INFLUENZA INFECTION MODIFIES HOST BRONCHOEPITHELIAL CELL

EXOSOME-ASSOCIATED MICRORNA EXPRESSION

by

JAROD MICHAEL HANSON

(Under the Direction of Ralph Tripp)

ABSTRACT

The role of exosomes in cellular communication is one of highly specific cell signaling and transport involved in various cellular processes. In order to better understand the role of basally excreted exosomes in relation to intracellular microRNA (miR) signaling during influenza A virus (IAV) infection, a miR screening panel was used to identify and evaluate exosomal-associated miRNA expression patterns across mock and IAV-infected cells.

Expression of miRs was also evaluated intracellularly via use of a novel P19 protein-based staining technique. P19 is a 19 kDa protein that binds double-stranded 21-25 base miRNAs with high affinity in a size dependent, but sequence independent, fashion making it a pan-miR marker of cellular processes. Additionally, basally-derived miRs were evaluated for their potential to regulate signaling via their addition to undifferentiated immortalized (Calu-3) or primary (human or swine) bronchoepithelial cells where basally secreted exosomes were examined from the recipient cell population. The presence of exosome-associated miRs in fetal bovine serum (FBS) was also evaluated in
relation to their effects on influenza infection studies as well as cell growth characteristics. Our studies indicated that in the case of certain miRs, exosomes not only acted as carriers for the miR, but that the miR itself was vital to propagating a cellular signal that can affect not only cell growth, but cellular responses during influenza infection.

INDEX WORDS: Influenza, exosomes, microRNA, miR, FBS, vRNP
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DEDICATION

This work is dedicated to my wife Dr. Corry Yeoukis, and my son Maxwell, for tolerating this endeavor.
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CHAPTER 1

INTRODUCTION

Influenza is a single-stranded, negative-sense, segmented RNA virus in the Orthomyxovirus family. Three of the six genera in the Orthomyxoviridae family include the influenza variants A, B, and C. Of the three variant viruses, influenza A viruses (IAV) infect humans, pigs, horses, dogs, cats, birds, ferrets, and guinea pigs, influenza B virus primarily infects humans, ferrets, and seals, while influenza C virus infects humans, dogs, pigs, and cows although it has recently been proposed that the C variant initially identified in swine, which mainly affects cattle, be reclassified as influenza D [1].

IAVs are further subdivided based on hemagglutinin (HA) and neuraminidase (NA) receptor types with 18 HA and 11 NA variants currently identified that can be combined through viral reassortment into nearly 200 unique combinations yielding ever more unique virus subtypes. Although multiple, temperature-sensitive, live-attenuated vaccine variants are available, constant virus evolution via antigenic shift and drift lead to ongoing vaccine failure in not only humans, but also pigs and horses which are the only two species outside of humans where influenza vaccination is routinely practiced.

Several studies have shown that IAV modifies the host immune response to infection, a feature proposed to contribute to immune evasion and viral
evolution which may further amplify and/or exacerbate viral resistance to host adaptive and innate countermeasures. IAV infection has been shown to induce microRNA (miRNA, miR) expression following infection both in vitro and in vivo, in various species including humans, swine, and mice [2]. IAV is among the most important zoonotic respiratory diseases due to its propensity for both inter- and intra-species transmission [3], as well as constant mutation and periodic reassortment events leading to antigenic shift and drift.

Antigenic drift primarily occurs through proof-reading errors in the IAV polymerase mechanism leading to subtle, but often insignificant, alterations in the viral genome [4]. Antigenic shift occurs when entire segments from different viruses reassort with one another leading to the generation of a novel virus incorporating genetic material from the distinct viral lineages, as well as interspecies reassortment seen among humans, swine, and avian viruses. Although vaccination can induce protective antibodies, the frequency of IAV mutation antigenic shift and/or drift may result in vaccine failure due to not only mutation, but also the lack of cross-protection among closely related IAV strains in many cases [5, 6].

Biomarkers are needed to identify infection and degrees of pathogenesis, such as how the virus first interacts with the bronchoepithelial host cells it initially infects. Biomarkers may also be useful in improving multiple IAV outcomes via providing a better understanding of the modulation of host immune responses to vaccination or during infection, or recovery from infection.
Air-liquid interface (ALI) transwell culture systems consisting of undifferentiated and/or fully differentiated cells such as primary normal human bronchoepithelial (NHBE) cells or primary normal swine bronchoepithelial (NSBE) cells were utilized to determine the importance of exosomal miR regulation during IAV infection, as well as cell growth. Additional studies in Calu-3 cells, which are transformed human respiratory epithelial cells [7, 8], and clinical swine serum samples, were utilized to assist aspects of validation of miRs to be used for transfection studies. The cell lines emulate normal respiratory epithelium, as they can be grown to yield undifferentiated cells, or differentiated, polarized, ciliated pseudostratified columnar epithelium [9, 10]. The use of differentiated cells to model airway epithelia is significant, as differentiated bronchoepithelial cells are more resistant to viral infection than the undifferentiated underlying cells and may be partly responsible for innate immune function differences seen among species [10].

Equally as important are the cell types that produce mucous which mimics one of the protective measures against influenza infection found in normal respiratory epithelium [11-15]. The transwell system provides an apical compartment representing the lumen of the airway, and a separate basolateral compartment which provides nourishment to the culture. This enables media collection from the basal side of the cell allowing for the capture of exosomes/miRNA excreted by the (un)differentiated apical cell layer which in an in vivo system would potentially affect the undifferentiated cell layers found beneath the apical cell monolayer [16]. The makeup of the media utilized in the
basal chamber of the transwell system to support cell growth during infection can also be modified to include components such as fetal bovine serum (FBS), exosome-free FBS, etc. all of which may modulate the dynamics of the IAV infection and are likely responsible for many of the differences observed in miRs between similarly executed studies.

**Specific Aims**

The *central hypothesis* of the proposed research is that IAV modulates miR expression in the host cell. Once modified by miRs, the exosomes from infected cells are used to signal adjacent cells to induce an anti-viral response. A *secondary premise* is that miRs in the exosomes are responsible for modulating the pro- and anti-viral signals between cells, and that the dysregulation in the cargo content of the exosomes is responsible for differences in viral infections among species where swine have a poorer antiviral response than humans to IAV [10, 17, 18]. It is presumed that swine cell lines exhibit differential miR expression patterns than that from humans and human-derived cell lines, and miRs modulate and regulate the innate immune response to IAV. Given the above, it is also imperative to understand how experimental conditions and reagents alter the assessment of outcomes via the presence of endogenous exosomal miRs found in fetal bovine serum (FBS), which is a key component of media used to propagate cells in vitro.
**Specific Aim 1.** To identify and evaluate differences in exosome miR-associated expression patterns in IAV-infected cells.

**Specific Aim 2.** To evaluate miRs in exosomes, from FBS and from vaccinated and non-vaccinated swine pre- and post-IAV challenge, to determine which miRNAs are dysregulated.

**Specific Aim 3.** To validate miRs using exosomes from IAV-infected cells, and IFN lambda, and/or miRNA inhibitors and mimics, to determine whether exosome-associated miRNAs can be utilized to modulate cell or virus growth *in vitro.*
References


Influenza Overview

Influenza infection requires the virus to gain entry into respiratory epithelial cells of a susceptible host. Relative to influenza infection, a susceptible host is one which possesses the requisite receptors in its respiratory tract necessary to establish patent infection. During the initial stage of influenza infection hemagglutinin (HA) attaches to sialic acid receptors on the host cell. Sialic acid, NAG, and GAL-2 together form the typical receptor found on a host human or avian epithelial cell [1]. The sialic acid receptor preferred by avian origin viruses is typically an α-2,3-linked sialic acid receptor while human origin influenza viruses prefer α-2,6-linked sialic acid receptors [2].

