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Long Non-coding RNAs in the Pathophysiology of Multiple Myeloma: New Insights on the Role of CRNDE

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Long Noncoding RNAs in Multiple Myeloma

Over the past 15 years, long non-coding RNAs (lncRNA) have emerged as an important class of regulatory molecules. The currently accepted definition is that lncRNA refers to RNA molecules with little or no protein-coding potential, and which are greater than 200 nucleotides in length, a size cut-off chosen largely to distinguish them from the more-extensively characterised group of small non-coding regulatory RNAs, which includes micro (mi)RNAs, small inhibitory (si)RNAs and PIWI-interacting (pi)RNAs [1]. A recent compilation of annotations from diverse sources identified nearly 57,000 genes encoding lncRNAs in the human genome [2].

While the cellular role of the vast majority of these is still unknown, the picture that has emerged from characterisation of some important examples suggests that lncRNAs often act as regulatory molecules to control the expression of downstream target genes. Some lncRNAs regulate gene transcription, often by recruiting chromatin modifying proteins to control the epigenetic state of nearby or distal target genes, or by coordinating long-range chromosomal loops and regulatory domains [3-5]. Other lncRNAs influence post-transcriptional stages of gene expression, by affecting mRNA splicing, or by acting in the cytoplasm to sequester and dampen the activity of miRNAs and RNA binding proteins and thereby modify mRNA stability [3-5]. Cytoplasmic lncRNAs can also affect the translation and post-translational modifications of downstream proteins [5]. In keeping with this emerging role as important regulators of gene expression, altered expression of lncRNAs is increasingly implicated in the onset and evolution of a variety of malignant diseases (for examples [6-9]). The altered expression of lncRNAs can lead to downstream changes in a variety of cancer-associated pathways, including dysregulation of p53 and other cell signalling pathways, control of hypoxia and the Endothelial-Mesenchymal Transition (EMT) as well as epigenetic dysregulation [10].

One malignant disease in which several recent studies have revealed important roles of lncRNAs, is multiple myeloma (MM), a tumour of immunoglobulin-secreting plasma B cells in the bone marrow. MM is primarily a disease of the elderly (median age at diagnosis of 69 years) which, together with its pre-malignant precursor stage disease (monoclonal gammopathy of undetermined significance; MGUS) is the most common haematological malignancy, accounting for ~20% of all haematalogical cancer [11,12]. Although survival times are gradually increasing with recent advances in treatment regimes [12], disease relapse occurs frequently and MM is still considered essentially incurable [13, 14]. Over the last five years, several microarray- and RNA-seq-based transcriptomic studies have documented widespread changes in the expression profile of lncRNAs in tumour plasma cells of MM patients relative to plasma cells from healthy individuals [15-19]. The expression of some lncRNAs was also dynamically regulated during the course of disease evolution [16], and upon acquisition of drug-resistance phenotypes by MM cell lines [20]. Together these studies led to the identification of prognostic lncRNA expression signatures which were predictive of event free- or overall survival, indicating that lncRNAs exert an important effect on disease progression and outcome in MM.

Despite the accumulating evidence of their important role in MM, at present only a few of these lncRNAs have been individually investigated to determine to what extent and by what molecular pathways they influence MM cell growth, tumour progression, treatment-response

and disease outcome. Among the few lncRNAs which have been extensively investigated in MM, important examples include the well-established oncogenic lncRNAs MALAT1, H19, TUG1, UCA1 as well as the known tumour-suppressor lncRNA MEG3 (for recent reviews see [21-23]). Importantly, these studies revealed that lncRNAs often affect MM cell growth and tumour progression by different pathways to those observed in other malignancies, suggesting that the molecular mechanisms involved are likely to be disease- and context-dependent.

