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The human cytomegalovirus non-coding Beta2.7 RNA as a novel therapeutic for Parkinson's disease – Translational research with no translation

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ABSTRACT

Human cytomegalovirus (HCMV) encodes abundant numbers of microRNAs (miRNAs) and other non-coding RNAs (ncRNAs) whose functions are presently under intense investigation. In this chapter, we discuss the function of one of the more well characterised virus-encoded ncRNAs, derived from the viral major early gene (Beta2.7). This RNA plays an anti-apoptotic role during infection by directly interacting with mitochondrial complex I to help maintain high levels of ATP production and by preventing the stress induced re-localisation of retinoid/interferon-induced mortality-19 protein, GRIM-19. We then go on to describe how an 800 nucleotide sub-domain of the Beta2.7 transcript, p137, has been exploited in the development of a novel therapeutic for the treatment of Parkinson's disease.

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1. Introduction

Human cytomegalovirus (HCMV) is member of the betaherpesvirus sub-family which can cause significant morbidity and mortality in the immune compromised or immune naive. However, primary infection of the healthy immune competent is generally subclinical. Like all herpesviruses, the ability of HCMV to enter a latent life cycle, after primary infection, helps the virus persist for the life time of the host and this persistence likely includes periodic reactivation events which, again, rarely result in disease. HCMV has co-evolved over millions of years with its human host which has led to the adaptation of the virus to co-exist in human populations with extreme efficiency – the seroprevalance of HCMV can range from 60% to 90% in the developed world.

HCMV is the largest known herpesvirus with a double stranded DNA genome encoding approximately 250 open reading frames (ORFs) which encode functions to facilitate efficient latent and lytic infection cycles. The lytic lifecycle can be observed after primary infection in vivo in a variety of cells types; such as fibroblasts, macrophages and endothelial cells. In these cells, lytic infection is associated with a temporal cascade of, so-called, immediate early (IE), early (E) and late gene expression which culminates in the production of infectious virions. A substantial number of genes

expressed during the virus lytic cycle have immune-modulatory functions which are believed to enable the virus to evade the host immune system and establish latency in cells such as CD34+ myeloid progenitor cells. During latency, the lytic transcription programme is suppressed, resulting in the expression of a much more restricted spectrum of latency-associated genes and this is characterised by the absence of production of infectious virions. Only following terminal differentiation of the latently infected progenitor CD34+ cells to macrophages or dendritic cells, does virus reactivation occur resulting in activation of IE gene expression and re-entry into the lytic lifecycle.

2. HCMV non-coding RNAs

It is now becoming clear that, in addition to ORFs which result in expression of mRNAs encoding proteins, HCMV also encodes a substantial number of ncRNAs. Although the functions of many of these ncRNAs are currently not well defined, roles for some of them have been identified.

2.1. HCMV-encoded microRNAs (miRNAs)

miRNAs are short single stranded RNA molecules which down-regulate expression of transcripts which contain complementary nucleotide sequences. Since their first identification as regulators of larval development in nematodes (Lee et al., 1993), they have been described to play key roles in the regulation of almost every

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important biological process in eukaryotes across the metazoan kingdom (Lu and Cullen, 2004).

