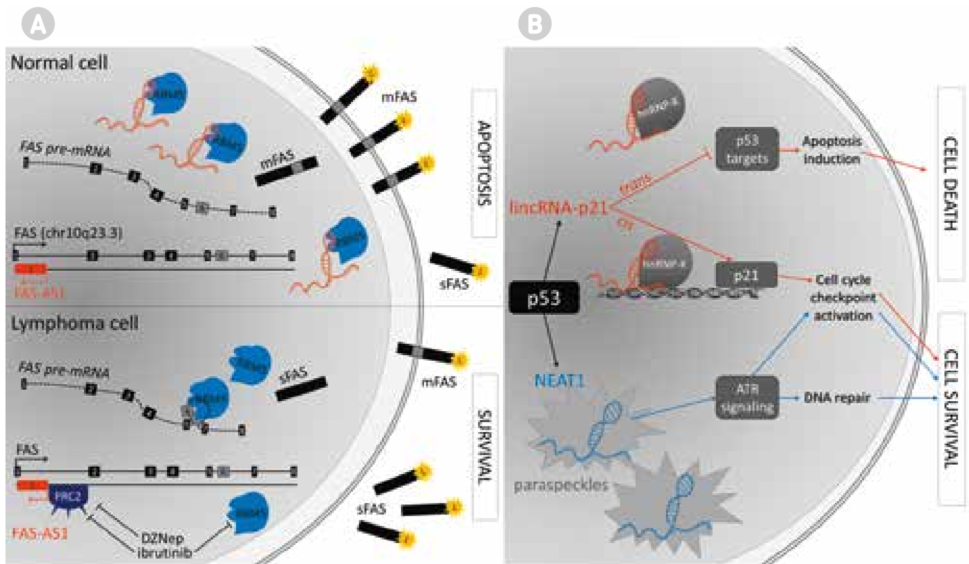

FIGURE 1 LncRNAs involved in apoptotic pathways. (A) LncRNA FAS-AS1 is required for efficient FAS-FASL-induced apoptotic signaling. In normal cells FAS-AS1 is expressed (top), acting as a decoy for RNA binding motif protein 5 (RBM5). This prevents interaction of RBM5 with FAS pre-mRNA and leads to production of membrane-bound FAS (mFAS) and effective triggering of apoptosis upon binding to the FAS ligand. In lymphoma cells (bottom), FAS-AS1 transcription is shut down by polycomb repressive complex 2 (PRC2) enabling interaction of RBM5 with FAS pre-mRNA, which mediates exon 6 exclusion, leading to production of soluble (s)FAS and inhibition of apoptosis. DZNep and ibrutinib can both re-activate FAS-AS1 expression and sensitize cells to apoptosis. **(B)** Functional mechanisms of p53-induced lincRNA-p21 (red) and NEAT1 (blue). Interaction of LincRNA-p21 with heterogeneous nuclear ribonucleoprotein K (hnRNP-k) results in (1) downregulation of p53-repressed target genes in trans, thereby inducing apoptosis and (2) enhances p21 transcription in *cis*, resulting in G0/G1 cell cycle arrest. NEAT1 (blue) is essential for the formation of nuclear paraspeckles, which leads to activation of ATR signaling and thereby activates G2/M and inter-S phase checkpoints and promotes DNA repair.

5.1 Apoptotic pathways

Functional FAS signaling is crucial for effective treatment response in lymphoma patients[61, 62]. Sensitivity to FAS ligands is regulated through alternative splicing; producing either the membrane bound FAS receptor (mFAS) or a soluble decoy receptor (sFAS). LncRNA FAS-AS1 was shown to be crucially involved in regulating this process by acting as a decoy for RNA binding motif protein 5 (RBM5), a protein that promotes the alternative splicing to sFAS (**FIGURE 1A**, top). Thus, high FAS-AS1 levels inhibit alternative splicing of the FAS transcript and thus the production of sFAS[63]. In lymphoma cells, FAS-AS1 expression levels were decreased due to polycomb repressive complex 2 (PRC2)-mediated histone methylation. As a consequence sFAS levels increase preventing FAS-ligand induced apoptosis (**FIGURE 1A**, bottom). Inhibition of EZH2, the catalytic

component of PRC2, using DZNep resulted in de-repression of FAS-AS1 and sensitized lymphoma cells to FAS-mediated apoptosis by upregulation of mFAS. The BTK inhibitor ibrutinib similarly increased FAS-AS1 levels and apoptosis through downregulation of EZH2 and RBM5 transcript levels[63].