CRNDE: An Oncogenic lncRNA Implicated in the Progression and Outcome of MM

Within this context, findings from our group [24] and others [25] have recently shed new light on the role of the oncogenic lncRNA Colorectal Neoplasia Differentially Expressed (CRNDE) in the pathophysiology of MM. CRNDE was originally identified as an overexpressed transcript in colorectal carcinoma (CRC) [26] and has since been shown to be upregulated in a range of other solid and haematological tumours (including glioma, hepatocellular carcinoma, non-small-cell lung cancer, renal cell carcinoma, bladder cancer, breast cancer and acute myeloid leukemia), where high levels of expression correlate with poor prognosis (recently reviewed in [27,28]). CRNDE is expressed from a gene located at human chr16q12.2, which is comprised of 5 core exons, including a complex first exon resulting from the use of alternative transcription start sites, as well as an additional annotated exon (exon 3) which is infrequently used [29] (Figure 1A). The CRNDE transcript is subject to complex alternative splicing events, with at least 10 splice variants documented [26,30]. Incomplete splicing particularly affects the 3' end of the transcript, and most often leads to the retention of intron 4, which contains a discrete region of ~600 nucleotides that has been strongly conserved [29-31] (Figure 1A). While the functional significance of the alternative CRNDE transcript isoforms remains unclear, fractionation experiments have revealed that different isoforms are located in distinct sub-cellular compartments, suggesting that they are likely to exert different molecular functions [24,31]. Observations in different malignancies suggest that CRNDE can influence tumour growth and disease evolution in a variety of ways, including effects on tumour cell proliferation and apoptosis, affecting adhesion, migration and invasion, by enhancing resistance to chemo- and radio-therapies, and even influencing inflammatory responses and tumour cell metabolism [28].

Consistent with these observations in other malignancies, we recently reported that expression of CRNDE is elevated in tumour plasma cells ($CD138^+$) isolated from a cohort of newly-diagnosed MM patients, compared with plasma cells of healthy individuals [24]. Within the patient group, higher levels of CRNDE correlated with poor disease outcome. Expression of CRNDE also increases during progression from early (MGUS) to late stages of the disease. Upregulation and prognostic significance of CRNDE has also been observed in an independent MM patient cohort [25]. By using CRISPR-deletion and CRISPR-inhibition approaches to disrupt expression of the major CRNDE transcript isoforms, we found that depletion of CRNDE reduced the proliferation of MM cell lines and increased their sensitivity to dexamethasone, a therapeutic cornerstone of MM treatment regimes [24]. Deletion of the *CRNDE* gene also reduced the tumorigenic potential of MM cells in a mouse xenograft model, confirming the relevance of this lncRNA for progression of the disease in an *in vivo* setting.

CRNDE Affects the IL6 Signalling Pathway in MM Cells

Transcriptomic profiling of CRNDE-deleted MM cells revealed an interesting link between this lncRNA and IL6 signalling, a pathway critical for MM cell proliferation and survival [32]. Autocrine- and paracrine-IL6 signalling, which activates the downstream JAK/STAT3, p38/MAPK and PI3K/AKT/mTOR pathways [33], promotes survival of MM cells by inducing expression of the anti-apoptotic genes MCL1 and BCL-XL [34,35]. Due to its critical role in MM disease progression, drug resistance and relapse, the IL6 signalling pathway is frequently targeted by MM therapeutic strategies [36]. Our transcriptomic analysis revealed that MM cells lacking CRNDE expressed reduced levels of *IL6R*, the gene encoding the gp80 subunit of the IL6 receptor complex, which correlated with a transcriptionally repressive epigenetic state at the *IL6R* promoter [24]. Consistent with impaired IL6 signalling, CRNDE-deleted MM cells showed reduced phosphorylation of STAT3 and decreased proliferation in response to IL6 *in vitro*, together with gene expression suggesting perturbation of downstream JAK/STAT, PI3K-AKT and MAPK pathways. Since IL6 signalling is known to protect MM cells against therapeutic agents, and in particular from the effects of glucocorticoids [37-39], it is likely that impaired IL6 signalling contributes to the Dexamethasone-sensitivity observed in the CRNDE-deleted MM cells. Expression of *CRNDE* also correlated with that of *IL6R* in our cohort of primary tumour plasma cells, supporting the idea that increased levels of CRNDE leads to transcriptional activation of the *IL6R* gene in MM patients [24]. Together these observations indicate that CRNDE impacts upon MM progression and outcome by sensitizing malignant plasma cells to pro-tumorigenic IL6 signalling due to enhanced levels of the IL6 receptor (Figure 1B).