Biochemical and bioinformatic analysis, as well as next generation sequencing, has now identified at least 20 mature HCMV encoded miRNAs derived from 13 pre-miRNAs (Fu et al., 2014; Meshesha et al., 2012; Stark et al., 2012). Whilst the functions of a number of these virally encoded miRNAs are still unclear, some targets, which include both viral and cellular mRNAs, have been identified and these are believed to be involved in orchestrating changes in the infected cell to optimise lytic infection. Perhaps unsurprisingly, a good number of viral miRNAs have been demonstrated to modulate the expression of cellular mRNAs which encode proteins that play a role in cell death or immune evasion. For instance, HCMV miR-UL170-3p and miR-UL148D have been reported to play a role in the regulation of apoptosis by targeting the pro-apoptotic proteins MOAP1, PHAP, and ERN1 (Babu et al., 2014). In addition to targeting anti-apoptotic proteins, a separate study demonstrated that UL148-D also targets CCL5 (RANTES) (Kim et al., 2012) which could contribute to a reduction in leukocyte chemoattraction to the infected cell. Another immune evasion strategy is the ability of viral miR-US4-1 to target ERAP-1. ERAP-1 is an amino peptidase which functions to regulate MHC class I presentation, thus perturbation of this gene contributes to the evasion of CD8+ T cell responses (Kim et al., 2012). The viral miR-UL112-1 targets IL-32 (Huang et al., 2013). Since IL-32 is a pro-inflammatory cytokine which can induce the expression of a number of pro-inflammatory cytokines in myeloid cells such as monocytes and macrophages (a site of latency and reactivation, respectively), targeting of this gene could potentially enhance carriage of the virus.

In addition to modulation of immune responses, HCMV miRNAs can also fine tune the intracellular environment by a number of mechanisms. There are a number of viral miRNAs that regulate the expression of cellular genes. For example, HCMV miR-UL148D targets the cellular immediate early gene X-1 (Wang et al., 2013) and the viral miR-US28-2-3p targets eIF41a (Qi et al., 2013). Viral miRNAs have also been reported to modulate the expression of HCMV genes. Viral miR-UL-112-1 targets the HCMV viral IE1 gene (Grey et al., 2007), viral US7 is targeted synergistically by two viral miRNAs, miR-US5-1 and miR-US5-2 (Tirabassi et al., 2011). HCMV viral miRNAs have also been implicated to play specific lifecycle-specific roles. For example, HCMV miR-UL36 targets the latency-associated viral gene, UL138, during lytic infection and thus decreases expression of this gene during the lytic cycle (Huang et al., 2013).

Although viral miRNAs have been shown to play important roles during latency in other herpesviruses (Pfeffer, 2007; Pfeffer et al., 2005; Samols et al., 2007), there are, as yet, no definitive reports of latency-associated functions of HCMV miRNAs. Although, it is likely that HCMV encoded miRNAs are also likely to be involved in the modulation of viral and cellular functions during latent infection. Indeed, viral miRNAs are well-suited to the demands of the latent life cycle; they are small non-immunogenic molecules that will minimise detection and eradication of the latently infected cells by the immune system. This is a particularly important consideration during latency as the majority of the immune evasion genes encoded by the virus are likely not expressed during this part of the viral life cycle. Furthermore, as individual miRNAs are able to concurrently modulate the expression of multiple targets (Zhu et al., 2013), their expression enables the alteration of gene expression at a global level. Thus, the known profound effects of viral miRNAs on cellular and viral gene expression are also likely to be mirrored during latency and could have significant roles in the establishment and maintenance of latency and reactivation. For example, the ability of HCMV miR-UL112-1 to target the viral major immediate early gene could serve to enhance the maintenance of latency by silencing any 'leaky' transcription from the major immediate early promoter of lytic IE gene expression (Murphy et al., 2008;

O'Connor et al., 2014). Similarly, as mentioned above, HCMV miR-UL36 targets the latency-associated viral gene, UL138, during lytic infection and thus decreases expression of this gene during the lytic cycle (Huang et al., 2013).

2.2. Long non-coding RNAs

In addition to miRNAs, HCMV is also known to encode other RNAs which are polyadenylated but do not appear to function as protein coding RNAs. These include long non-coding RNAs (lncRNAs) such as RNA2.7, RNA1.2, RNA4.9 and RNA5.0 which can be expressed at extraordinarily high levels during lytic infection (Gatherer et al., 2011; Kulesza and Shenk, 2004) although deletion of RNA5.0 did not result in any growth defects of the virus in fibroblast cells (Kulesza and Shenk, 2004). Although none of these overlap with established annotated protein coding regions, they may produce polypeptides as a result of non-canonical translation of these RNAs (Ingolia et al., 2014).