LincRNA-p21 (~17kb upstream of p21) and NEAT1 were identified as p53-responsive transcripts by analyzing expression levels in primary CLL cells with functional (p53WT) and disrupted p53 signaling (p53mut/del or ATMdel)[64]. Upon p53 stimulation both lncRNA loci showed increased expression and occupancy by p53 in p53WT CLL cells, but not in p53mut/del cells and ATMdel cells. LincRNA-p21 and NEAT1 affect different p53-mediated processes, i.e. inducing apoptosis or cell cycle arrest and DNA repair (**FIGURE 1B**). Three independent studies showed a direct interaction of lincRNA-p21 with heterogeneous nuclear ribonucleoprotein K (hnRNP-K) in murine embryonic fibroblasts (MEFs)[14, 15, 65]. hnRNP-K is a transcriptional co-factor thought to act in apoptosis induction via p53-dependent gene repression[66], a process less well-characterized than p53-mediated gene activation. Furthermore, hnRNP-K was shown to assist p53 recruitment to the p21 promoter[67], thus supporting cell cycle checkpoint integrity. In vitro lincRNA-p21 knockdown in MEFs caused activation of many p53-repressed target genes and decreased apoptotic levels, without affecting p21[65]. However, conditional knockout of lincRNA-p21 in murine embryonic fibroblasts (MEFs) diminished p21 levels thereby causing checkpoint defects and increased proliferation, while no changes in apoptosis were observed[15]. In line with a *cis* induced p21 activation by lincRNA-p21, its expression positively correlated with p21 levels across 34 CLL cases[64]. In contrast to the proposed *cis* effect of lncRNA-p21 on p21[15] it was shown that ectopic expression of lncRNA-p21 in a DLBCL cell line caused an increase in p21 and G1 cell cycle arrest[40]. To assess feasibility of therapeutic targeting of lincRNA-p21, it is important to study which downstream p53-responses are affected in B cells and whether low lincRNA-p21 levels are solely caused by *TP53* mutations or also by epigenetic or (post)-transcriptional mechanisms. The p53-induced lncRNA NEAT1 was shown to take part in the p53-induced DNA repair response pathway[68]. Opposed to its role in apoptosis induction, the DNA repair response triggered by p53 may support cell survival and hamper the effects of genotoxic and p53-reactivation therapeutics. The formation of paraspeckles (i.e. nuclear substructures involved in RNA metabolism/translation regulation) is induced by replication stress and dependent on p53 activation as well as NEAT1 expression[12, 68]. Interestingly, depletion of NEAT1 in cancer cell lines sensitized them to replication stress-inducing agents such as genomycin, doxorubicin and PARP inhibitors. Mechanistically, NEAT1 depletion compromised activation of DNA repair-promoting ATR signaling[68]. Inhibition of ATR signaling in CLL and MCL cells that strongly depend on this pathway (i.e. ATMmut cells) increased chemosensitivity[69, 70]. Future work should address the precise role of NEAT1 in p53 and ATR signaling in normal and malignant B cells.

Knockdown of the lncRNA HULC in a DLBCL cell line resulted in increased apoptosis via downregulation of the anti-apoptotic BCL2 protein[38]. HULC was previously shown to

protect from apoptosis through direct repression of the tumor suppressor EEF1E1 (P18/AIMP3), and subsequent prevention of the DNA-damage induced activation of p53[71,72].

