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RSV Induced Changes in miRNA Expression in Lung

Shirley R. Bruce and Joseph L. Alcorn
Department of Pediatrics, University of Texas Health Science Center at Houston, Houston, TX
USA

1. Introduction

The ability of Respiratory Syncytial Virus (RSV) to exist within the host cells of the lung depends on its ability to evade host antiviral defenses. Indeed, suppression of host cell immune defenses is not restricted to RSV but is a common mechanism of survival employed by most viruses. Viruses utilize a number of mechanisms to evade detection by the immune response including translational repression of host cell mRNA. One mechanism to alter translation of a specific mRNA is through binding of microRNAs (miRNAs) within the 3'-untranslated region (3'-UTR) of the target gene. Here, we will discuss mechanisms of translational repression by miRNAs during viral infection and will provide data on changes in miRNA expression during RSV infection of the lung.

1.1 Functions of the lung

The lung is a complex multicellular organ that has two main responsibilities; air exchange and pulmonary defense. The first responsibility is for exchange of oxygen with CO_2 between epithelial cells and capillaries in the lung alveolus. As oxygen enters the nasal passages, it passes through the trachea into the bronchi of each lung. From the bronchi, oxygen passes through much narrower bronchioles until it reaches the terminal alveoli, where actual exchange takes place. The alveolar surface is composed primarily of type I and type II alveolar epithelial cells which assist in gas exchange and in preventing alveolar collapse upon exhalation (West, 1990). Type I alveolar epithelial cells are large, thin cells that possess long cytoplasmic extensions. The cytoplasmic extensions allow type I cells to stretch along the large alveolar surface area, forming the air/blood barrier necessary for gas exchange. While type I cells cover 90% of the surface area within the alveoli, they only represent 8% of the total cells in an adult lung (Crapo et al., 1982). Type II epithelial cells are cuboidal in shape and much smaller than type I epithelial cells. They are typically found at the alveolar-septal junction and represent 15% of the cells lining the alveoli (Crapo et al., 1982). To prevent alveolar collapse upon exhalation, type II cells synthesize and secrete surfactant, which is composed of approximately 90% lipid and 10% protein (Haagsman & Diemel, 2001). The protein portion of surfactant is composed of four different surfactant proteins, A (SP-A), B (SP-B), C (SP-C) and D (SP-D), and these proteins are critical for the lungs primary functions of mediating gas exchange and the removal of infectious agents. The hydrophobic proteins SP-B and SP-C, along with the lipid portion of surfactant, function to reduce surface

tension at the air liquid interface of the lung thereby maintaining alveolar stability and allowing gas exchange to occur (Haagsman & Diemel, 2001). The importance of SP-B in normal lung function is demonstrated by prematurely-born infants and by humans carrying genetic mutations of the SP-B gene that result in the lack of appropriate levels of the SP-B protein (Beers et al., 2000; Nogee et al., 1994). SP-B protein is produced late in the third trimester and infants born prior to 31 weeks of gestation are subject to respiratory dysfunction and respiratory distress syndrome (RDS) leading to increased morbidity and mortality of the neonate (Pryhuber et al., 1991). Full term infants born with a genetic defect in SP-B, which results in decreased expression of the SP-B protein, experience respiratory failure and lethal RDS succumbing by 6 months of age (Beers et al., 2000; Nogee et al., 1994).

The second responsibility of the lung is the removal or neutralization of infectious agents via the pulmonary innate and adaptive defenses (Wilmott et al., 1998). As a natural consequence of gas exchange, the lung is exposed to numerous environmental stimuli. Suspended within the inhaled air is an array of particulate matter and infectious agents that result in many respiratory illnesses. These infectious agents include bacterial and viral agents, as well as inert irritants found in the environment. The hydrophilic surfactant proteins SP-A and SP-D, produced by type II epithelial cells, aid in removal of infectious agents (see below) (Crouch & Wright, 2001). In addition, goblet cells within the lung produce a thin layer of mucous which helps trap foreign particles found in the air we breathe. Once foreign particles are trapped within the mucous, ciliated epithelium in the bronchi and bronchioles function to move mucus out of the lung and towards the pharynx (Murray, 2010). Also within the lung are a variety of secreted factors which target microbial agents for lysis and destruction as part of the innate immune response (Ganz, 2002).

1.2 Respiratory syncytial virus pathogenesis

One pathogenic agent encountered during breathing is RSV. RSV is a negative sense, single stranded, enveloped RNA virus of the family *Paramyxoviridea* (Domachowske & Rosenberg, 1999; Hacking & Hull, 2002). RSV infects epithelial cells of the lung, resulting in lower respiratory tract disease that can be particularly problematic for children, elderly adults and individuals with compromised immune systems (Englund et al., 1988; Griffin et al., 2002). A highly ubiquitous and contagious virus, RSV is the major respiratory pathogen in young children <1 year of age and virtually every individual has been infected with RSV at least once by the age of 3 (Black, 2003; Centers for Disease Control [CDC] 2004; Denny & Clyde 1986; Foy et al., 1973; Mufson et al., 1973; Parrott et al., 1973). In the United States up to 2.5% of children require hospitalization for RSV infections and epidemiological studies in children suggest an association between lower respiratory infection and persistent or recurrent respiratory obstruction (Mufson et al., 1973; Parrott et al., 1973). Prematurely-born infants have immature lungs and inadequate host defense mechanisms, resulting in increased susceptibility to pneumonia and bronchiolitis as a result of RSV infection.

When RSV infects airway epithelial cells in culture, no virus is produced in the initial 12 hours. An exponential growth phase follows this latent period, reaching a slight plateau at 34 hr, and then further exponential growth occurs until 48 h. Ultimately, lysis of the cell occurs with complete cellular destruction by 96 hr. During this relatively slow life cycle, most of the infectious virus remains cell-associated, with extracellular viral release upon death and lysis of the cell (Richman & Tauraso, 1971). In the lung, RSV infections generally last 8-15 days. Despite the prevalence of RSV infections, there are currently no adequate

treatments. Palivizumab a monoclonal antibody directed against RSV surface fusion (F) protein (Subramanian et al., 1998), pharmacologic options (Frogel, 2008), and the antiviral drug Ribavirin show limited effectiveness (Ventre & Randolph, 2007).