Effects of CRNDE on Interactions with the Bone Marrow Niche

The progression and evolution of MM is critically influenced by interactions between malignant plasma

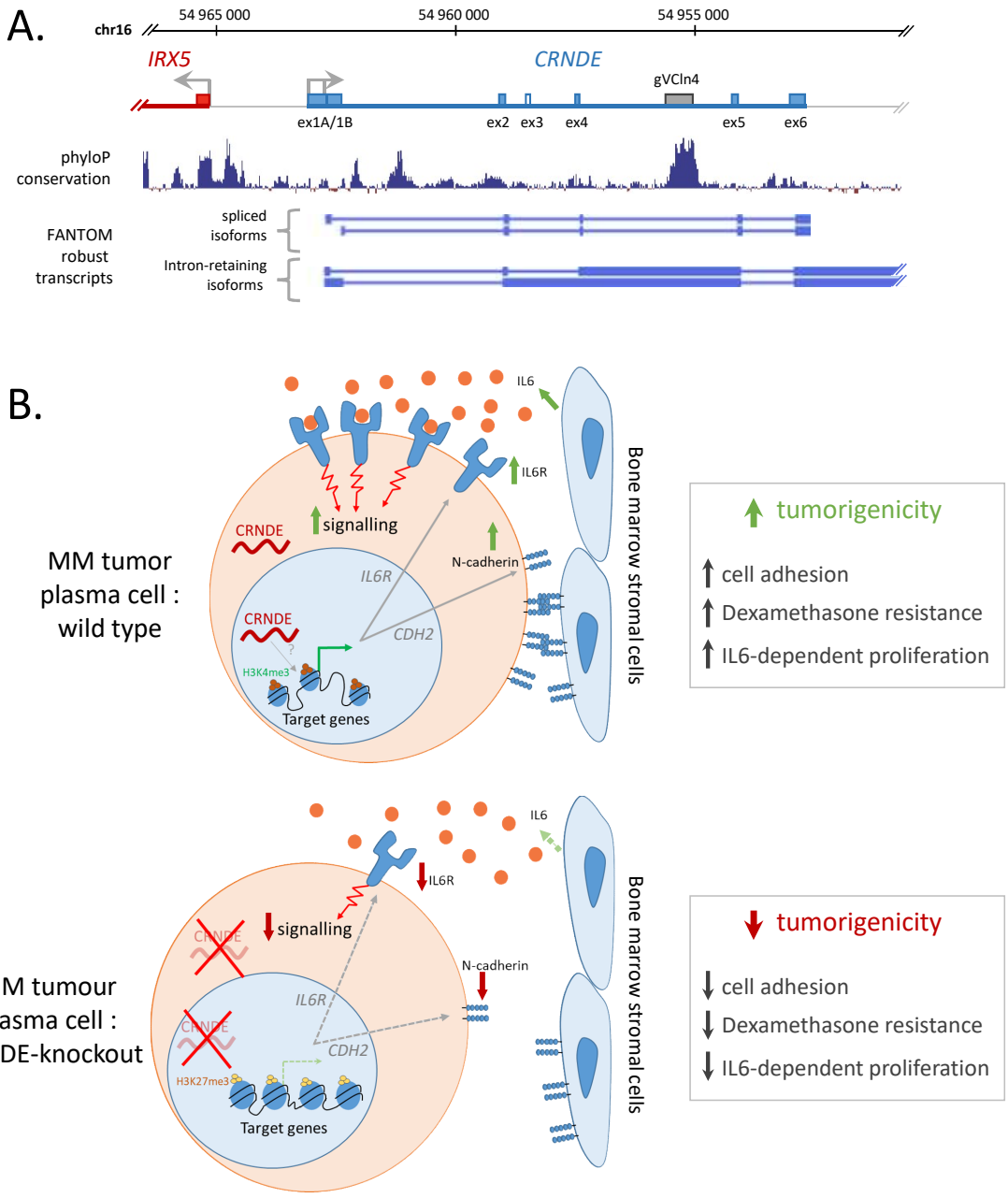

Figure 1: (A) The CRNDE locus at human chromosome 16p: The *CRNDE* gene and upstream antisense gene *IRX5* are indicated in blue and red respectively, with Refseq-annotated exons shown as boxes. CRNDE exon 3 is rarely detected in mature transcripts (28). Below is shown the phyloP conservation track, demonstrating a highly conserved element within intron 4 of *CRNDE* (gVC-In4, indicated as a grey box). At the bottom are shown transcripts representative of fully-spliced and intron-retaining isoforms of CRNDE (representative transcripts taken from the FANTOM robust transcript annotations). **(B) Model of the effects of CRNDE in MM cells :** High levels of CRNDE in wild type MM cells (upper panel) increase expression of several target genes, including *IL6R* (encoding a component of the IL6 receptor complex) and *CDH2* (encoding the adhesion molecule N-cadherin). In CRNDE-deleted cells (lower panel), these target genes are epigenetically less active. Decreased IL6R at the cell surface dampens IL6 signalling responses. Altered cell adhesion properties affect interaction with bone marrow stromal cells and stimulation of IL6 secretion. Collectively these changes decrease IL6-dependent proliferation and the *in vivo* tumorigenic potential of MM cells.

cells and the bone marrow microenvironment in which they reside [40-42]. The bone marrow milieu provides cytokines and chemokines which are critical for growth, survival and chemo-resistance of tumour plasma cells. Conversely, the tumour cells also influence the surrounding microenvironment, which is remodelled and adapted to establish a highly supportive neoplastic niche [40]. Our studies of CRNDE-deleted MM cells suggest that, as well as regulating the IL6 signalling pathway, this lncRNA also affects disease progression and outcome by influencing the adhesion properties of MM tumour cells and their interactions with the bone marrow niche. MM cells lacking CRNDE were less adhesive to an extracellular matrix substrate in *in vitro* assays, and gene expression profiles revealed dysregulation of pathways related to cell-cell and cell-substrate