5.2 Proliferation and growth

HOTAIR acts as a cofactor in PRC2-mediated repression via induction of H3K27me3 at specific gene loci[13]. Multiple lines of evidence suggest an important oncogenic role for PRC2 in lymphoma (reviewed in[73]): (1) The presence of recurrent activating EZH2 (i.e. the catalytic component of PRC2) mutations in GCB DLBCL, FL and BL leading to a global increase in H3K27me3 levels[74-76]; (2) the association of high EZH2 and H3K27me3 levels with aggressive clinicopathological features in both ABC and GCB DLBCL[77,78]; and (3) accelerated Myc-driven lymphomagenesis by EZH2 activating mutations[79]. In addition, increased HOTAIR expression has been observed in 23% of DLBCL cases (n = 164) and positively correlated with high EZH2 expression, but not with global H3K27me3 levels[77]. This was used as an argument to support a specific rather than a global manner of H3K27me3 regulation by HOTAIR in DLBCL. Interestingly, increased HOTAIR correlated with low Myc levels[77]. As Myc was previously shown to indirectly repress genes, including its own transcription, via EZH2[80] it is intriguing to speculate that HOTAIR may be involved in this autoregulatory process. Another study suggested an oncogenic role for HOTAIR in DLBCL by activation of NF-kB and phosphorylation of PI3K and AKT[37]. Activation of NF-kB by HOTAIR was also described in murine cardiac muscle cell lines[81]. All together these data support a crucial role of HOTAIR in the pathogenesis of lymphoma.

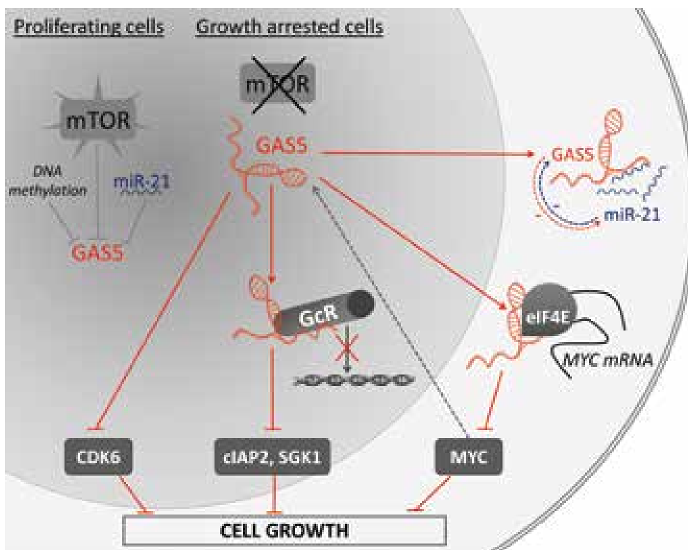

FIGURE 2 GAS5 mediates growth arrest. In proliferating cells, GAS5 is repressed by active mTOR, miR-21 and DNA methylation. In growth arrested cells, GAS5 levels are increased and negatively influence growth and apoptosis-associated genes, such as MYC, CDK6 (G1-to-S transition) , cAIP2 and SGK1 (anti-apoptotic) as well as known oncomiR microRNA-21. GcR – glucocorticoid receptor.