The RSV genome is composed of 11 primary RNAs which encode major viral proteins (Domachowske & Rosenberg, 1999). Two of these proteins, the G protein and the F (fusion) protein, transmembrane proteins found within the envelope of the RSV viron, are important for viral infection. The G protein is the major attachment protein and the F protein is important for fusion between the viral envelope and the host membrane (Domachowske & Rosenberg, 1999; Hacking & Hull, 2002). The F protein is also involved in syncytial formation between adjacent cells, giving rise to the multinucleated cell-like structures that are characteristic of RSV infection (Domachowske & Rosenberg, 1999; Hacking & Hull, 2002). In addition to the G and F proteins, a small hydrophobic (SH) protein of unknown function is also located within the viral envelope. The matrix (M) protein is located in the inner layer of the viral envelope and although the function of the M protein is not know, it is presumed to play a role in virus assembly and budding similar to the M protein of other members of the *Paramyxoviridea* family (L. Rodriguez et al., 2004). The nucleocapsid (N), nucleocapsid phosphoprotein (P), and the polymerase (L) proteins form a single complex and this complex is important for RSV replication and transcription (Cowton et al., 2006). The RSV genome also contains several proteins that can inhibit or ameliorate the host response to infection. Two nonstructural proteins (NS1,NS2) are the most abundantly expressed proteins of the RSV genome, and these proteins have been shown to inhibit the response of host cells to interferon by inhibiting the activation of interferon regulatory factor 3, a transcription factor involved in IFN-β promoter activity (Bossert & Conzelmann 2002; Bossert et al., 2003). In addition, the G protein can be expressed in a truncated, secreted form that acts as a chemokine mimic. Secreted G protein competes with the chemokine fractalkine (Fkn) for binding to the CX3CR1 receptor (Tripp et al., 2001). Binding of Fkn to the CX3CR1 receptor induces migration of leukocytes to the site of infection and this migration can be subverted by the RSV G protein. While some RSV proteins can mitigate detection by the host cell immune response, components of the lung innate immunity, such as SP-A, can directly bind to the RSV F protein and mediate uptake of the virus by resident macrophages (Barr et al., 2000; Ghildyal et al., 1999).

1.3 Innate immune environment of the lung

As the first line of defense against invading pathogens, the innate immune system acts in an immediate non-specific manner to a variety of organisms. A physical barrier to pathogens is established by the airway epithelium and the cell-cell junctions between neighboring cells. Overlaying the airway epithelium is a layer of mucous which entraps foreign particles inhaled during breathing (Knowles & Boucher, 2002). The mucous forms a semi-permeable barrier that is not permeable to many pathogens however, still allows for exchange of gas and other nutrients. Mucins are the major component of respiratory mucous and have antiviral and anti-inflammatory properties (Thornton et al., 2008). Located within the mucous are polypeptide components including lysozyme, lactoferrin, protease inhibitors, defensins, and members of the collectin family of surfactant proteins SP-A and SP-B, all of which function to neutralizing foreign particles (Ganz, 2002; McCormack & Whitsett, 2002). Monocytes, macrophages, neutrophiles and epithelial cells secrete the enzyme lysozyme which lysis the bacterial cell wall of gram positive bacteria (Laible & Germaine, 1985). Lactoferrin is also secreted by neurophiles and binds iron in the environment thus depriving bacteria of essential nutrients

(Arnold et al., 1977). Defensins are a group of proteins produced by neutrophiles and epithelial cells of the respiratory system. They can bind to bacterial cells and some enveloped viruses forming pores in the microbial membrane. Formation of these pores compromises the integrity of the cell membrane allowing for the efflux of essential nutrients (Ganz, 2003). Finally, the hydrophilic proteins SP-A and SP-D, produced by type II epithelial cells, are also part of the lung's innate defense mechanism and serve to neutralize foreign particles and stimulate the immune response (Tino & Wright 1996; Wright & Youmans 1993).

The SP-A and SP-D proteins belong to a family of C-type lectins called collectin that function in the innate host defense of the lung (Crouch & Wright, 2001; Holmskov et al., 2003). Members of the collectin family of proteins are important in the innate response within the lung where they opsonize pathogens by non-specific binding to glycoconjugates and lipids present on the cell surface, facilitating phagocytosis by resident macrophages within the lung (Tino & Wright, 1996; Wright & Youmans, 1993). In addition, SP-A and SP-D can bind to receptors on the surface of macrophages to enhance phagocytosis of nonopsonized particles (Crouch & Wright, 2001). The importance of SP-A in the innate immune response to foreign pathogens is illustrated by transgenic mice that are deficient in SP-A. These mice were unable to efficiently clear bacterial infections, such as group *B streptococcus* and *Pseudomonas aeruginosa* (LeVine et al., 1997; LeVine et al., 1998). Furthermore, SP-A deficient mice infected with RSV exhibited increased RSV plaque forming units when compared with control mice (LeVine et al., 1999). These data suggest that SP-A is important in the lung's defense against RSV infections. Previous studies from our laboratory using alveolar type II cells in primary culture and the lung adenocarcinoma cell line NCI-H441 have shown that RSV infection alters SP-A protein production (Alcorn et al., 2005; Bruce et al., 2009). In the presence of RSV, SP-A mRNA increases approximately 3-fold however, the level of cellular and secreted SP-A protein decreases. The decrease in SP-A protein expression in lung epithelial cells during RSV infection results from translational inhibition of SP-A mRNA leading to reduced *de novo* SP-A protein synthesis (Bruce et al., 2009).