Although the functions of many of these lncRNAs during HCMV infection is far from clear, recently, some have been shown to target specific gene promoters mediating transcriptional silencing, often by epigenetic targeting. For instance, one of the HCMV encoded lncRNAs, RNA4.9, has been proposed to play a role in transcriptional repression of viral IE gene expression during latency (Noriega et al., 2014; Rossetto et al., 2013a). Identified during both experimental and natural latent infection (Rossetto et al., 2013a), the RNA4.9 transcript has been shown to associate directly with the polycomb repressor complex 2 (PRC2) in experimentally latently infected CD34+ cells and this has been suggested to enhance silencing of the viral MIEP during latent infection by recruitment of histone modifiers so resulting in histone H3 tri-methylation at lysine 27 around the MIEP (Rossetto et al., 2013a). Viral lncRNAs are not specific to HCMV, other herpesviruses also encode them. Interestingly, the KSHV encoded PAN RNA also binds to PRC2 and is proposed to play a similar role to that of HCMV RNA4.9 (Rossetto et al., 2013b) during KSHV latency (Rossetto and Pari, 2014).

Arguably the most enigmatic HCMV lncRNA, is expressed from the viral Beta2.7 gene. Present as two copies in clinical isolates of HCMV (McSharry et al., 2003), it is the most abundant viral RNA produced during lytic infection – some 20% of all viral RNAs expressed at early times of lytic infection derive from the Beta2.7 genes (Gatherer et al., 2011). A potential coding frame within Beta 2.7 was identified in the highly passaged strain AD169 and this potential mRNA was termed RL4 (Bergamini et al., 1998). Similarly, the coding potential of Beta2.7 has been recently been re-assessed (Stern-Ginossar et al., 2012). However, sequencing of all other strains of virus demonstrated that this potential coding region is disrupted (McSharry et al., 2003). Further to this, although in vitro studies demonstrated that a short 24 kDa protein could be expressed from the RL4 region of Beta2.7, intracellularly the RNA was found to be nucleolar and its expression was post-transcriptionally inhibited by the 5' sequence of the transcript (Bergamini et al., 1998). Taken together, therefore, it seems unlikely that the Beta2.7 encodes a polypeptide.

3. Anti-apoptotic functions of the non-coding HCMV RNA p137

By early times (12–24 h) of the HCMV lytic cycle, the transcript, represents approximately 20% of the total viral RNA expressed in the infected cell and is known to be associated with mitochondria (Gawn and Greaves, 2002). Since the Beta2.7 RNA does not encode any protein, potential cellular protein partners which may interact directly with this RNA were identified by screening a human cell cDNA expression library in lambda phage with an RNA probe

derived from the transcript by northwestern analysis. This screen identified one interaction partner of the viral RNA as being NADH Ubiquinone Oxidoreductase, a subunit of mitochondrial Complex I (Reeves et al., 2007). Inhibition of Complex I by reactive oxygen or nitrogen (O or N) species as well as by environmental toxins such as rotenone is known to result in apoptosis (Green and Kroemer, 2004; Li et al., 2003) and interaction of the transcript with Complex I was found to inhibit rotenone-induced apoptosis in neuronal cells (Reeves et al., 2007). HCMV is known to employ a number of mechanisms to avoid apoptosis during both lytic and latent infection (McCormick, 2008; Arnoult et al., 2004; Terhune et al., 2007; Michaelis et al., 2004; Chan et al., 2010; Poole and Sinclair, 2015). For example, during lytic infection, HCMV expresses a number of gene products which inhibit both intrinsic and extrinsic apoptotic signalling pathways and these include the viral mitochondrial inhibitor of apoptosis (vMIA) – a Bcl2 homologue which inhibits Bax (Arnoult et al., 2004; Ma et al., 2012; McCormick et al., 2003, 2005, 2008; Pauleau et al., 2007; Smith and Mocarski, 2005).