GAS5 was discovered as a transcript specifically expressed in growth-arrested cells[82] and was shown to induce growth arrest in normal T lymphocytes[83]. Its expression is tightly regulated by mTOR, where high mTOR levels (i.e. growing cells) shut down GAS5 expression post-transcriptionally[84]. mTOR signaling plays pivotal roles in B-cell differentiation[85] and the pathway is activated in B-cell malignancies[35, 86]. Depletion of GAS5 in MCL cell lines and leukemic T cells inhibits the apoptotic response to multiple therapeutic compounds[87], such as mTOR antagonists[88] and dexamethasone[83]. The various molecular mechanisms described for GAS5 (**FIGURE 2**) (reviewed in[87]) are in line with its broad effects on cell growth: (1) GAS5 knockdown increased levels of CDK6, a protein involved in G1/S transition, thereby causing a more rapid cell cycle and increased proliferation[89, 90]; (2) GAS5 acts as a decoy for the glucocorticoid receptor, preventing transcription of target genes including the apoptosis inhibitors cIAP2 and SGK1[16]; (3) GAS5 downregulated miR-21, a known oncomiR in B-cell lymphoma[91], and miR-21 targets GAS5, thus forming a reciprocal feedback loop[20], and (4) GAS5 downregulates Myc at the translational level via interaction with the translation initiation factor 4E (eIF4E)[18]. The reported induction of GAS5 by Myc[42, 43] could indicate presence of a negative feedback loop to regulate Myc levels (**FIGURE 3**). These studies showed that GAS5 is a potent tumor suppressor lncRNA acting as a master regulator of growth and apoptosis in B-cell lymphoma.

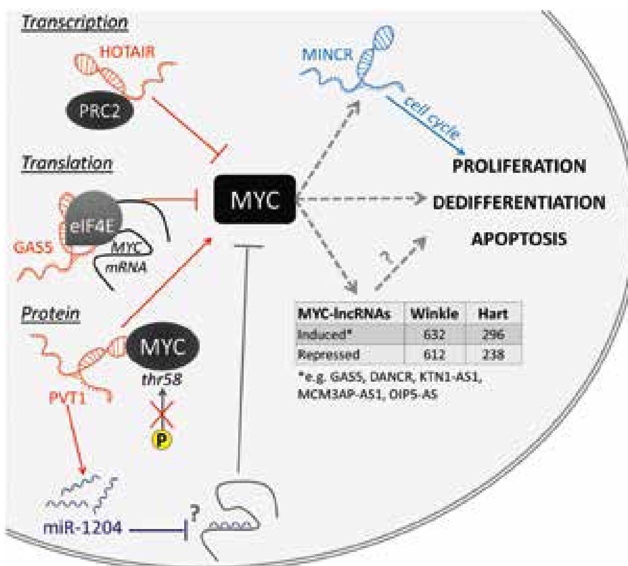

FIGURE 3 LncRNAs in the Myc pathway. Different lncRNAs were implied in the regulation of Myc at the transcriptional (HOTAIR), translational (GAS5) and protein (PVT1) level. In addition, miR-1204 encoded within the PVT1 transcript may induce Myc indirectly. The Myc-induced MINCR lncRNA affects proliferation through regulation of cell cycle genes. Several Myc-responsive lncRNAs are likely involved in the Myc-induced effects on proliferation, de-differentiation and apoptosis.

PVT1 (located downstream of MYC) has been implicated in B-cell lymphoma based on the association of several SNP alleles within the PVT1 locus with the risk on FL[92], DLBCL[93] and cHL[94]. In addition, PVT1 is co-translocated with MYC to the immunoglobulin locus in many BL cases[95] and in part of the DLBCL cases[96]. In MM, the PVT1 locus is translocated to non-immunoglobulin loci[97]. In addition, PVT1 is frequently co-amplified in cancers with MYC copy number increase. In mice, amplification of a larger genomic region (i.e. MYC, PVT1, Ccdc26 and Gsdmc) significantly increased tumor incidence compared to amplification of MYC alone[17]. Functional studies in other cancer types have revealed three possible modes of action for PVT1 (reviewed in[98, 99]) (**FIGURE 3**). First, the PVT1 transcript encodes for the miR-1204-1208 cluster and miR-1204 was found to be upregulated B and T cells highly expressing PVT1[100, 101]. Ectopic expression of murine miR-1204 indirectly increased Myc levels in pre-B, but not in pro-B cells[100]. Second, PVT1 was shown to interfere with Myc phosphorylation at threonine 58, a modification that promotes Myc degradation[17]. This may result in a positive feedback mechanism, as expression of PVT1 is induced by Myc[42, 102]. Third, PVT1 was shown to activate TGF-β signaling and to mediate stabilization of the proliferation-associated protein NOP2[103]. TGF-β signaling in non-Hodgkin lymphoma cells may lead to exhaustion of the T cell effector response through upregulation of CD70[104].