1.4 Host/virus interactions

Viruses are obligate, intracellular parasites with the sole purpose to replicate itself, usually at the expense of the host cell, and viruses are not considered to be of any great benefit to humans in general. The viral genomes of RNA viruses reside in the cytoplasm of host cells while the host genomes reside within the nucleus. The nuclear membrane physically separates the cellular location of these two genomes. However, the protein and RNA products produced from each genome can interact with one another, having consequences for the replication and survival of the virus and the host cell. Viruses have developed a number of mechanisms to circumvent detection by the innate and adaptive immune system of the host, in order to assure survival of virus progeny. Alternatively, the innate immune system of the host contains a number of mechanisms designed for early detection of viral infections. The constant evolutionary pressure upon the virus and the host for survival leads to different, novel and more effective strategies to win the race for continued existence. Viruses can mutate quickly in order to survive within the environment (Holmes, 2011). Their small genome and small number of proteins would allow the viruses to adapt quickly. Humans have a much larger genome and their ability to adapt to the environment would presumably occur at a much slower rate. Since viruses generally replicate at the expense of the host cell, the host cell must spend energy and resources to combat the virus. The host cell innate immune response is an early detection method used for this purpose.

The innate immune system of the host cell contains a number of mechanisms designed for early detection of viral infections. Through pattern recognition receptors (PRR) located within host cells, viral components such as DNA, RNA and proteins are recognized and the innate immune response activated (Akira et al., 2006). PRRs recognize pathogen associated molecular patterns (PAMPs) which distinguish host molecules from pathogen associated molecules (Kawai & Akira, 2009). Binding of PAMPs to toll-like receptors (TLR) are one mechanism by which pathogenic microorganisms are recognized. TLRs are transmembrane proteins that recognize ssRNA, dsRNA, DNA and viral envelope proteins. Activation of TLRs initiates production of type I Interferons (IFNs) which lowers the susceptibility of the cell to infection by altering metabolism and upregulating the expression of MHC class I and MHC class II molecules (Bonjardim et al., 2009). Retinoic acid-inducible gene I (Rig-I)-like receptors (RLRs) are a family of cytoplasmic proteins that recognize viral RNA (Kang et al., 2002; Yoneyama et al., 2004, 2005). As with TLR receptor signaling, RLR proteins also initiate production of type I IFNs. Finally, the cytoplasmic nucleotide oligomerization domain (NOD)-like receptors (NLRs), also recognize viral DNA and regulate interleukin-1β maturation (Muruve et al., 2008).

Viruses utilize a number of mechanisms to evade the host immune response. For example, some viruses are able to inhibit the action of interferons. Interferon expression inhibits protein synthesis and destroys RNA in infected cells, up-regulates MHC class I and MHC class II expression and stimulates the innate immune response by activating macrophages and natural killer cells (Fensterl & Sen, 2009). Human cytomegalovirus (HCMV), Varicella-Zoster virus (VZV), Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV) contain proteins expressed during the lytic cycle, which disrupt interferon signaling (Abendroth et al., 2000; Grundhoff & Sullivan, 2011; Sedmak et al., 1994). Secondly, viruses can alter MHC class I expression. For example, HCMV produces two proteins US2 and US11 which "dislocate" newly synthesized MHC class I molecules from the endoplasmic reticulum to the cytoplasm, where the MHC molecules are targeted for degradation by the proteosomal pathway (Wiertz et al., 1996a, 1996b). Here the virus interferes with the process of antigen presentation and downregulates the adaptive immune response. Some viruses produce their own cytokine mimics which can alter the immune response. Interleukin-10 (IL-10) is an anti-inflammatory cytokine which inhibits pro-inflammatory cytokines, including interferon, and suppresses antigen-presentation (Moore et al., 1993). EBV and HCMV produce homologous of IL-10 which can bind to IL-10 receptors and dampen the immune response during viral infection (Hsu et al., 1990; Raftery et al., 2004). In addition, both HIV-1 and RSV infection were shown to induce expression of IL-10 in the host cell, thus suppressing release of early immunoregulatory cytokines (Panuska et al., 1995; Schols & De Clercq, 1996). Decoy receptors for the human cytokines are produced by some viruses which effectively dilute the amount of cytokines available for the immune response. The molluscum contagiosum virus (MCV), a human pox virus, produces two proteins that bind the human cytokine IL-18. IL-18 is a pro-inflammatory cytokine which induces the innate immune system. Binding of IL-18 by these decoy receptors prevents IL-18 from binding to its normal receptor on the cell surface and prevents activation of the interferon pathway (Xiang & Moss, 1999). Some viruses are able to inhibit activation of the pattern recognition receptors on host cells. Influenza virus for example, has adapted to circumvent the activation of the RLR pathway of innate immune system activation. The influenza genome contains a nonstructural protein (NS1) which specifically inhibits TRIM25 ubiquitination of RIG-I, a member of the RLR pathway. In the absence of ubiquitination, RIG-I fails to activate type I interferon production which increases virulence of the virus (Gack et al., 2009). HCMV has incorporated a mimic of a MHC class I molecule. The HCMV UL18 protein

looks like a MHC class I molecule and is displayed on the surface of cells infected with HCMV (Beck & Barrell, 1988). Once on the cell surface UL18 binds the LILRB1 inhibitory receptor on Natural Killer cells (NK) with higher affinity than the host cell MHC class I molecule (Chapman et al., 1999). During viral infection, many viruses have developed a mechanism to suppress the appearance of MHC class I molecules on the host cell surface. NK cells seek out cells which lack high levels of MHC class I molecules, which are presumably virally infected, and targets these cells for destruction. Since the UL18 protein interactions with the NK LILRB1 receptor, the cell is not targeted for destruction and the HCMV virus can complete its lifecycle. Finally, some viruses contain microRNAs within their genome which can target host cell genes involved in the immune response (discussed below).