Subsequent to attachment, the HA fuses with the endosomal membrane, and via its unique trimer ring structure creates a pore for entry into the host cell [3]. The pore serves as a pathway for the viral particle to empty the contents inside the viral envelope directly into the host cell and thereby avoid the host immune response [4]. Once inside the cell, the virus uses the host cell’s own machinery to manufacture more virus and ultimately exits the cell using neuraminidase (NA), where the virus is attached to the same type of sialic acid
receptor used by hemagglutinin to exit the cell [5]. The cell is killed upon exit of the newly created virus particles.

However, the receptor preference noted previously is not strictly adhered to as has been seen with the recently identified human H7N9 virus that exhibited increased affinity for the human α-2,6-linked sialic acid receptors while maintaining its affinity for avian origin α-2,3-linked sialic acid receptors [2]. This is abnormal in that influenza virus typically shows an affinity for one species' receptor type over all others, but has been seen previously in viruses of swine origin which had equal affinity for multiple receptors [6]. The binding specificity for sugar moieties is also thought to influence the host range of a particular virus which would explain why some viruses cross species more easily than others [6].

A myriad of environmental [7, 8] and physical factors including particle size [9], the number of infectious virions in the particle [9-11], ambient temperature [12], sunlight [13], relative humidity [14, 15], and proximity to the infectious source [16] all have a role in the success or failure of a virus to infect a naïve host. Temperature and pH changes also greatly affect the activity of hemagglutinin [3]. Membrane fusion potential and conformational changes occur with pH and temperature changes in that low pH and high temperature typically trigger fusion and/or conformational changes [17], likely due to the fact that neutral pH conformations are inherently unstable, but become stable as the pH decreases to that of virus’s specific fusion pH (slightly acidic) [3]. Also of note is the fact that viruses grown at high pH, in the presence of anti-viral drugs, or in cell lines, often develop adaptations to growth in those specific conditions [3].
This poses unique challenges because the simple act of culturing the virus for laboratory studies can induce changes in the base structure of the virus yielding a virus with characteristics different from that of the initial field virus.

Also important are the host defenses that preclude infection (reviewed in [18, 19]), an example being the full complement of innate host defenses. Of particular interest is the ability of infected cells to establish pro- or anti-viral states in neighboring cells via intercellular communication, endocytic, and/or fusion pathways including pores [4] and filopodia [20-22] as well as through endocytosis of exosomes [23].

The role of exosomes during viral infections, including signal carrying agents for things such as microRNAs (miRs) is underappreciated [24-27]. Given the preponderance of exosomes and microvesicles commonly found in cell culture media containing fetal bovine serum [28] and/or platelet-derived lysates [29], as well as in the sera of host species, it is imperative to examine and understand the potential implications of exosomes and exosome-associated miRs to better understand the potential implications not only for host defense against IAVs, but also as they relate to cell growth, IAV cell permissive to infection, and/or virus growth.

The Airway

Airway bronchoepithelium is the first line of defense against pathogens. Mammalian bronchoepithelium is composed of several different cell types (reviewed in [30, 31]) including ciliated cells, Goblet cells, Clara cells, serous
cells, and basal cells. Goblet cells are mucous-secreting and responsible for the large variety of mucins seen both in vitro and in vivo along with serous cells and other mucous-secreting cells found in the underlying submucosal glands. They are also responsible for the differences in mucin type, structure [32], quantity, and functionality [32, 33] that occurs between species, and cancerous versus non-cancerous cell lines [34]. Goblet cell quantity and capacity directly impact not only mucous levels, but also mucous consistency via variations in the components forming the mucous [35].

The bronchoepithelial cell layer lining the respiratory tract is typically coated with a thin layer of mucous, secreted by local goblet cells and submucosal glands. In addition to protecting the underlying cells from irritants, pathogens, etc. the mucous also acts as a lubricant for the cells and helps prevent desiccation of the tissues [32]. Mucins (MUC), when mixed with other secreted proteins and fluid, make up the primary component of mucous [32]. Mucins also have a role in the defense of the host against IAV although the virus has adapted neuraminidase activity to help penetrate mucins via the degradation of sialic acids [36].

Mucous, often associated debris and particulates, is removed from the local area via a mucociliary clearance mechanism [37] whereby mucous is moved outward via beating cilia (also referred to as the mucociliary escalator mechanism) with the loss of cilia leading to hyper reactive airways [38]. The mucin profile generated in vitro can be varied depending on the differentiation status of the cells used with differentiated cells producing mucins more like those
seen in actual airway secretions, especially if used in conjunction with retinoic acid to restore mucous production in retinoid-deficient bronchoepithelial cultures [35, 39, 40].

Mucins can be divided into two groups based on the layer to which they associate in vivo: 1) the transporting mucous layer (TML) on the apical cell surface, and 2) the periciliary layer (PCL) located above the TML and spanning the distance between the TML and the apical portion of the cilia. The TML is rich in two polymeric mucins, MUC5AC and MUC5B while the PCL is rich in keratin sulfate and MUC1 that is involved in exosomal membrane trafficking [41], MUC4, and MUC16 [42]. While these mucins may be enriched in their respective layers, they are not solely found there.

Mucins and exosomes are intertwined in several ways. MUC1, a transmembrane glycoprotein, has been shown to be incorporated into the surface of exosomes via lipid rafts while still in the multivesicular body (MVB) facilitating its export out of the cell via the exosome [41, 43]. Tethered mucins have been shown to compromise nearly half the diameter of tracheobronchial exosomes [44] isolated from lung washes indicating that even if exosomes are secreted free of mucins during exocytosis, they quickly acquire a mucin coating in the extracellular lung environment which may be a protective mechanism, or aid exosome penetration through the relatively impermeable TML into the more fluid-like PCL layer. Enhanced mucous penetration/solubility would allow exosomes to act as more distant signaling molecules versus if they were otherwise trapped in
the mucous layer at which point they could only exert a localized effect, primarily on cells directly adjacent to their point of origination.

Although exosomes are produced within the cell and released via exocytosis, they often contain cell-derived constituents on their external surfaces such as aminophospholipids, ceramide, membrane-bound mucins [45, 46]. Additionally, exosomes released from tracheobronchial epithelial cells have the multivesicular and late endosomal markers TSG101 [47] and CD63 [44], as well as MUC1, MUC4, and MUC16 on their surfaces [44]. MUC1 in particular has been implicated in T cell responses as well as a modulator of the airway during inflammation and infection [44]. This would imply a potential immunomodulatory effect of exosome preparations themselves which is a reason for including representative mock controls when studying these features so as to account for any variation induced by the exosomes alone.

Additionally, several natural inhibitors containing sialic acid motifs including several mucin derivatives [44] have been postulated to help neutralize influenza virus [48]. However, this effect is likely transient as neuraminidase has been shown to aid mucous penetration via the cleavage of sialic acid molecules, although a study showed greater than 80% neutralization of influenza virus in vitro by exosome preparations containing MUC1 [44].

There is evidence of mucins on exosomal surfaces, but it is less clear if mucins are regularly transported as exosomal cargo. It is unlikely large mucins are present intra-exosomally due to their large size (1um) relative to smaller exosome (0.1um). As such if mucins are transported internally, this presumably
happens as smaller monomers likely associated with other lipid-like cargo given their typical associations with lipid rafts on the exosome surface. As exosomes are released via exocytosis it is probable that mucin granules [49] and exosomes are released simultaneously which would allow for both to be in close proximity and allow the exosomes to rapidly acquire additional mucins not added during their formation. The inclusion of MUC1, MUC4, and MUC16 on a large percentage of exosomes suggests their inclusion is an important modification since the first mucins the exosomes typically encounter during exocytosis are the MUC5 variants found in the TML [44].