Knockdown of the Myc-regulated lncRNA MINCR caused a partial G0/G1 cell cycle arrest in hT-RPE-MycER cells[44]. RNAseq analysis upon MINCR depletion revealed downregulation of a significant number of genes involved in cell cycle regulation. Several of these genes were directly regulated by Myc and their knockdown phenocopied the effects of MINCR knockdown in BL cells[44]. These data suggest that MINCR positively regulates Myc binding to promotor regions of important cell cycle regulators. The MINCR knockdown-induced effect on cell cycle arrest was observed in both Myc high and Myc low cells, suggesting that MINCR also mediates Myc-independent effects. MINCR knockdown affected a total of 1,227 genes[44], suggesting a much broader function of this lincRNA (**FIGURE 3**).

LUNAR1 was first described as a NOTCH1-induced lncRNA in T-ALL. The NOTCH1 enhancer element of LUNAR1 is located in the last intron of the downstream IGF1R gene[56]. LUNAR1 was shown to positively regulate expression of IGF1R in *cis*. Accordingly, LUNAR1 depletion decreased cell viability in T-ALL cell lines, while ectopic IGF1R re-expression restored normal growth. This indicates that LUNAR1 mainly functions through the IGF1R pathway[56]. Aberrant expression of both NOTCH1[105] and IGF1R[106] has been reported in cHL, while but LUNAR1 was not expressed in HL cell lines[28]. LUNAR1 was expressed in DLBCL cases[39], but not in primary CLL or BL cases[42]. In DLBCL increased LUNAR1 levels were linked to cell cycle progression[39]. As inhibition of IGF1R in T-ALL[56] and DLBCL[107] cell lines decreased cell viability, it would be interesting to determine whether LUNAR1 is involved in this phenotype.

MALAT1 is a proliferation and metastasis-inducing oncogene involved in many solid cancers (reviewed in[108]). MALAT1 has broad functions and acts through regulation

of transcription and alternative splicing[108]. Although high MALAT1 expression was a good prognostic factor in DLBCL patients[51], its knockdown in DLBCL cell lines lead to decreased viability[41]. Specifically, MALAT1 knockdown caused increased apoptosis, cell cycle arrest and autophagy[41]. Mesenchymal stem cells (MSCs) of MM patients consistently showed increased MALAT1 expression compared to MSCs of normal controls[55]. This was proposed to support tumor cell survival and bone destruction by regulating TGF-β bioavailability. This was achieved by interaction of MALAT1 with the SP1 transcription factor, which induced expression of LTBP3 (ca. 30kb downstream of MALAT1) in *cis*[55]. LTBP3 regulates the bioavailability of TGF-β in the extracellular environment[109]. Accordingly, MALAT1 levels strongly correlated with both LTBP3 and TGF-β levels in MM patient-derived MSCs[55]. Thus, this study is the first to reveal a significant role of lncRNA overexpression in the MSCs present in the microenvironment of the MM cells.

6 Conclusions and future perspectives

Clearly, evidence is accumulating that lncRNAs are significantly involved in B-cell development, lymphomagenesis and lymphoma cell biology. Their high cell type specific expression pattern makes them potentially novel biomarkers for different clinical applications in the coming years. In addition, modulation of lncRNAs might improve therapy response, for example by modulating FAS-AS1 levels through inhibition of EZH2 using DZNep or ibrutinib[68], and modulating GAS5 levels using demethylating agents[110, 111]. In relation to novel or improved therapeutic approaches, lncRNAs situated in the p53 pathway or in the Myc pathway are also of particular interest.