1.5 miRNA processing and function

MicroRNAs (miRNAs) play an increasingly important role in gene expression and act at the level of mRNA stability and mRNA translation. Located within the genome, microRNAs are small, single stranded, noncoding RNAs of ~21-22 nt that regulate gene expression by altering mRNA stability and mRNA translation (R.C. Lee et al., 1993). In mammalian cells, the genes for miRNAs are found in intergenic chromosomal regions between known genes or in the intronic regions of mRNAs (Lagos-Quintana et al., 2001; A. Rodriguez et al., 2004) and are transcribed by RNA polymerase II and in some cases RNA polymerase III (Borchert et al., 2006). Furthermore, many miRNAs have been organized in clusters (Ambros, 2004). In some cases a single promoter can drive expression of several miRNAs, resulting in a polycistronic transcript which would presumably allow for their coordinated regulation (Aravin et al., 2003; Y. Lee et al., 2002). miRNAs are initially transcribed as a long primary transcript (pri-miRNA) that can fold into a hairpin secondary structure; the transcript is cleaved within the nucleus to a 70-100 nt pre-miRNA by the RNAse III Drosha (Y. Lee et al., 2003). Subsequently, the pre-miRNA is transported to the cytoplasm via the exportin 5 pathway where the pre-miRNA is further processed into an approximately 22 nt dsRNA by the RNAse III Dicer (Hutvagner et al., 2001; Yi et al., 2003). One strand of the dsRNA is incorporated into the multi-protein RNA induced silencing complex (RISC), where the miRNA exerts its effect on gene expression (Hutvagner & Zamore 2002; Mourelatos et al., 2002). The purpose of miRNAs is to inhibit protein expression of a gene by either targeting the mRNA for degradation or by preventing translation of the target mRNA (Ulvila et al., 2010). Degradation of the mRNA occurs when the miRNA forms a perfect RNA/RNA hybrid with its target mRNA. In mammalian cells however, miRNAs generally form an imperfect RNA/RNA hybrid within the 3'-UTR of its target mRNA. This imperfect binding within the 3'-UTR interfers with and inhibits translation of the mRNA presumably by sequestering the mRNA away from the translational machinery in translationally silent regions of the cytoplasm known as p-bodies (Pillai et al., 2005). While miRNAs are generally thought to inhibit protein expression, recent evidence shows that in some cases, miRNAs can also increase protein production (Vasudevan et al., 2007). During cell cycle arrest miR-369-3 and let-7 were shown to stimulate translation of their target mRNAs while the same miRNAs inhibited translation of their target mRNAs during cellular proliferation. This suggests that the function of at least some miRNAs can be altered during the cell cycle, oscillating between a repressive and a stimulatory function. While miRNAs act as fine tuning regulators of important biological processes such as apoptosis, proliferation, and differentiation miRNAs can also regulate interactions between host and virus. miRNAs involved in regulating host-virus interactions can be derived from the virus, in the case of nuclear DNA viruses, or derived from the host cell (Ulvila et al., 2010).

2. miRNA expression during viral infection

The use of small non-coding RNAs to interfere with gene expression is observed in diverse organisms, from mammals to drosophila to plants (Stram & Kuzntzova, 2006). In plants, these small RNAs are vital to the plant's immune response to viruses and bacteria, and some plant viruses have evolved mechanisms to downregulate the inductive effect of these small RNAs on antiviral innate immunity (Lucy et al., 2000; Voinnet, 2001). The role of small RNAs in the innate immune response of mammals is still poorly understood although, great progress has been achieved over the past few years. Our understanding of how miRNAs aid in the innate immune response is enhanced by the use of microarray miRNA analysis.

2.1 Changes in host miRNAs

The first report of these small non-coding RNAs in animals was in the nematode *Caenorhabditis elegans (C. elegans)* where these non-coding RNAs were shown to be important for proper development (R.C. Lee et al., 1993). In *C. elegans* proteins produced from the *lin-14* and *lin-28* mRNAs must be decreased in order for proper development and specification of cell fates during the early larval stages. The *lin-4* miRNA directed against the 3′-UTRs of these two mRNAs can repress protein production and was shown to do so after translation initiation. *C. elegans* that contain *lin-4* miRNA mutations are unable to downregulate protein expression from *lin-14* and *lin-28* mRNAs resulting in reiteration of early larval developmental stages (R.C. Lee et al., 1993; Wightman et al., 1993).

Not only do miRNAs regulate gene expression of endogenous mRNAs, but endogenous human miRNAs can interact with some viral genomes to alter viral gene expression. In one report examining the liver specific human miR-122, it was shown that miR-122 binds to the 5′ region of the hepatitis C genome (HCV), that mutations within the HCV miR-122 binding sites reduced translation of the HCV mRNA, and that binding of miR-122 within the HCV genome increased the yield of infectious HCV virus (Jangra et al., 2010). Since viral genomes are by necessity small, this would suggest that at least some viral genomes can utilize endogenous miRNAs to their advantage, decreasing the need to incorporate the sequences for some translational control elements within the viral genome. Alternatively, in a human hepatoma cell line, treatment with Interferon β (IFNβ) identified eight IFNβ-induced miRNAs with complementarity to the HCV genome (Pedersen et al., 2007). Five miRNA mimics corresponding to the IFNβ induced miRNAs were able to inhibit HCV replication and thus infection by HCV. Additionally, miR-122 which was previously shown to increase translation of HCV, was downregulated by IFNβ treatment (Pedersen et al., 2007). These results indicate that an increase in IFNβ production induced by the cells immune response to pathogens can abrogate HCV infection.