It appears that distinct mucins may afford the advantage relative to development of mucous penetration, or travel throughout the lung milieu that is not available to exosomes that do not contain similar functional groups, tethered mucins. Mucin-exosome interactions are important both apically and basally as exosomes from hepatitis B or C virus-infected cells have been implicated for signaling adjacent naïve cells to induce pro- or anti-viral states in those cells via the expression of IFNs or other anti-viral molecules [50, 51]. Apical exosomes from systems using mucous secreting cells is challenging [44, 46] as reagents to remove mucous are not readily available and/or efficacious due largely to the poor solubility of the mucins. Of note is the potential for mucins to confound *in vitro* studies as many of the cell lines used produce variable amounts of MUC5AC and MUC5B [34]. The mucin expression may lead to differential exosome features, and exosomes derived from mucin-secreting cells are larger
in diameter than primary cell derived exosomes which is likely a function of which mucins coating the exosomes.

The glycocalyx is a secondary barrier that is attached to the epithelial cell surface. It is an organized mesh consisting of various glycoproteins, and glycolipids that is typically secreted from the apical surface of cells lining the respiratory tract [42, 52]. Given its makeup, the glycocalyx can act as a physical barrier against airway trauma, and repel pathogens such as viruses and bacteria by trapping them before they are able to reach the apical surface of respiratory cells [53].

Bronchoepithelial cells lining the airways also possess an additional barrier against entry of pathogens, irritants, and the like via intercellular junctions which acts as exclusionary mechanisms against pathogen penetration between cells, i.e. from the airway lumen into the underlying cell layers [54]. Since invading pathogens often use host cell surface markers/receptors to gain access to the host cell, tight junctions can prevent a pathogen from invading the cell as long as the necessary marker is located basolaterally where the tight junctions prevent access by the pathogen. Additionally, the tight junctions prevent virus entry into the undifferentiated underlying cell layers via physical exclusion of the virus forcing the virus to attempt to gain access to the differentiated cell to establish an infection.

Normal or immortalized bronchoepithelial cells from different species have been used experimentally with variable results [55-61]. Not unexpectedly, there are differences in cytokines expressed among the various bronchoepithelial cell
types [56, 62]. Interestingly, respiratory syncytial virus (RSV) can infect basal cells underlying the primary differentiated cell layer and influence cellular differentiation towards a more mucous-producing phenotype which aids RSV survival [63]. Thus, the ability of viruses to modulate epithelial cell responses to infection is critical to virus survival, although the permissiveness of the host cell to viral modulation varies greatly depending on things such as cell type, number of cell layers, species, and/or co-infections.

Modeling the Airway In Vitro

Historically, in vitro cell culture models utilizing primary or immortalized cell lines have been used to emulate the airway with varying success. Transwells allow cells to be grown on an inner porous membrane separate from the underlying culture media, or in the presence of another cell layer grown in the basal well. Additionally, transwells can be utilized to establish an air-liquid interface (ALI) which can mimic respiratory tract cellular architecture especially when used in conjunction with differentiable cell lines capable of producing multiple distinct cell types to include ciliated cells [61, 64].

Calu-3, NHBE, and NSBE cell lines can be differentiated using in vitro cell culture media and obligatory components (e.g. retinoic acid), the introduction of an air interface, and/or fetal bovine serum (FBS) concentrations as Calu-3 cells can be differentiated via decreased FBS levels in conjunction with the establishment of an ALI system [55, 56, 65]. Calu-3 cells are derived from non-small-cell human lung cancer, adherent in culture, and morphologically display
epithelial characteristics including microcilia when fully differentiated [64, 66, 67].

The added benefit of a transwell model is the ability to not only differentiate the cells, but also polarize the cell surfaces so that one surface becomes apical and the other basal which can be utilized experimentally via apical budding of viruses such as influenza, and thus the generation of a virus-free basal surface and/or media chamber [64, 68, 69].

Exosomes

Exosomes are small (30-150nm) vesicles derived from the inward budding of cellular compartments producing multi-vesicular bodies (MVBs). These MVBs fuse with the plasma membrane causing the vesicles inside the MVB to be released as free exosomes [70, 71]. Once released from the plasma membrane, the exosomes can freely travel throughout the host system affecting a multitude of host physiological, signal transduction, and cellular responses [44, 72]. Exosomes can carry a variety of contents taken up from inside the cell such as RNA, DNA, lipids, etc. and have also been found to mediate immune effects such as inducing IFN-λ activity [51].

Historically, exosomes were thought to contain mainly cellular trash/debris, but that view has shifted radically as much of that debris can actually act as a signaling system both locally and regionally once released from the exosome [44, 51, 70, 73]. Interestingly, miRNAs have been found to be concentrated in exosomes further indicating their potential biological significance [74]. For instance, miRNA contained in exosomes can have transcription level
effects often at multiple locations in the genome. Emerging consensus is that exosomes serve not only as a signaling system, but also as important players in maintaining overall cellular fitness during stress, viral infection, and other cellular destabilizing events [75]. Exosomes can also have source-dependent functions such as the suppression of Th 1 cells elicited by exosomes derived from T regulatory cells [76].

**Exosomes as Virus-Like Particles (VLPs) or Adjuvants**

Exosomes have the capacity to function as an innate vaccine-like entity in terms of displaying virus antigens while not actually spreading the virus. This would serve as a signal to adjacent cells in terms of antigen recognition which may be a very early evolutionary tactic to halt the spread of infectious agents. Although exosomes are produced within the cell and released via exocytosis, they often contain cell-derived constituents on their external surfaces to include aminophospholipids, ceramide, and membrane-bound mucin [45, 46]. Since exosomes display common cell surface markers including various mucins, CD10, CD63, alpha-2,6- and alpha-2,3-linked sialic acids, it is expected that exosomes can bind viruses externally and potentially act as infectious delivery systems to adjoining cells, a phenomenon that has been observed with HCV [77] and with Enterovirus [78].

As noted previously, exosomes released from tracheobronchial epithelial cells have the multivesicular and late endosomal markers, TSG101 and CD63, as well as MUC1, MUC4, and MUC16 on their surfaces [44]. MUC1 in particular
has been implicated in T cell responses, as well as being a modulator of the airway during inflammation and infection [44]. This would imply a direct immunomodulatory role of exosome preparations themselves which could allow them to be specifically prepared to function as cell-derived adjuvants. Others have shown the inclusion of interferon-inducible transmembrane proteins (IFITMs) on the exosome surface if derived from certain cell lines with the IFITMs capable of inducing anti-viral effects [25]. This could also potentially eliminate or reduce the need for more controversial adjuvants in vaccines, such as aluminum salts, which have garnered scrutiny by the public at large.

However, the role of exosomes as adjuvants requires further scrutiny with respect to IAV. Several of the mucins have alpha-2,6-linked sialic acid associated with them which has been postulated to help neutralize influenza virus with at least one study showing greater than 80% neutralization of influenza virus in vitro by exosome preparations containing MUC1 [44]. Additionally, the effect is transient as neuraminidase (NA) has been shown to aid mucous penetration via the cleavage of sialic acid molecules meaning any binding of exosomes to influenza is likely short lived as NA cleaves sialic acids.

This potential neutralization could also be beneficial to an influenza virus in terms of the exosome carrying it in close proximity to the host cell potentially allowing it to bind the cell and escape immune detection, or be directly taken into the cell with the exosome as extra-exosomal cargo. This offers particularly novel opportunities to use exosomes as vaccines in that IAV could be bound to exosomes which have themselves been modified to display multiple HAs, and
NAs, so that when the virus-exosome fusion is processed by APCs, multiple antigens could be displayed inducing much broader immunity.