adhesion [24]. CRNDE-deleted MM cells were also less able to induce IL6 secretion by bone marrow stromal cells in co-culture assays, suggesting that the influence of CRNDE on the adhesive properties of tumour cells could also feedforward to further enhance IL6 signalling. Of particular note, our gene expression profiling revealed that CRNDE activates expression of *CDH2*, encoding the cell adhesion molecule N-cadherin. It has previously been shown that CDH2 is a negative prognostic indicator [43] and is increased in plasma cells of approximately 50% of newly-diagnosed MM patients where it facilitates interaction with the cells of the bone marrow niche [44]. Furthermore, knockdown or inhibition of CDH2 reduced both the adhesive properties and *in vivo* tumorigenicity of mouse MM cells [45]. These observations therefore suggest that *CDH2* could be another downstream target by which CRNDE impacts upon MM progression, by affecting the interaction between tumour plasma cells and their bone marrow niche (Figure 1B).

Future Perspectives

As in other diseases, the emerging importance of lncRNAs for the aetiology of MM has generated significant interest in these molecules both as prognostic markers, and as targets for the development of novel therapies. As discussed above, prognostically predictive signatures have been identified from lncRNA expression profiles in MM, and several individual lncRNAs, including CRNDE, are markers of poor disease outcome. Interestingly, for several of these lncRNAs, prognostically informative levels can be detected in circulating serum [46-48], suggesting the potential to develop lncRNA signatures as useful and easily accessible biomarkers of disease outcome and treatment response. Beyond their utility as biomarkers in malignant disease, there is expanding research interest in the potential to therapeutically target lncRNA activity, with several molecules already in the development pipeline within the pharmaceutical industry [49]. While there has been little investigation to date of the therapeutic value of lncRNAs in the treatment of MM, the link between CRNDE and the critical IL6 signalling pathway in MM cells suggests that this lncRNA may present an interesting target to attenuate IL6 signalling and improve or prolong drug sensitivity in some MM patients.