HCMV also inhibits apoptosis during latent infection by modulating expression of a number of cellular proteins involved in the regulation of, again, both the intrinsic and extrinsic apoptosis pathways (Poole and Sinclair, 2015). Similarly, latency-associated changes during latent infection also prevents FAS-mediated apoptosis by a number of mechanisms (Poole et al., 2011; Seirafian et al., 2014).

In more detail, the mechanism by which the Beta2.7 RNA enhances cell survival during lytic HCMV infection appears to be via a direct interaction with the cellular gene associated with retinoid/interferon-induced mortality-19 (GRIM-19). This interaction, between Beta2.7 RNA and GRIM-19 appears to be important for stabilizing mitochondrial membrane potential and results in continued ATP production, which is critical for the successful completion of the virus' life cycle. Thus, by targeting Mitochondrial complex I, the Beta2.7 RNA represents a refined strategy by which the virus is able to regulate the metabolic viability of the infected host cell (Reeves et al., 2007).

4. Therapeutic application of the non-coding HCMV beta 2.7 RNA in the treatment of Parkinson's disease

Parkinson's disease (PD) is a clinical condition that is characterised by the gradual onset of a series of motor features including tremor, bradykinesia, rigidity, gait dysfunction and postural instability. Pathologically PD is defined by the loss of nigral dopaminergic neurons and the formation of alpha synuclein Lewy bodies. In addition to the motor dysfunction, PD patients also manifest multiple non-motor problems including cognitive impairment and dementia, mood disorders and autonomic dysfunction. These latter problems reflects a pathology that extends out of the substantia nigra and into a number of other CNS sites as well as the enteric nervous system (Ford et al., 2013; Greenbaum and Lerer, 2015; Lin and Wu, 2015; Stebbins et al., 2013; Yarnall et al., 2013).

As yet, there is no disease-modifying therapy for PD although a number of symptomatic treatments are available which help some of the motor features of the condition. These include drugs such as Levodopa which is transported from the peripheral circulation across the blood–brain barrier and then is converted to dopamine centrally, thus replacing the known decrease in dopamine resulting from the death of nigral dopaminergic neurones (Fedorova et al., 2012; Sourkes, 1971). There are also a number of other drugs available which promote the delivery of L-dopa to the brain by blocking its peripheral catabolism such as catechol-O-methyltransferase inhibitors. Post synaptic receptors in the striatum can also be targeted by dopaminergic agonists and in addition anticholinergic drugs are also occasionally used in younger onset, tremor

dominant patients and amantadine in more advanced patients with L-dopa induced dyskinesias (Del Dotto et al., 2001). In contrast to the number of drugs which target deterioration in motor functions associated with PD, there are far fewer available therapies for the non-motor features of the disease. Studies have indicated that 46% of patients develop Parkinson's disease dementia within 10 years of diagnosis and 80% by 20 years leading to significantly increased morbidity, a decrease in quality of life and a greatly increased probability of being placed in a nursing home (Lin and Wu, 2015). Thus, a significant amount of current research has concentrated on better understanding the development of a dementia in PD and its underlying pathological substrate. Clinically patients who are older at disease onset with cognitive deficits involving semantic fluency and visuospatial function tend to develop the dementia earlier, especially if they possess the more common H1/H1 tau haplotype (Goris et al., 2006; Williams-Gray et al., 2009) or a heterozygote GBA mutation (Winder-Rhodes et al., 2013). Pathologically the main finding is cortical alpha synuclein positive Lewy bodies, although whether there is also an amyloid pathology associated with it is still unclear (Alves et al., 2013; Braak and Braak, 1990; Compta et al., 2014; Ho et al., 2014; Jendroska et al., 1996; Maetzler et al., 2014; Mastaglia et al., 1989).