Of interest from a vaccine standpoint is that both Hepatitis C virus (HCV) and Enteroviruses have been shown to be transported inside exosomes, and/or phosphatidyl serine vesicles, with both being able to induce infection via the exosome carrier [78, 79]. This finding is substantial as several other viruses including IAV, Epstein-Barr virus, Coxsackie virus, and HIV have also been implicated as potentially having exosome-mediated transport of virus or virus components [80]. As such, part or all of an influenza virus could be hidden inside an exosome or vesicle as part of a traditional vaccine preparation with the hidden virus inducing prolonged antigen exposure compared to naked, inactivated virus quickly taken up by the cell. The membrane protection afforded to the virus would result in a prolonged exposure to vaccine antigens stimulating a prolonged immune response and potentially simulating a vaccine response more typically seen with multiple vaccine doses over 3-4 weeks.

Exosomes can also be used to modulate vaccination responses via non-virus/virus component cargo. Porcine reproductive and respiratory syndrome virus (PRRSV) has been studied to determine if exosomes can deliver miRs to modulate disease outcome. It was shown that exosomes could be loaded with miRs and transfected into five different porcine cell lines and retain the miR target effect in the transfected cells [81]. Another study showed that exosomes carrying miR-155 could effectively regenerate miR-155 activity in hepatocytes and in vitro in macrophages in miR-155 knockout mice [82]. It was
recently shown that not only could Epstein-Barr virus (EBV)-infected B cells generate exosomes, but those exosomes contained miRNAs helpful to viral replication including miRs BHRF1-1, BHRF1-2, BHRF1-3, 1-5p, 1-3p, 3*, and 3, and that those exosomes could transmit those pro-viral miRs to uninfected human epithelial cells in vitro [26, 27].

One option for using exosomes would focus on using them as carriers for miRNAs, siRNAs, or shRNAs, such as miR-155, in order to modulate host cell processes. miR-155 has been implicated in enhanced dendritic cell (DC) maturation, recruitment, and activation [83]. As such including native miR-155 as exosomal cargo in a killed vaccine could help bolster the immune response to said vaccine via the enhanced recruitment of dendritic cells to the vaccination site which would allow for increased antigen uptake and presentation. This would be applicable to intramuscular (IM) vaccines where a depot effect may occur whereby the vaccine is not distributed in the tissue, but rather as a bolus, with the resultant antigen presentation less optimal compared to intradermal (ID) administration, in part because the vaccine dose may exist outside of the actual muscle in the tissue plain. miR17-92 could also be considered in certain populations to encourage cell proliferation and minimize apoptosis, both of which can be problems in elderly patients due to immune cell aging and apoptosis induced by telomere shortening [84]. Thus, there appear to be multiple avenues whereby exosomes alone or in conjunction with miR cargos could be incorporated into killed or live influenza vaccines with their inclusion dependent in part on the specific vaccine configuration (live versus killed), the target vaccine...
population (children, elderly, healthy adults), as well as the way the vaccine administration method (IM, intranasal, ID, etc.)

As exosomes are approximately the same size as influenza virions they make ideal VLPs from an antigen presentation standpoint, especially if they could be functionalized to display multiple antigenic epitopes in the form of HAs, NAs, M1s, etc. This would allow the targeting of many variable regions in order to elicit maximal cross-protection against circulating virus strains. This sort of system may also eliminate the need for egg-based vaccine production as it would be performed exclusively in cell culture systems. Much as the IAV backbone is used in conjunction with reverse genetics to update strains, the base cell line could be transformed with additional HAs in order to continually maximize antigenic inclusion in the vaccine. The primary disadvantage of this approach over conventional vaccines would be cost of exosome-VLP isolation relative to growing virus in eggs. However, over time the cost of isolation would likely decrease substantially especially if other surface antigens such as CD63 could be used in high-throughput separation systems.

**microRNA (miRNA)**

microRNAs (miRs) are small, non-coding single-stranded RNA molecules 18-23 nucleotides in length which function to regulate host gene expression in eukaryotic organisms. They are one of the most abundant gene families and are evolutionarily conserved among eukaryotes with similar if not identical miR sequences as well as between closely related species [85, 86]. Additionally, both
miRs and smaller piwi RNAs exist across multiple phyla, suggesting they have been evolutionarily conserved as gene expression regulatory systems [86]. miRs have also been found to have significant roles in stem cell differentiation in vivo [87]. miR regulation can occur via up- or down-regulation of miR expression with only small changes in relative expression leading to large changes in protein expression. As such, miRs function much like a thermostat in maintaining homeostatic control [88] whereby host genes can be dysregulated or affected via miR expression in response to a stimulus.

miR genes are found in the sense:antisense strands in a genic-intergenic fashion, as well as in the introns as independent transcription units termed mirtrons [89, 90]. The parental genes are normally transcribed by RNA polymerase II [91], and sometimes RNA polymerase III [92], but these enzymes also transcribe elongated primary transcripts termed pri-miRs. Interestingly, human helicase senataxin (SETX) has been shown to attenuate RNA polymerase II activity after viral infection which can then modulate the host response to the pathogen and/or affect viral replication [93]. SETX has also been shown to be involved in the targeting of exosomes to sites of DNA damage which combined with its attenuation of RNA Pol II makes it an attractive target for anti-viral strategies [94].

Pri-miRs are subjected to a processing phase via Drosha which results in a hairpin structure termed a pre-miR that is approximately 60 nucleotides in length [95, 96]. Pre-miRs travel out of the nucleus via Exportin 5 where they can then be further processed by the Dicer/Argonaute complex to produce so-called
mature miRs that are 18-23 nucleotides in length as noted previously [97-102]. Interestingly, Dicer has also been implicated as having potential anti-influenza activity, presumably through miR modulation [103-105].

The mature miR has both a guide strand and a passenger strand as the guide strand is used to exert the effect of the miR via binding of a seed site on the strand to a similar but not necessarily identical sequence on the target gene [106]. This seed site binding leads to the miR’s effect on transcription which occurs via transcript decay or a block in translation [107]. This binding and blockage is termed RNA induced silencing complex (RISC) and is the primary function of miR in vivo [107]. Since the miR uses a short seed site (5-8 nucleotides) to initiate binding, and requires only moderate sequence complementarity for binding, miRs can regulate many different genes. As such, altering a single miR can have many unintended effects on the host cell due to off-target effects not anticipated when only evaluating the primary binding site in the gene of interest.

Many miRs detectable in serum and other clinical samples are concentrated in exosomes lending to the theory that exosomes do in fact function in a messenger capacity [74, 108]. For example, the expression of miR-4276, as well as several other miRs, has been shown modulate the expression of cytochrome c oxidase which can help respiratory epithelial cells resist influenza A infection [109, 110].

Other miRs have been shown to induce or interfere with IFN production, modulate NF-kappa B signaling, modulate immune responses via antigen binding
to Toll-like receptors [111-116]. miRs also have a significant role in sepsis depending on whether they are located intra- or extra-cellularly [117]. The immune altering effects of miRs have been observed in multiple species indicating this is a genetically conserved messaging system that has been retained across many species [118-124]. Others have shown that with Enteroviral infection inhibiting a certain miR can actually restore IFN production and limit Enterovirus-induced cell death [112]. The inhibition of miR-34c has been shown to reduce the replication of IAV [125] while both mir-29c and mir-342-5p can actually inhibit innate immunity and enhance viral replication via inhibition the of NF-kappa B and sterol pathways respectively [111, 126]. Additionally, herpes simplex virus 1 has been shown to export exosomes containing viral mRNAs and miRs to neighboring uninfected cells [127]. Virally induced miRs clearly are not all pro- or anti-viral in nature (reviewed here [128]), but both effects must be considered when evaluating miRs as biomarkers or targets for interventions.