One of the most studied viruses, protein expression from the human immunodeficiency virus type 1(HIV-1) is regulated by host cellular miRNAs during their latency period. Resting CD4+ T cells exhibit increased levels of human miR-28, miR-125b, miR-150, miR-223 and miR-328 when compared to activated CD4+ T cells. These cellular miRNAs are predicted to target the 3′ ends of HIV-1 mRNAs. Huang, et.al showed that that inhibition of these miRNAs resulted in increased HIV-1 protein translation in resting CD4+ T cells transfected with HIV-1 (Huang et al., 2007). Their data suggests that cellular host miRNAs contribute to the latency of HIV-1 in resting CD4+ T cells. Type III latency of EBV, but not

type I latency, showed increased expression of host cell miR-155 in B cells (Jiang et al., 2006; Kluiver et al., 2006). Furthermore, miR-155 was shown to target a number of transcriptional regulatory factors (Yin et al., 2008). In addition to miR-155, EBV was shown to increase expression of cellular miR-146, miR-200, miR-429, miR-29b, and miR-10b (Anastasiadou et al., 2010; Cameron et al., 2008; Ellis-Connell et al., 2010; G. Li et al., 2010). Kaposi's sarcoma-associated herpesvirus (KSHV), herpes simplex virus-1 and HCMV induce expression of miR-132 in their host cells and increased expression of miR-132 decreases expression of interferon-stimulated genes (Lagos et al., 2010), indicating that the viruses are attempting to suppress the initial innate immune response. Alternatively, increased host cell miRNA levels have been shown to decrease viral replication; the replication of primate foamy virus was repressed by human miR-32 (Lecellier et al., 2005). In addition, depletion of Dicer function, a protein involved in miRNA processing, in mice increased their susceptibility to infection with Vesicular stomatitis virus, likely from improper processing of miR-24 and miR-93 (Otsuka et al., 2007). Host cellular miRNAs can modulate expression of protein components of the innate immune response and in some cases, viruses have adapted ways to either exploit these miRNAs for their own survival or to counteract the effects of these miRNAs.

2.2 Virally encoded miRNAs

Not only are miRNAs found within the genome of the host cell, but DNA viruses which replicate in the nucleus, such as members of the herpes virus and polyomavirus families, have been found to encoded viral miRNAs (vmiRNAs) within the viral genome. RNA viruses, which replicate in the cytoplasm, have not been found to contain vmiRNAs within their genomes. The most likely explanation for the presence of vmiRNAs within the genome of DNA viruses, but not RNA viruses, is the need to initially process the pre-miRNAs by the nuclear enzyme Drosha. These vmiRNAs target components of the innate immune system, apoptotic factors and regulators of the host cell cycle (Boss & Renne, 2010). Inhibition of these pathways promotes viability and proliferation of the host cell, allowing for continued viral replication and synthesis. Moreover, production of vmiRNAs directed against host cell components illustrates the complex relationship between viruses and their host cells as each tries to ameliorate the damage caused by the other.

miRNAs derived from the viral genome are processed by the same pathway and in the same manner in which host cell miRNAs are processed. The first viral vmiRNAs were discovered in the genome of EBV (Pfeffer et al., 2004). Latent infection of a Burkitt's lymphoma cell line with EBV revealed the presence of five EBV encoded small RNAs with the characteristic stem-loop structure observed with mammalian miRNAs. Also, these vmiRNAs have been show to target the human mRNAs encoding the pro-apoptotic protein Bim and the p53 up-regulated modulator of apoptosis (PUMA) (Choy et al., 2008; Marquitz et al., 2011). Currently, at least 44 mature vmiRNAs have been identified in the EBV genome (Grundhoff & Sullivan, 2011). The human adenovirus genome, which produces a mild respiratory infection, contains several small vmiRNAs, one of which neutralizes the anti-viral action of interferon (Kitajewski et al., 1986; Reich et al., 1966). In addition, HCMV is reported to contain at least 11 vmiRNAs precursors which putatively target genes involved in T-cell activation and other inflammatory reactions (Grey et al., 2005; Stern-Ginossar et al., 2007) and the Kaposi Sarcoma Virus encodes at least 12 pre-miRNAs which target genes involved

in the immune response and in cellular proliferation (Cai et al., 2005; Samols et al., 2007). In these examples, the viral genome contains vmiRNAs which allow the virus to escape detection by the host immune response. Thus, virally encoded or cellular miRNAs, altered after infection, may modulate viral replication.

3. miRNA expression profile in the lung during RSV infection

Our laboratory is interested in the expression of SP-A during RSV infection. SP-A is important in the innate immune response against invading pathogens and recognizes glycoconjugates and lipids present on the cell surface of these organisms. Through opsinization, SP-A targets these organisms for destruction by resident macrophages. Once these pathogens are phagocytosed by macrophages and dendritic cells within the lung epithelium, the pathogens can be processed and their peptides presented to components of the adaptive immune response (Tino & Wright, 1996; Wright & Youmans, 1993). The inability of SP-A to target these organisms early in the infection could lead to increased pathogenicity, prolonged infection and increased morbidity. We found that RSV infection of type II pulmonary epithelial cells results in a 3-fold increase in SP-A mRNA, an attempt by the host cell to upregulate the innate immune response to this pathogen. However, the amount of SP-A protein produced during RSV infection decreases up to 60% when compared to uninfected cells. This decrease in SP-A protein resulted from translational inhibition of the mRNA which was specific to the SP-A mRNA and not a global translational repression of all cellular mRNAs (Bruce et al., 2009). The disparity between increase SP-A mRNA and decreased SP-A protein is puzzling and suggests that during viral infection, RSV is blocking enhanced expression of this component of the innate immune response. How this translational block is occurring is currently unknown. RSV is an RNA virus and not likely to contain conanical miRNAs that could target host mRNAs that encode components of the innate immune response. For example, virally encoded proteins could potentially act as transcriptional factors that change cellular miRNA levels, which could alter the course of infection by RSV. Since miRNAs play pivotal roles in development and in the innate immune response and miRNAs exert their function through translational inhibition, we investigated whether RSV infection would alter the miRNA profile of primary type II epithelial cells derived from human fetal lung explants. Using miRNA microarray analysis performed by LC Sciences, Houston, TX, we compared the miRNA profile of primary type II epithelial cells from fetal lung extracts treated in the absence and presence of RSV infection.

3.1 miRNAs normally expressed in lung type II cells

Previously, the miRNA profiles of the mouse lung and of the human A549 cell line, a human lung carcinoma cell line, have been reported (Jiang et al., 2005). However, the miRNA expression profile of primary human fetal lung type II epithelial cells has not been reported. Baseline expression of the miRNA profile of type II epithelial cells from fetal lung explants were performed using Affymetrix chips that contained probes for 856 mature human miRNAs from the miRBase version 12 database. The florescent expression results of miRNAs from type II epithelial cells from three fetal lung samples (gestational age 19-21 weeks) were averaged. To rule out any dye labeling bias, two of the samples were Cy3 labeled and one of the samples was Cy5 labeled. These studies revealed the presence of 184 miRNAs with an average florescent signal strength ranging from a high of 48,314 to a low of 50, from all three samples. The top 25 highest expressed miRNAs, are shown in Figure 1.