There are now known to be a number of Mendelian forms of PD (Kruger et al., 1998; Martinez et al., 2004; Polymeropoulos et al., 1997; Singleton et al., 2003) which has elucidated some of the pathways that may underlie the development of this condition. While this includes over production of alpha synuclein and abnormalities of autophagy, another common intracellular problem seems to lie within Complex I of the mitochondria (Martin et al., 2010; Schapira et al., 1990). Indeed all these pathways may interact, so for example, alpha synuclein overproduction may compromise mitochondrial function which in turn may promote alpha synuclein aggregation. As such, any agent which counteracts such mitochondrial impairment could be appealing therapeutically and the discovery that the HCMV encoded Beta2.7 RNA enhances cell survival by increasing ATP levels in neuronal cells led to research on the ability of Beta2.7 to enhance cell survival during PD.

Delivery of functional ncRNAs, perhaps, has a number of obvious advantages over drug-based therapies. For instance, delivering an RNA molecule might be predicted to have less cytotoxicity than chemical drugs. Interestingly, initial observations showed that a small domain of the transcript called p137, which contained the putative TLR4 subdomain (Bergamini et al., 1998), was as capable of protecting cells stressed by rotenone as the full length RNA (Kuan et al., 2012). However, in order for it to have real therapeutic potential for e.g. PD, it would be necessary to be able to deliver the RNA specifically to the degenerating neuronal cells.

The ability to deliver small RNAs to specific target cells has been a topic of considerable interest in recent years, especially following the advent of RNAi technology. With this as a driving force, a number of delivery systems for siRNAs have been tested. These have included the use of various viral vector delivery systems. Viral vectors, including lentiviruses, retroviruses and adenoviruses, have all been proposed and used to deliver genes to dysfunctional cells which can then serve as treatments for a number of disorders including cancer, diabetes and asthma (Kay et al., 2001). For example, the delivery of the insulin gene to islet cells may be used for the treatment of type I diabetes. However, to deliver RNA specifically to neuronal cells involved in PD would require the development of a viral vectors which target at least the dopaminergic neurones known to be affected by PD and, so as not to depend on more invasive treatments such as intracranial injection, would have to have the ability to cross the blood brain barrier.

To side step the need to deliver therapeutics directly into the brain, single or multiple times, a number of alternative delivery system have been investigated. These include polymers, liposomes,

nanoparticles and peptides (Zhang et al., 2012). One of these peptide-based approaches is based on a small peptide (RVG) from the rabies viral glycoprotein which not only crosses the blood brain barrier but binds specifically to neuronal cells via the acetyl choline receptor, exclusively expressed in central nervous system cells (Son et al., 2011).

Elegant studies by Kumar and colleagues (Kumar et al., 2007) showed that modification of RVG to contain a tail of 9 arginine residues (RVG-9R) resulted in a peptide not only capable of efficiently binding small RNAs (through the 9 arginine residues) but also, after transvascular delivery, able to cross the blood brain barrier resulting in specific delivery of these RNAs to neuronal cells in the brain (Kumar et al., 2007).

Based on this, our own work showed that a complex of p137 RNA and RVG-9R peptide (p137/RVG-9R) protected neuronal cell lines from rotenone induced cells death as well as protecting primary neurons from death using the catecholaminergic neurotoxin, 6 hydroxy dopamine (6-OHDA) in culture (Kuan et al., 2012). Our subsequent studies went on to show that not only was p137/RVG-9R able to protect dopaminergic neurons from death in an acute 6-OHDA rat model of PD by protecting mitochondrial Complex I activity when injected directly into the substantia nigra of the brain but also when administered into the blood stream. Consistent with this protection of dopaminergic cells from death, rats treated with p137/RVG-9R performed better in a battery of standard behavioural assessments (Kuan et al., 2012).