**miRs as Biomarkers and Diagnostic Tools**

miRs have the potential for use not only as biomarkers and diagnostic tools, but also as potential therapeutic agents. As miRs can exhibit pro-viral or anti-viral effects in many cases, their potential use as therapeutics should be apparent. miR 29-c is induced by influenza virus infection and has a significant role in cellular apoptosis, thus could serve as an interesting drug target [129]. miR-323, miR491, and miR-654 have all been shown to inhibit IAV replication [110]. However, due to the many functions and binding sites they utilize, the
potential for off-target effects by altering a specific miR level via a mimic or inhibitor is possible.

miRs, and to a lesser extent exosomes or exosome-associated miRs, have been investigated as biomarkers for viral infections of the respiratory tract. Both Hepatitis C Virus and Enterovirus have been shown experimentally to be transported inside exosomes or phosphatidyl serine vesicles with both being able to induce infection via the exosome [78, 79]. This finding is significant as several other viruses including influenza, Epstein-Barr virus, Coxsackie virus, and HIV have also been implicated as potentially having exosome-mediated transport of virus or virus components [80]. Thus, exosomes appear to have the potential to offer definitive diagnosis of viral infection with certain viruses. A decline in exosome production during HCV infection has been previously observed and was postulated to be due to the virus needing the same cellular ESCRT machinery used to process MVBs into exosomes in order to produce virions [130]. MUC1 on the exosome surface has a role in the immune response to various agents and as such, looking at the percentage of MUC1 on the exosome surface, may be a simple biomarker for infectious/inflammatory processes [44].

While studies have examined media and cellular culture components, and in vivo serum and tissue miR levels during respiratory viral infections in the attempt to find potential biomarkers, relatively few have examined exosome-associated miRs [74, 108]. This is related to difficulties isolating useable quantities of miRs from exosomes, as well as the perception that looking at total
miR levels in a given tissue/media will yield comparable levels and/or ratios to the combination of exosome and supernatant.

However, this has been shown to be untrue, in part because miRs can be concentrated in exosomes and may be lost in the processing of whole samples [26, 74, 131, 132]. Also worth noting is the longevity of a miR outside a protective entity such as an exosome, as this alone would necessitate the evaluation of specific miR fractions. Even the method of exosome isolation can generate differential results with respect to miR abundance, in part due to which surface markers are present on the exosome surface as many exosome isolation kits use CD63 or CD10 conjugates to pull down exosomes while failing to capture any exosomes not containing those markers.

Although one may readily attain total miR data for viruses including IAV, RSV, measles, metapneumovirus, including Varicella, and Vaccinia viruses, little information is available for exosome-derived miRs. In addition to host-generated miRs, virus-associated miRs are also potential biomarkers although this may be somewhat limited to viruses that have a DNA component in their life cycle as they appear to be more likely to have viral miRs and include viruses such as Herpesvirus, Polyomavirus, Ascovirus, Baculovirus, Iridovirus, and Adenovirus [128].

PRRSV has also been studied to determine if exosomes can deliver miRs to modulate disease outcome. It has been shown that one could load exosomes with miRs, and transfect these into five different porcine cell lines, and retain the miR target effect in the transfected cells [81]. Another study showed that
exosomes carrying miR-155 could effectively regenerate miR-155 activity in vivo in hepatocytes and in vitro in macrophages in miR-155 knockout mice [82]. While not biomarker studies, these approaches show that exosomes can be used to carry miRs and effectively target different cell populations both in vivo and in vitro, which is one of the outcomes we would expect from any potential miR biomarker in vivo.

It is known that Epstein-Barr virus (EBV) can replicate in type II alveolar cells leading to alveolitis [133]. It was found that not only could EBV-infected B cells generate exosomes, but those exosomes contained miRNAs helpful to viral replication including miRs BHRF1-1, BHRF1-2, BHRF1-3, 1-5p, 1-3p, 3*, and 3, and that those exosomes could transmit those pro-viral miRs to uninfected human epithelial cells in vitro [26, 27]. They were also able to show differential miRNA levels between the exosomes and liquid cell culture lysate proving miRs can concentrate in exosomes, reinforcing the notion that looking at miR concentrations in their cellular carrier may be more relevant than evaluating total miRNA levels in a given sample (at least when evaluating culture media or serum exosomes/miRs) [26]. Herpes simplex virus 1, has been shown to export exosomes containing viral miRs (miRs H5, H5, and H6), to neighboring uninfected cells in vitro as well using human cell lines [127]. While limited, these exosome-miR data support the hypothesis that exosomes are the relevant carrier of miRs when outside the cell. Others have shown the transduction efficiency of miRs encapsulated in exosomes far surpasses the transfection efficiency of liposome carriers, and occurs in minutes as opposed to hours [23]. This further
supports the importance of exosomes as message carriers as opposed to unprotected miRs transmitting similar messages given the relative lack of transfection efficiency when using RNA without transfection reagents, or the specialized reverse transfections with transfection reagents more typically utilized to transfect respiratory cell lines [134].

**P19 Binding Protein to Assess Total miR Distribution**

P19 binding protein, a tombusvirus-derived protein, is able to suppress RNA silencing as well as bind miRs [135] and can act as a reagent for miR identification and as an inhibitor of miRs and siRNAs, allowing one to tease out the miR-mediated viral defenses [136-138]. Since P19 binds very specifically to short 21-23 nucleotide double-stranded RNA sequences, its use to block all miR-mediated effects if transfected into cells has interesting ramifications for use as a labeling agent in conjunction with a discrete binding domain to which antibodies can be targeted [139]. Additionally, P19 has also been shown to increase virus yield in viral culture systems via complete miR silencing, limiting the effects of any anti-viral effects generated by host miRs [136, 137, 140]. If this same effect could be demonstrated with influenza virus, it may have important implications for vaccine generation that is affected by miR regulation.

**miRs During Influenza Infection**

miR mimics or inhibitors have several roles during influenza infection. Mimics or inhibitors could be used to modulate the immune response during the
initial infection. This could include therapeutic use as well as prophylactic use where one may temper the miR response to a broadly affected anti-viral immune response, or attempt to break the replication or transmission cycle of the virus. This would be important with the continued emergence and subsequent circulation of influenza strains that are resistant to one or more antiviral pathways [141, 142], such as for the A/California/04/2009 (H1N1) strain which was highly resistant to amantadine and has also shown resistance to oseltamivir [143]. For those with clinical disease, multiple miRs have been implicated in influenza pathogenesis and replication, and by targeting these specifically one may be able to affect the outcome of disease.

For instance, miR-150 was overexpressed in critically ill patients infected with A/H1NI virus, while miR-29c, miR-145, and miR-22 were all dysregulated in patients with severe disease compared to those with more moderate disease [144]. Another human study showed dysregulation of miR-101, mir-193b, miR-30e, and miR-23b with three different influenza subtypes (pandemic H1N1, H5N1, and H3N2) [145]. As such, using a miR antagonist or silencing RNA (siRNA) against miR-150 would allow modulation of the immune response and potentially shift the progression of disease from severe to a more moderate form via reduced expression of Type II IFNs, IL-2, IL-6, etc. [144].

miRs have the potential to regulate several areas in an epidemic or pandemic situation. miRs could be used prophylactically across the population to broadly upregulate immune system function (IFN Type I and III). In the case of those exposed to, but not infected with the virus, several miRs (miR 29-c, miR-
323, miR491, and miR-654) have been shown in vitro and/or in vivo to severely decrease the virus’s replication in the host. All of the aforementioned features are important in the initial stages of a new virus introduction to prevent it from being an epidemic or pandemic, as well as important to the initial host in terms of minimizing clinical signs and associated pathologic changes that often induce secondary bacterial pneumonia, ARDS, etc.

Secondly, miRs could be used as traditional therapeutics for those already infected, either as an adjunct to antiviral drugs or when antiviral drugs have failed. This would be especially important during situations such as acute respiratory distress syndrome (ARDS) where conventional antiviral drugs have minimal to no effect and necessitate the administration of steroids, etc. to downregulate the immune response and prevent death from ARDS due to respiratory system collapse [146]. However, the administration of steroids can actually worsen the long-term outcome of the influenza infection via immune suppression. As such, miRs could be utilized to specifically upregulate certain immune responses (Type I and III IFN expression) necessary to combat influenza while still allowing for the immune suppression necessary to treat ARDS.