No.	miRNA	Average Signal Strength
1	miR-21	48314
2	let-7a	27336
3	let-7f	26117
4	miR-26a	26102
5	let-7d	21538
6	let-7c	20250
7	miR-200c	19115
8	let-7e	17697
9	miR-23b	17694
10	miR-23a	16717
11	let-7g	15064
12	miR-92a	14256
13	miR-200b	14217
14	let-7b	13890
15	miR-26b	13226
16	miR-125a-5p	12878
17	miR-1246	12147
18	miR-125b	10021
19	let-7i	9929
20	miR-27b	9067
21	miR-1826	9045
22	miR-30d	8613
23	miR-15b	8417
24	miR-30b	8010
25	miR-92b	7820

Fig. 1. Average signal strength of the highest expressed miRNAs in human alveolar type II epithelial cells.

Several miRNAs from the let-7 family (let-7a, let-7f, let-7d, let-7c, let-7e, let-7g, let-7b and let-7i), known to be involved in development, differentiation and cell proliferation were highly

expressed from the fetal lung explants (Peter, 2009). The let-7 family members are expressed late in embryonic development and regulate a number of genes involved in early embryonic development (Schulman et al., 2005). Previous miRNA results using the A549 cell line did not reveal high level expression of the let-7 miRNAs consistent with higher expression in developing tissue as compared with cancer cell lines which are often times dedifferentiated (Jiang et al., 2005). In addition to the let-7 family, several members of the miR-200 family, miR-200b and miR-200c, were also highly expressed. Members of the miR-200 family play a key role in the epithelial-to-mesenchymal transition (S.M. Park et al., 2008). This suggests that the human type II epithelial cells grown in primary culture more faithfully represent the miRNA expression profile seen during fetal lung development, as opposed to cell lines, and serves as the basis for examining changes in miRNA expression of human type II cells during different treatments.

3.2 miRNAs whose level increase during RSV infection

To determine the differential miRNA expression profile of fetal human type II epithelial cells to RSV infection, cells were treated in the absence or presence of RSV (M.O.I.=1) for two hours and RNA harvested 24 hrs later. RSV infection resulted in a significant increase (p-value <0.01) in 37 miRNAs with 11 showing a two-fold or greater increase (Figure 2).

No.	miRNA	Human Pulmonary Type II Signal Strength	RSV Treated Pulmonary Type II Signal Strength	Fold Change Increase
1	miR-654-5p	19.62	427.98	21.8
2	miR-663	508.08	4,709.58	9.3
3	miR-149*	141.47	811.71	5.7
4	miR-29c	27.63	144.46	5.2
5	miR-638	1,482.68	4,636.37	3.1
6	miR-1469	232.43	587.35	2.5
7	miR-19b	165.53	413.60	2.5
8	miR-15a	271.71	646.77	2.4
9	miR-181d	165.22	377.95	2.3
10	miR-30e	203.56	411.82	2.0
11	miR-1915	369.23	723.68	2.0

Fig. 2. Human alveolar type II epithelial cell miRNAs that increase 2-fold or greater during RSV infection.

miR-654-5p showed the greatest change at an approximate 22-fold increase followed by miR-663 with an approximately 9-fold increase in the presence of RSV. Using the miRBase databank, we determined the chromosomal location of the miRNAs that showed a 2-fold or greater change in the presence of RSV (Figure 3) (Griffiths-Jones et al., 2008). Seven of the miRNAs were located in intergenic regions or within the intron of processed transcripts.

UPREGULATED miRNAs

miRNA	Chrom	Region	Gene	
miR-654-5p	14	Intergenic	None	Cluster
miR-663	20	Intronic	Processed Transcript	
miR-149*	2	Intronic	Glypican-1	
miR-29c	1	Intergenic	None	Cluster
miR-638	19	Intronic	Dynamin 2	
miR-1469	15	Intronic	Nuclear Receptor subfamily 2	
miR-19b	13	Intronic	Processed Transcript	Cluster
miR-19b	X	Intergenic	None	Cluster
miR-15a	13	Intronic	Processed Transcript	Cluster
miR-181d	19	Intergenic	None	Cluster
miR-30e	1	Intronic	Putative Protein Coding	Cluster
miR-1915	10	Intronic	Uncharacterized Protein	

DOWNREGULATED miRNAs

miRNA	Chrom	Region	Gene	
miR-122	18	Intergenic	None	Cluster
miR-421	X	Intergenic	None	Cluster
miR-183	7	Intergenic	None	Cluster
miR-1268	15	Intergenic	None	
miR-182	7	Intergenic	None	Cluster
miR-331-3p	12	Intergenic	None	Cluster

Fig. 3. Genomic location of human miRNAs that change during RSV infection.