Besides the above-discussed studies there are also other lines of evidence that point towards important roles for lncRNAs in lymphomagenesis. Firstly, GWAS studies revealed associations for SNPs within chromosomal regions referred to as 'gene deserts'. Such SNPs may be associated with the functionality of lncRNAs as shown in a recent study linking 26% of investigated disease associated SNPs (n = 11,194) to lncRNA genes[2]. The most evident lymphoma associated SNPs are located in the PVT1 locus at 8q24[92, 93, 112-114]. Secondly, the act of lncRNA transcription has recently been connected to recurrent genomic translocations introduced by AID and RAG1 involved in lymphomagenesis (affecting e.g. Myc, BCL-6, BCL-2). Specifically, it was shown that recurrent AID translocation hotspots are situated in genomic regions where convergent sense mRNA and antisense lncRNA transcription occurs[115, 116]. The antisense transcription was initiated from gene-overlapping super-enhancers. Notably, such convergent transcription also occurred at the PVT1 locus[115-117], possibly explaining its frequent involvement in genomic rearrangements in B cells[95-97]. In addition, the lncRNA RP11-211G3.3.1-1 transcribed antisense of intron 1 of the BCL6 gene was shown to

encompass the exact chromosomal breakpoint region in >87% of primary DLBCL cases[118]. The GAS5 locus was also found to be amongst the AID-induced translocation hotspots[117]. In a single DLBLC case the first exons of GAS5 were fused to the BCL6 gene, which resulted in increased BCL6 expression[119]. Whether AID is responsible for translocations between different AID off-target (i.e. non-Ig) genes requires further investigation. Lastly, RAG1-induced breakpoints in pre-B-ALL were also enriched in enhancer regions showing convergent transcription[120]. Thus, these studies support a significant role for lncRNA transcription in genome integrity in B cells.

A major factor hampering current lncRNA research is their limited evolutionary conservation[5], which greatly restricts the use of available animal models to study their functions. In line with this, the best-characterized lncRNAs are the most conserved ones, such as MALAT1, NEAT1, XIST and PVT1. In this respect, it would be of interest to study the role of OIP5-AS in lymphoma as this lncRNA is conserved even in zebrafish (called 'cyrano'). Its high expression observed in proliferating B cells[26], as well as its induction by Myc[42, 43] are typical features of proliferation-associated lncRNAs. Notably, OIP5-AS was shown to act as a sponge for HuR[66], a RNA-binding protein that plays an essential role in B-cell development[121] and regulates Myc[122]. The study of less conserved lncRNAs calls for the use of primary cells, humanized animal or primate models to improve our insights into their functions and interactions in vivo.

In the past decade vast advancements have been made regarding the toolbox for lncRNA research. Novel techniques such as global run-on (GRO)-sequencing[123] give a detailed picture of nascent RNA transcription. In addition, lncRNA annotation databases are constantly growing (e.g. Broad Institute human lincRNA catalog, LNCipedia)[1, 124], and ensure the integration of lncRNAs into systems biology, e.g. by assessment of co-expression networks with known proteins. In this regard, it is of utmost importance that raw as well as analyzed lncRNA expression datasets are made publically available to allow cross-comparisons. As lncRNA transcription is often directly linked to their functionality, the use of technologies such as CRISPR-Cas9 or antisense oligonucleotides (ASOs) to achieve (co-)transcriptional knockdown should be preferred over conventional RNAi methods. LncRNA-protein interactions may be studied using either conventional protein-IP or novel lncRNA-IP techniques (e.g. ChIRP, CHART, RAP)[125-127]. Lastly, RNA-FISH technology has been developed to visualize single lncRNA molecules in living cells[128].

In conclusion, lncRNAs are quickly advancing as main players in the pathogenesis in B-cell malignancies. Some lncRNAs show potential as therapeutic (co-)targets, particularly those that are actively involved in the mediation of chemotherapeutic effects. To this end, efforts are made to develop small molecule inhibitors that specifically disrupt lncRNA-protein interactions[129]. Furthermore, as antisense oligonucleotide-based therapies gradually make their way to the clinic[130], new ways to therapeutically target specific lncRNAs seem just a grasp away.

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