To date, most strategies have focused on targeting pathogenic lncRNAs for degradation using small interfering (si)RNAs to trigger the RNA interference (RNAi) pathway. Indeed, knockdown of CRNDE by RNAi has been successfully carried out in pre-clinical models of diverse tumours, including MM [25,27,28]. However, since the RNAi machinery is located in the cytoplasm, these approaches are often less successful at degrading nuclear lncRNAs, such as the intron 4-retaining isoform of CRNDE. This is a serious limitation both in the laboratory and in the clinic. More recent approaches invoke the use of chemically modified antisense oligonucleotide (ASO) reagents, which bind to the target lncRNA by Watson-Crick base pairing and trigger endonucleolytic cleavage by endogenous RNAseH (reviewed in [50]). Since RNAseH is present in both the cytoplasm and the nucleus, ASO technology promises to be more generally effective against lncRNAs, many of which display a nuclear localisation [51,52]. ASO reagents have been used to inhibit the pro-tumorigenic and metastatic potential of the nuclear-located lncRNA MALAT1 in *in vivo* models of breast and lung cancer [53, 54]. Nevertheless, several challenges remain in order to fully exploit the therapeutic potential of these molecules to inhibit lncRNAs in the clinic. These challenges include minimizing off-target effects, optimizing delivery technologies and strategies in order to limit on-target but off-tumour silencing of the lncRNA in healthy tissues, as well as improving the pharmacokinetics, stability and bioavailability of these reagents in the face of cellular nucleases and innate immune responses [50].

An alternative approach to impede the activity of lncRNAs is to inhibit the interactions between lncRNAs and the partner proteins through which they exert their biological activity. High-throughput molecular screens have successfully identified small molecules able to block the interaction between two lncRNAs (BDNF-AS and HOTAIR) and EZH2, a histone methyltransferase component of the Polycomb Repressive Complex 2 (PRC2), thereby upregulating expression of target genes normally repressed by these lncRNAs [55]. As for many other lncRNAs, one of the principle bottlenecks for this approach with respect to CRNDE is a current lack of understanding of its mechanisms of action and partner proteins. While several studies have described a mechanism of action in which CRNDE acts as a sponge to inhibit the activity of various miRNAs [27,28], miRNA sponging by CRNDE appears to be highly cell-type specific and is unlikely to account for the full range of its observed effects. Other evidence suggests that CRNDE (also called lincIRX5) can physically interact with members of the PRC2 complex

to repress downstream target genes [56,57]. However, in MM cells, CRNDE is a transcriptional activator of *IL6R* and *CDH2*, suggesting that it interacts with other partner proteins in some circumstances. To fully understand the cellular functions of CRNDE, it will be important to extend beyond candidate approaches by carrying out holistic profiling of its effects on the miRnome, and using unbiased screens to identify the full range of CRNDE-interacting proteins. As well as mass spectrometry approaches to identify proteins co-purified with a lncRNA of interest [58], other screening approaches, such as that reported recently which detects in-cell interactions using lncRNAs that have been tagged with the MS2 coat protein, promise to improve the sensitivity and resolution of these analyses [59].

In summary, CRNDE adds to a growing list of lncRNAs implicated in MM, with recent studies furthering our understanding of the important role played by CRNDE in this currently incurable malignancy. As a result of rapid evolution in the field of antisense-based therapeutics, as well as the improved characterisation of lncRNA partners and molecular function, there is a clear potential for the development of new therapeutic molecules targeting lncRNAs that could complement existing treatment regimes. Further, the link between CRNDE and the IL6 signalling pathway observed in MM may have more general implications for other malignancies in which CRNDE is involved.

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