Additionally, miR-29 could be used to treat patients who are at increased risk if they develop (enhanced) pulmonary fibrosis secondary to influenza such as chronic obstructive pulmonary disease (COPD) patients, smokers/others with emphysema, asthmatics, cystic fibrosis patients where the direct introduction of miR-29 into the lungs via a liposome carrier or similar carrier could arrest
collagen deposition and induce apoptosis of infected cells, and prevent fibrosis thereby preserving maximal lung function following resolution of the infection. In addition to those at risk of fibrosis due to underlying lung pathology, both the elderly and children could be targeted with specific miR-122 to enhance their generally poor immune responses (compared to healthy adults) to vaccine. In the case of the elderly miRs could also be utilized to maximize the functional components of their immune system while minimizing aberrant responses seen due to faulty T cell maturation and/or improper/insufficient DNA methylation [147].

While limited data exist for using miRs as therapeutics, there are several examples of their use in current literature. It was shown that miR-122 could limit Hepatitis C virus (HCV) both in vitro and in vivo via the down regulation of heme oxygenase [148]. Others have shown that depletion of miR-122 and Hepatitis B virus (HBV) via the introduction of its anti-sense inhibitor enhanced HBV replication while transfection of its mimic led to significant declines in HBV replication in vitro [149]. This is important as HCV has both acute and chronic disease presentations with the former often transitioning to the latter. If miR-122 can limit viral replication during an acute HCV infection, it is possible that it could prevent chronic HCV which often leads to liver cirrhosis and subsequently necessitates a liver transplant. It has been shown that miR-145 expressed ectopically is able to prevent invasion, metastasis, and angiogenesis of gastric cancer cells through the inhibition of the oncogenic erythroblastosis virus E26 [150]. While not directly affecting the virus, they were able to show that the virus
mediates metastasis and as such miR-145 was able to prevent this spread which occurs over weeks to months.

Others have shown that incorporating miR-143 or mir-145 into Herpes Simplex Virus 1 (HSV1) can itself act as an anti-cancer agent against highly invasive and rapidly spreading castration resistant prostate cancer both \textit{in vitro} and \textit{in vivo} [151]. Recently, miRs and anti-sense RNAs were introduced into lentivirus vectors and used to attack the 5' ends of the viral polymerase gene segments (PA, PB1, and PB2) \textit{in vitro} and showed that this approach could decrease influenza virus replication [152]. The existing \textit{in vitro} and \textit{in vivo} evidence supports the further development of the miR-based approach especially in cases where current anti-viral drugs are ineffective or where the development of chronic lesions post-infection may be detrimental to future host function.

Another promising strategy for using miRs to enhance influenza vaccine efficacy would be to include miRs as self-replicating adjuvants in live attenuated vaccines (LAVs) whereby the miR is included in the live virus genome and replicates concurrently with the virus as was done with miR-155 [153]. The selected miR(s) would ideally stimulate the desired immune response and could include miRs such as miR-29 (generally miRs in the same family, and miR-29a and miR-29b in particular, have similar 5' seed sites, and different downstream sequences, but still recognize the same mRNA sequences) [154] which has been shown to be involved in the stimulation of type III IFN (IFN lambda) production as well as the stimulation of T cell maturation, T helper cytokine production, etc. Any miR that can enhance IFN lambda production could help to inhibit influenza virus
replication. However, the primary drawback of this approach is that the current live, attenuated influenza vaccine virus is only designed to undergo a single round of replication which may not yield adequate miR numbers to effect the necessary immunologic change, but that may depend on the miR(s) used and route of administration.

There are miRs that may be problematic as well, such as miR-146 which has been shown to be upregulated not only during IAV infection, but also during Enterovirus (EV) and Dengue virus infections [155, 156]. miR-146 can facilitate viral pathogenesis via the down regulation of IFN production leading to enhanced virus replication; the anti-viral effects of miR-146 were subsequently validated via the use of antagomiRs via phenotypic expression of decreased EV propagation, increased type I IFN production, and improved survivability during in vivo mouse studies [156]. Since IAV also significantly upregulates miR-146, it represents a target for upregulation during live virus vaccination as it would help delay cellular apoptosis until maximal virus replication has occurred thereby achieving a maximal viral “dose” before replication is halted due to the temperature sensitivity of the virus induced by the mutations in the PB2 portion of the genome [157].

In contrast, miR-155 has been implicated in enhanced dendritic cell (DC) maturation, recruitment, and activation, a feature which can be used to induce gene targets via viruses encoding the necessary miRs [83, 153, 158]. As such including native miR-155 in a killed vaccine or in the genome of a live vaccine could help bolster the immune response to either type of influenza vaccine via
the enhancement of DCs to the vaccination site which would allow for increased antigen uptake and presentation.

This would seem particularly applicable to intramuscular (IM) vaccines where a depot effect may occur whereby the vaccine is not distributed in the tissue, but rather as a bolus, with the resultant antigen presentation less optimal compared to intradermal (ID) administration, in part because the vaccine dose may exist outside of the actual muscle in the tissue plain. miR17-92 is another that could also be considered in certain populations to encourage cell proliferation and minimize apoptosis, both of which can be problems in elderly patients due to immune cell aging and apoptosis induced by telomere shortening [84].

Thus, there appear to be multiple avenues whereby miRs could be incorporated into killed or live influenza vaccines with their inclusion dependent in part on the specific vaccine configuration (live vs. killed), the target vaccine population (children, elderly, healthy adults), as well as the way the vaccine administration method (IM, intranasal, ID) miRs could be incorporated either as a part of an adjuvant system, or more likely into the vaccine virus itself as an example in live attenuated virus vaccines, whereby the replicating virus actually produces the desired miR(s) as part of its replication cycle. This self-production of the desired miRs is already under investigation by several labs and has shown promising results thus far in a study using miR 155 to modulate SOCS1 mRNA levels to increase IFN-β[153].
It is likely that any vaccine or standalone miRs are destined to face heightened regulatory and safety scrutiny especially with the potential for off-target effects. Any miR-based adjuvant or miR-containing virus would be subject to more robust clinical trials until their safety could be demonstrated, which continues to be a major financial hurdle to their widespread implementation. However, miRs can offer several potential benefits as they can be manufactured synthetically and thus not contain issues of concern that include mercury, aluminum, etc. all of which have come under scrutiny for their inclusion in vaccines.

Importantly, miRs can be synthesized for very minimal cost, and may be able to minimize some of the reaction site lesions that are associated with vaccines. A promising aspect of miR-containing viruses is that many viruses, including influenza, and HCV induce miRs as part of regulating the host response to infection and disease pathogenesis. As such, modulating miR expression is potentially easier to justify from a regulatory standpoint than the inclusion of an adjuvant, or protein that is not naturally occurring in a vaccinee, especially if the miR is already present in the host and we are only altering its basal expression temporarily.

**Fetal Bovine Serum-derived miRs as Cell Growth Modulators**

Many in vitro cell culture-based experiments involve the use of fetal bovine serum (FBS) as a media component to sustain cell viability. However, the largely undefined composition of FBS includes variables such as quantities of proteins,
growth factors, exosomes, miRs, and other intrinsic molecules. There is always inconsistencies between lots and suppliers regardless of the species of origin as serum supplements are available from human [159], bovine, caprine [160], porcine [161, 162], equine, and other species.