Those miRNAs found in intergenic regions are miR-654-5p, miR-29c, miR-19b and miR-181d. Located on chromosome 14, miR-654-5p is found in an intergenic region comprising a cluster of 16 different miRNAs. All of the miRNAs located in this cluster are expressed at low or undetectable levels in human lung epithelial type II cells and miR-654-5p is the only miRNA in this cluster upregulated during RSV infection, suggesting that this miRNA has its own promoter and is not transcribed as part of a polycystronic mRNA. Several of the miRNAs identified in our differential microarray analysis reside in intron containing, processed transcripts that do not contain open reading frames and are thus not translated. The three miRNAs located in the introns of processed transcripts are miR-663, miR-19b and miR-15a. Previously, the presence of processed yet untranslated mRNA transcripts has presented a conundrum as to their physiological importance however, the identification of miRNAs within the introns of these transcripts could suggest that these types of transcripts may be "hot spots" for other unidentified miRNAs. Two copies of miR-19b are found in the human genome, one on chromosome 13 and this copy is found in an intron of a processed transcript and the second copy is located on the X chromosome in an intergenic region. Three of the miRNAs, miR-149*, miR-638 and miR-1469 are located in intronic regions of known genes and one miRNA, miR-1915 is located in the intron of an uncharacterized protein. miR-149* is located on chromosome 2 in the intron of the glypican 1 (GPC-1) gene which encodes a cell surface proteoglycan. GPC-1 is important for TGF-beta1 induced cell growth inhibition (J. Li et al., 2004). Located within an intron of the dynamin 2 (DPN2) gene is miR-638. DPN2 is present in many different cell types, is a GTPase and is involved in endocytosis (Durieux et al., 2010). Finally, miR-1469 is located on chromosome 15 in an intron of the nuclear receptor subfamily 2, group F, member 2 (NR2F2) gene. NR2F2 is a ligand inducible transcription factor of the steroid thyroid hormone superfamily and plays an important role in lung development. In the mouse, NR2F2 is detected in both the proximal and distal lung (Kimura et al., 2002). Whether the upregulation of the three miRNAs, located in the introns of known genes, is a consequence of increased transcription of these genes during RSV infection is unknown. It would be interesting to determine whether the mRNAs of the GPC-1, DPN2 and NR2F2 genes also increase during RSV infection.

3.3 Viral targets of miRNAs increased during RSV infection

The increase in miRNA expression during RSV infection could serve to regulate viral and/or host cell mRNA expression. For example the PB1 mRNA of the H1N1 influenza A virus was recently shown to be a target of miR-654. The influenza PB1 protein is part of a complex involved in transcription initiation and elongation of viral genes (Ulmanen et al., 1981). Binding of miR-654 to the PB1 mRNA was through imperfect base pairing between the miRNA and the mRNA which typically results in translational repression in mammalian cells. However, binding of miR-654 to the PB1 mRNA resulted in mRNA degradation rather than translational repression (Song et al., 2010). In this example, miRNAs are acting as anti-viral agents by inhibiting viral expression. Similarly, increased expression of miR-149* and miR-638 was observed during HCV infection of a hepatoma cell line and inhibition of miR-149* and miR-638 increased HCV entry into the cell line as measured by luciferase assay. In addition, inhibition of miR-638 resulted in increased HCV mRNA but inhibition of miR-149* decreased HCV abundance (Liu et al., 2010). Finally, in concordance with the RSV results, a keratinocyte cell line infected with human papillomavirus type 11 (HPV-11) also showed upregulation of miR-149*, miR-638, and miR-663 (Dreher et al., 2010).

3.4 Cellular targets of miRNAs that increase during RSV infection

Several algorithms are available for predicting cellular targets of miRNAs however, relatively few confirmed mRNA targets have been identified. RAC-alpha serine/threonine-protein kinase (Akt1) and E2F1 (a transcription factor) both play a role in apoptosis (Liu et al., 2001; Polager & Ginsberg, 2008). Overexpression of miR-149* was shown to directly regulate the 3′-UTR of these two mRNAs resulting in decreased mRNA and protein levels and increased apoptosis in cell lines (Lin et al., 2010). Apoptosis is an important host cell mechanism to regulate viral infection and the increase in miR-149* is one avenue to increase apoptosis of infected cells. Activator protein 1 (AP-1) a transcription factor which regulates genes in response to various stimuli including cytokines, bacterial infections and viral infections is a direct target of miR-663 (Hess et al., 2004; Tili et al., 2010a) and miR-663 also targets the 3′-UTR of transforming growth factor beta 1 (TGFbeta1) (Tili et al., 2010b) a cytokine involved in cell growth, differentiation and proliferation. Cyclin E expression, which is necessary for cell cycle progession from G1 to S phase, is regulated by miR-29C (Ding et al., 2011). In addition, miR-29C directly suppresses p85 alpha (regulatory subunit of PI3 kinase) and CDC42 (Rho family GTPase), two proteins that negatively regulate p53 another cell cycle regulatory protein (S.Y. Park et al., 2009). B-cell lymphoma 2 (BCL-2) a protein involved in regulating apoptosis, breast cancer type 1 susceptibility (BRCA1) a protein involved in the repair of DNA double strand breaks and BMI1 a protein involved in transcriptional repression are directly regulated by miR-15a (Bhattacharya et al., 2009; Cimmino et al., 2005; Zhu et al., 2009). Finally, ubiquitin-conjugating enzyme E2I (UBE2I or UBC9) an enzyme responsible for targeting proteins for degradation is targeted by miR-30e (Wu et al., 2009).

3.5 miRNAs whose level decrease during RSV infection

Thirty four miRNAs were shown to significantly decrease (p-value <0.01) in the presence of RSV infection with six showing a decrease of 2-fold or more (Figure 4).

Unlike what we observed with miRNAs that increased in the presence of RSV, none of the miRNAs which decreased by 2-fold or more were located within introns of known genes. Indeed, all of the miRNAs were located in intergenic regions (Figure 3). The largest decrease was with miR-122 which showed a 7-fold decrease followed by miR-421 with a 4-fold decrease. The decrease in miR-122 is interesting as decreased expression of miR-122 was also observed during HCV infection of a hepatoma cell line (Liu et al., 2010) and during Borna disease virus (BDV) infection of human oligodendralglial cells (Qian et al., 2010). miR-122 is abundantly expressed in the liver and plays a role in HCV replication by binding to sites within the HCV 5′-UTR and stimulating HCV accumulation in vivo. When miR-122 was sequestered in liver cells, replication of HCV RNAs was inhibited (Jopling et al., 2005). Conversely, overexpression of miR-122 blocked replication, transcription and protein synthesis of BDV in oligodendralglial cells (Qian et al., 2010) and inhibited Hepatitis B virus expression in hepatoma cells (Qiu et al., 2010). These data suggest that miR-122 can have contrasting effects on expression of different viruses. Several members of the let-7 family of miRNAs were decreased in the presence of RSV (data not shown). Whether this represents an attempt to inhibit cellular differentiation is unknown however, the amount of decrease in the presence of RSV, while statistically significant, was less than 2-fold.