These studies, therefore, clearly showed that this novel RNA-based therapeutic could prevent dopaminergic cell death when delivered before 6-OHDA lesion in a rat model of PD. However, a more clinically relevant question was whether it could also show protective effects after a lesion had been initiated. This is because patients typically only present clinically when they have lost over 50% of their nigral dopaminergic neurons – in other words neurodegeneration is already well advanced at the time of clinical diagnosis. Follow up experiments, using a progressive model of PD in which the 6-OHDA lesion was initiated before delivery of p137/RVG-9R, showed that p137/RVG9 complex delivered transvascularly also protected dopaminergic neurons in this progressive PD model and mediated a neuroprotective function to attenuate cell loss after the onset of lesion. These findings suggest that this novel therapeutic based on the Beta2.7 RNA of HCMV could be a very effective and non-toxic potential treatment for PD- not least because it should in theory target all dysfunctional neurons in PD not just the dopaminergic ones that we used in these model systems.

It should be said that, in these studies, delivery of p137 outside the brain after transvascular administration was not assessed. Consequently, further studies will be needed to assess potential off-targets effects perhaps resulting from irregular mitochondrial protection in other cell types.

5. p137 may have therapeutic applications in other neurodegenerative disorders

Parkinson's disease is not the only neurodegenerative disorder which is associated with mitochondrial abnormalities. Indeed, mitochondrial dysfunction has been proposed to be involved, at least to some extent in the observed neuronal cell death in a number of other neurodegenerative disorders.

Complex I function is reduced in the skeletal muscle of patients with Huntington's Disease (HD) and in the brain tissue of Alzheimer's Disease sufferers in addition to being reduced in the Substantia Nigra of PD patients (Johri and Beal, 2012) (Table 1). It is possible, therefore, that p137 may be used therapeutically for other neurodegenerative disorders. Consequently, it has been proposed that any therapeutic treatment which protects mitochondrial

Table 1

	Complex I (NADH dehydrogenase or NADH ubiquinone oxidoreductase) defect
Huntingdon's Disease	Reduced Complex I function in skeletal muscle but unaltered in the brain
Alzheimer's Disease	Complex I function is reduced in platelets and lymphocytes as well as mitochondria. Also reduced function in post mortem brain tissue.
Amyotrophic Lateral Sclerosis	Reduced function in skeletal muscle
Parkinson's Disease	Impaired Complex I activity in the substantia nigra, platelets and skeletal muscle of patients

Summary of Complex I defects in neurodegenerative disorders

function could prevent the onset of clinical features if the diagnosis could be made early enough and ahead of a clinical diagnosis as is possible in some disorders such as HD given its autosomal dominant basis and the fact that the gene is fully penetrant.

6. Further investigation into potential therapeutic functions of Beta2.7 and other non-coding RNAs

To date, a small domain of approximately 800 nucleotides of the Beta2.7 RNA has been identified which can mediate protection of dopaminergic neurons in rat models of PD by its ability to interact with mitochondrial Complex I. However, the generation of a tractable therapeutic will likely depend on the ability to identify a smaller minimum domain of this RNA which easily lends itself to production under good manufacturing practices (GMP).

Mitochondria clearly play an important role in the development of neurodegenerative disorders and it is not only Complex I which is affected. In PD the activity of both Complex IV (in platelet mitochondria and skeletal muscle) and complex V (in skin fibroblast) activity are reduced. In HD, Amyotrophic Lateral Sclerosis and Alzheimer's Disease complex II–V are all dysregulated (Johri and Beal, 2012). Given the ease with which small molecules can be delivered to neurons using this RVG peptide and the lack of toxicity and host immune reaction associated with RNA molecules it may be possible to use the p137-Complex I interaction as a model for predicting synthetic RNAs for targeting the other mitochondrial complex proteins. Structural analysis of the p137-Complex I interaction may give insights as to how these other RNA molecules could be designed. Indeed, recent technologies have been designed which allow for the visualisation of long non-coding RNAs within cells at a structural level using the 'ribozyme toolkit' (Pyle, 2014) and these types of analyses with the p137 molecule may lead to the development of other RNA-based therapeutics.

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