While chemically-defined media that does not contain serum is among the most common serum free media (SFM) alternative, it has been used with varying success compared to bovine serum-based media [163-166]. Non-serum based alternatives, such as royal jelly from honey bees [167], and platelet lysates from human [159, 168], have also been investigated as alternatives to FBS, but have not been widely adopted due to lot-to-lot variability, and less than optimal cell growth rates, and platelet lysates also contain both exosomes and miRs. Commercial SFM variants are also not without problems as at least one commercial variety has shown considerably altered phenotypes in both equine and canine stem cell lines compared to traditional FBS-based media which could be due to a variety of intrinsic factors including the lack of exosomes and miRs to help stimulate differentiation [169].

Although suppliers typically test individual lots of FBS for a variety of features, including chemical/physical composition, microbiological tests, and cell growth performance as part of their quality control process to try to ensure product consistency and minimize lot-to-lot variation, they do not typically evaluate exosome or miR content [162]. Additional treatments are often applied to the serum prior to sale including heat inactivation (HI), gamma irradiation, dialyzation, charcoal stripping, [162] to further define or enhance the
performance characteristics of the FBS relative to cell growth, contaminating virus content, virus viability, immune interference potential, or other potential features that may impact performance.

In addition to essential biomolecules and growth factors necessary for \textit{in vitro} cell culture, serum also contains extracellular vesicles (exosomes) involved in cellular communication, cell differentiation, and cell programming [170]. Exosomes are found in nearly all bodily fluids and have significant regulatory roles on a cellular and system-wide level as discussed previously [171]. As endocytotic vesicles, exosomes are known to carry a variety of proteins, lipids, and RNAs, the latter of which is believed to have an appreciable role in cell-to-cell communication. Exosomes have been shown to alter cell growth phenotypes [170, 172], impact cell growth [173], affect differentiation of skeletal muscle cells \textit{in vitro} [174], and inhibit the macrophage response to lipopolysaccharide (LPS) [28]. Others have shown that FBS can change the efficiency by which stem cells can be reprogrammed as well as their proliferation rate [175].

While human platelet lysates have been suggested as the new gold standard over FBs for human-origin cell culture [176], this strategy is not without related caveats to FBS for use. Platelet concentrates, the source of platelet lysates, have been shown to contain not only exosomes, but also low-density lipoprotein (LDL), which can mimic exosomes and purify concurrently with exosomes using commonly employed purification techniques [177]. The simultaneous purification of LDL with platelets can result in incorrect relative ratios of exosomal miRs as the LDL molecules do not contain miRs themselves.
Also, platelet lysates include platelet microparticles, an amalgamation of both exosomes and microvesicles. Others have shown that platelets contain large amounts of miRs as well as miR processing machinery (reviewed in [178]).

Platelet microparticles have been shown to positively impact angiogenesis [179, 180], as well as induce gene expression in monocytes [181] through largely unknown pathways. Purification methods to include activated charcoal sponging [179], heat inactivation [179, 182], and/or addition of antibodies [180], designed to remove growth factors or other molecules contributing to these traits are largely ineffective in altering the genotypic and/or phenotypic changes which would imply the presence of a mechanism that preserves functionality of the intrinsic growth factors, miRs, or other biomolecules. Since exosomes use host cell membranes as their exterior surfaces, it would seem apparent that they are the likely vehicle with miRs being the most likely cargo given their propensity to induce large changes within a cell in spite of only minute changes in absolute miR concentrations.

Also of interest is the relatively high level of transduction efficiency of exosomes, and consequently the potential for their miR cargo to regulate cell growth characteristics or differentiation status. As miRs are non-coding RNA that are involved in regulating upwards of 60% of all protein-encoding genes post-transcriptionally [183], the ability of exosomes to serve as miR transports that then affect cellular growth, and protein synthesis is extremely relevant to cell culture given the relative abundance of both exosomes and miRs in FBS.
Furthermore, several miRs have already been shown to impact cell growth and differentiation including miR-19a, miR-19b, and miR-26b affecting fibroblast differentiation [184], while miR-29b has been shown to inhibit osteosarcoma cell proliferation [185, 186]. Other studies have tied low *in vivo* expression of miR-26A and high expression of miR-27a with enhanced osteosarcoma development in human patients [187] which is of interest since many cell lines utilized for *in vitro* experiments are derived from cancerous tumors, and thus potentially under similar miR regulatory mechanisms. Although platelet lysates have gained favor over serum for some cell culture applications, it is important to understand intrinsic factors beyond growth factors in cell culture media that may impact cell growth rate and/or phenotype/differentiation.

**Influenza**

Influenza is a single stranded, negative sense, segmented RNA virus from the *Orthomyxoviridae* family, and is one of the most widespread respiratory viruses in the world [188]. It is among the most important zoonotic respiratory diseases due to its propensity for both inter- and intra-species transmission. It is further subdivided into three influenza subtypes (A, B, and C) along with the separate *Thogoto* and *Isavirus* genera. The Influenza A virus (IAV) family typically has eight RNA segments encoding eleven genes that produce eleven viral proteins [189]. The IAV subtype is regarded as the most common and most pathogenic of the three subtypes typically causing more severe disease as well as causing the majority of influenza related disease worldwide. While IAV affects
multiple species, the B and C viruses typically only affect humans although limited instances of infection in other species have been documented. Influenza B and C also differ slightly from IAV in their mechanisms of infection of host cells, which may explain why they affect less species and are less virulent than IAV.

Influenza is a viral pathogen of multiple species including humans, poultry, and swine. It causes primarily respiratory disease that can affect virtually any portion of the respiratory tract from the nasal turbinates to the epithelial cells lining the smallest airways in most species. Interestingly, an influenza virus outbreak was likely first documented by Hippocrates in 412 BC, demonstrating that the viruses have been with us for virtually all of recorded history [190]. Influenza infects millions of people worldwide each year and results in thousands of deaths, mainly among children, the elderly, and other immunocompromised groups.

Historically the Spanish flu pandemic of 1918 that originated in a Kansas military barracks holds the distinction as the most lethal pandemic in recorded history with between 20 and 50 million people dying from the infection and many times that number infected [191]. Multiple other less severe influenza pandemics have been seen with the most recent being the swine origin H1N1 virus outbreak that started in Mexico and proceeded to spread across the globe in rapid fashion [192]. Currently, emerging variants of H5N1 and H7N9 viruses have produced high case mortality with a low overall infection rate in the human population in China, but these variants have not spread rapidly outside that country except in rare instances usually involving travel to/from an affected area.
Influenza Structure

Two glycoproteins comprising the influenza virus exterior structure are hemagglutinin and neuraminidase, which are involved in entry and egress from the target cell, respectively. Hemagglutinin and neuraminidase coat the outer surface of the virus, which is called the viral envelope. The envelope serves to encapsulate the rest of the virus particles and RNA prior to their release into an infected cell after fusion with the host cell membrane has occurred. Currently there are eighteen different hemagglutinin subtypes (H1-H18) and eleven different neuraminidase subtypes (N1-N11).

Hemagglutinin is involved in the initial attachment and binding of the target respiratory cells on the host via a glycoprotein receptor that expresses sialic acid and is found on the host cell’s surface. Interestingly, while the influenza virus typically binds preferentially to cells in the mammalian respiratory tract, it has a unique tropism to cells in the gastrointestinal tract of birds and yields little respiratory system pathology. Subsequent to binding the host cell’s sialic acid receptors, hemagglutinin begins the process of membrane fusion which perforates the host cell allowing the contents inside the viral envelope to be emptied into the host cell effectively infecting that cell with influenza virus.

After infection has taken place the virus uses the host cell’s internal machinery to multiply and eventually exit the cell through the use of neuraminidase, which results in death of the cell. Paradoxically, both hemagglutinin and neuraminidase can bind to sialic acid which poses a problem in terms of the regulation of viral replication as hemagglutinin uses sialic acid as
a binding moiety while neuraminidase binds to and subsequently destroys sialic acid receptors.[2] Thus, overactive neuraminidase activity can actually be detrimental to virus survival by destroying receptors on uninfected cells necessary for hemagglutinin to bind, fuse, and subsequently replicated with the host cell.