No.	miRNA	Human Pulmonary Type II Signal Strength	RSV Treated Pulmonary Type II Signal Strength	Fold Change Decrease
1	miR-122	529.32	76.89	-6.9
2	miR-421	123.75	28.95	-4.3
3	miR-183	2,481.76	1,046.96	-2.4
4	miR-1268	177.12	76.53	-2.3
5	miR-182	4,513.70	2,127.39	-2.1
6	miR-331-3p	275.98	135.27	-2.0

Fig. 4. Human alveolar type II epithelial cell miRNAs that decrease 2-fold or greater during RSV infection.

3.6 Cellular targets of miRNAs that decrease during RSV infection

Two of the miRNAs that decrease 2-fold or more during RSV infection have known mRNA targets. First, miR-122 was shown to bind the 3'-UTR of cationic amino acid transporter (CAT-1) resulting in decreased protein abundance of CAT-1 protein (Jopling et al., 2006). In addition, miR-122 has been shown to directly target interleukin-1 alpha (IL-1A) (Gao et al., 2009). IL-1A activates the immune response shortly after infection stimulating proliferation of fibroblasts and lymphocytes as well as chemotaxis of neutrophils (Boraschi et al., 1990; Ozaki et al., 1987). Also, the CUTL1 mRNA which encodes a transcriptional repressor that regulates genes specifying terminal differentiation, is also directly regulated by miR-122 (Xu et al., 2010). Second, miR-421 was shown to regulate the androgen receptor (AR), a transcription factor important for male sexual characteristics (Ostling et al., 2011) and Ataxia-Telangiectasia mutated (ATM) a serine/threonine protein kinase involved in cell cycle arrest, DNA repair and/or apoptosis (Hu et al., 2010).

4. Conclusion

The innate immune response is our first line of defense against invading pathogens and is important for activating the immune response early during infection. Viruses, which are obligate intracellular parasites, must subvert the innate immune response in order to replicate and survive. The constant battle between the host and the virus for survival necessitates the continued evolution of new and better avenues of gene regulation. One fairly recent identified avenue of gene regulation is that of miRNAs which can regulate gene expression at the level of mRNA stability and/or translational efficiency (R.C. Lee et al., 1993). miRNAs can be encoded by the host genome or in some cases, within the viral genome (Boss & Renne 2010). In order to fully understand the intricate interaction of the host and the viral genomes during infection, the impact of miRNAs on changes in gene regulation is an important consideration. In the present study, we determined the miRNA expression pattern from primary fetal lung type II epithelial cells and identified changes

in miRNA expression that occurs during RSV infection. RSV infection significantly increased 37 miRNAs and significantly decreased 34 miRNAs. Suggesting that changes in miRNA levels could alter cellular protein levels and affect the course of pathogen associated infections.

One component of the innate immune system within the lung is SP-A. Because SP-A can bind to glycocongugates and lipids on the surface of pathogens, it plays an important role in eliminating these pathogens early during infection (Tino & Wright, 1996; Wright & Youmans, 1993). The divergent results between the increase in SP-A mRNA and the decrease in SP-A protein during RSV infection prompted us to investigates whether changes in miRNA levels occur during RSV infection (Bruce et al., 2009). Using the MicroInspector bioinformatics tool, we checked the 3'-UTR of the SP-A mRNA against the miRNAs that increased at least 2-fold, during RSV infection (Rusinov et al., 2005). Several putative miR-638 and miR-149* binding sites were identified. Whether these miRNAs directly regulate the SP-A mRNA is currently unknown and future directions within the laboratory, will seek to address this question.

The change in cellular miRNA expression profile resulting from several viral infections has recently been investigated. Here we add to this knowledge by investigating the change in cellular miRNA expression of type II lung epithelial cells in the presence of RSV infection. Comparing our results with the results of other viral infections has identified common miRNAs whose expression change in regard to these different viral infections. In the presence of infection with a DNA virus (HPV-11) or an RNA virus (RSV, HCV) miR-149* and miR-638 both increase in concentration within the cell (Dreher et al., 2010; Liu et al., 2010). When cells were infected with the RSV or HCV virus, the level of miR-663 increased within the cell (Liu et al., 2010). The increase in these miRNAs does not appear to be random, but rather a specific attempt by the cell to inhibit replication, transcription, protein synthesis or entry of the virus into the host cell. Commonalities in the changing expression of specific miRNAs during viral infection, suggests that these miRNAs may have antiviral effects. Augmenting expression of these antiviral miRNAs could be an avenue to treat viral infections and could have a global affect on viral infection, resulting in a broad-spectrum avenue to defend against viral invasion. Evaluation of changes in miRNA expression, resulting from additional viral infections, may clarify the miRNAs which exhibit anti-viral effects, and will further our understanding of the host/viral interactions that occur during infection.

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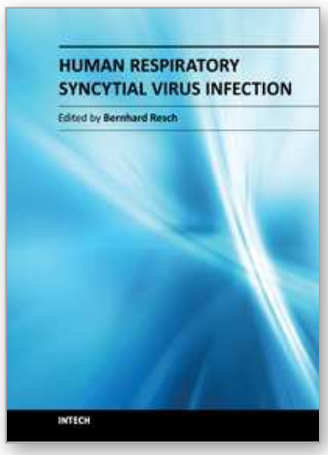

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In this online Open Access book on "Human RSV Infections", several distinguished authors contribute their experience in respiratory syncytial virology. A major focus lies on the fascinating pathophysiology of RSV and represents recent and actual work on different mechanisms involved in the complex pathogenesis of the virus. The second section elucidates epidemiologic and diagnostic aspects of RSV infection covering a more clinical view of RSV disease. At last, treatment modalities including the search for a vaccine that is still not in sight are discussed and conclude this book, thus building up a circle that runs from experimental models of RSV related lung disease over clinical aspects of disease to the latest news of therapeutic and prophylactic approaches to human RSV infection.

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
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