Structurally, IAV is composed of eight RNA segments including PB2, PB1, PA, HA, NA, NP, M(1/2), and MS1/NEP [193]. Small structural changes in these segments, or their reassortment with other influenza viruses, can have profound effects on virus pathogenicity, especially as related to interspecies transmission. As was seen with the 2009 pandemic virus, the inclusion of an NA and M segments from a Eurasian swine virus, as well as a minor change in the HA amino acid structure, allowed for efficient aerosol transmission [194]. Others have shown similarly altered phenotypes due to changes in PA and NA leading to enhanced respiratory disease and lung pathology [195]. The M segment has also been shown to be involved with contact transmissibility efficiency exchange between viruses, as well as morphological changes [196]. Interestingly, the route of infection also affects selective pressure in that direct transmission induces less selective pressure than aerosol spread [197].

Also of interest is that in swine, the 2009 pandemic virus NA and M genes cooperated with one another resulting in improve replication efficiency and transmissibility [198]. HA and NA segments from canine influenza virus (CIV) have also been shown experimentally to reassort with the 2009 pandemic virus leading to enhanced replication and pathogenicity which is an on-going concern
given the current outbreak of CIV that began in late 2014 and has continued to present [199]. Conversely, lack of a sufficient number of segments and/or defective segments can lead to the generation of defective virions known as defective interfering (DI) particles [200]. Since DI particles can often attach to host cells, it is possible they can also initiate cellular signaling, and as such their effect on miR genesis by the host cell must be considered.

In addition to enhanced transmissibility, the gene segments also confer other properties related to influenza virus assembly and pathogenesis. Specifically, virus assembly requires that the viral components be moved to the assembly location on the apical plasma membrane of polarized epithelial cells [201]. Neuraminidase not only helps with viral egress from cells, but is also integral to enhanced mucous penetration via the cleavage of sialic acids from glycans in the mucous as mentioned previously [36].

Vaccination has been shown to induce antibody formation, and improve disease outcomes across a variety of species. The frequency of viral mutation makes virtually all IAV vaccines prone to potential failure due to rapid mutation, but also the lack of cross-protection among closely related strains. Due to the time required to propagate and inactivate viruses for use in inactivated vaccines, currently circulating influenza strains may not be readily incorporated into a new vaccine for a period of time. Additionally, the use of killed autogenous influenza vaccines in livestock, a feature that requires the strain be procured from within the herd necessitating an active influenza strain be isolated before the strain can legally be included in that producer’s influenza vaccine. Commercial livestock
vaccines face the same hurdles as their human counterparts, but the time to include new influenza isolates is even longer, and one of the original isolates used when the vaccine was first licensed must always be included, or the vaccine must be completely relicensed.

The near or complete ban on the use of antiviral drugs in food-producing animals in many pork-producing countries, which was instituted to minimize the potential for the development of drug resistance, translates to limited treatment options for infected animals outside of supportive therapies. As such, alternative vaccines, adjuvants, and therapies are necessary that do not use traditional antiviral drug mechanisms for treatment. Instead they would presumably rely on priming the swine immune system to respond robustly and/or rapidly in order to improve influenza outcomes by limiting virus spread and/or pathology in an infected pig and/or to neighboring pigs.

**Influenza Viral Ribonucleoproteins (vRNPs)**

Although the vRNP complex has been studied for over 50 years, only recently have several key features regarding its structure and transmission been elucidated. These discoveries give insight into how vRNPs may be involved in both influenza transmission and reassortment in host species leading to novel virus development. Several recent observations regarding the transport of vRNPs between cells yield key clues suggesting the potential for exosome involvement in vRNP trafficking as well.
Influenza vRNPs are comprised of one of the eight viral RNA segments along with a viral polymerase and several copies of the nucleoprotein (NP), all of which are packaged together into a rod-shaped vRNP complex (reviewed here [202]). The vRNP contain an NP core, with the RNA wrapped around it in a helical arrangement [203]. However, if no viral RNA is present, M1 and NP are unable to self-assemble into the helical structure characteristic of the vRNP complex [204]. Live imaging techniques utilizing labeled vRNP complexes have elucidated key points in their trafficking from the nucleus out of cell [205] and vRNP purification protocols have allowed for the detailed studies on nuclear import and viral replication without other viral proteins contaminating the vRNP complexes [206].

Initial vRNP formation begins in the nucleus with polarized epithelial cells exhibiting unique asymmetric aggregation of either ribonucleoproteins (RNPs) or vRNPs at the inner nuclear membrane [207]. As the vRNP complexes emerge from the nucleus, they utilize Y-box binding protein (YB-1) as a carrier to move the vRNP to the microtubules where it subsequently enters the vesicular trafficking network to be carried to the plasma membrane of the cell for virus budding [208]. Additionally, Rab11 has also been shown to be involved with vRNP trafficking via its interaction with them at recycling endosomes [21]. The rod-like vRNPs are variable in length and position within the assembled virion, but the relative pattern of assembly implies the mechanism of assembly is quite specific [209].
Recent studies have more closely looked at the specific assembly of the vRNP components. A live cell imaging study showed that the PB1/PA heterodimer enters the nucleus via RanBP5 where it then joins with PB2, which is imported into the nucleus via a distinct importin alpha-linked mechanism [210]. The same group also observed the potential for abnormal formation of the polymerase trimer in the nucleus if nuclear import was blocked, which may be a potential route for incorporation of one or more members of the vRNP complex to be incorporated into exosomes if they are aberrantly formed in the cytoplasm [210].

The potential for exosomes to mediate viral transport have been discussed previously for several viruses including Enterovirus and Hepatitis C virus. Exosomes have also shown the ability to utilize virus entry routes for the delivery of exosomal contents to the cell in order to avoid degradation prior to releasing their cargoes (reviewed in [211]). However, the potential for influenza subunits such as vRNPs to be transported via exosomes and then mixed with other viral subunits in the cytoplasm during virus assembly requires further exploration. Exosomes and influenza viruses are taken into the cell via several of the same pathways (reviewed in [211-213]) and rely on much of the same cellular machinery for cellular uptake and egress so the possibility for exosomes to mediate reassortment is not withstanding.
Influenza Host Tropism

Multiple features of IAV and host cells influence tropism. These features can include viral and host glycoproteins, sialic acid residues which are involved in the binding of virions, host receptor morphology, internal viral genes such as the swine triple reassortant internal gene (TRIG) cassette [214], as well as many others that confer tropism of a virus to a particular host species and/or cell type.

Tropism influences infection, transmission, and clinical disease in several ways. Preferred hosts, or those to which the virus is adapted, generally experience less severe disease in part because it is against viral evolution to kill the host species if the virus is to survive more than one generation. Host species are often asymptomatic carriers as is observed with highly pathogenic avian influenza where the carriers experience mild clinical disease while aberrant hosts experience severe, and often lethal, clinical manifestations. Sometimes the non-adapted species are more permissive to infection where there is heightened virus replication resulting in more severe disease as well as the emergence of severe disease syndromes such as acute respiratory distress syndrome in humans which is not characteristic of the virus in the host-adapted species.

Multiple virus-specific factors influence viral tropism for specific hosts and/or cell types. HA, polymerase complex, NS1, and PB1 proteins are all implicated in enhanced pathogenicity of certain viruses over others, including potentially the 1918 pandemic virus as well as the various H5N1 avian influenza viruses. However, with the avian H5N1 variants, it is thought this enhanced tropism with its ensuing severe disease in non-adapted species is due
predominantly to the presence of the multibasic cleavage site. The multibasic cleavage site is an area of multiple basic amino acids around the HA cleavage site that lead to enhanced pathogenicity in some mammalian hosts compared with the original avian hosts due in part to several amino acid substitutions [215]. As such influenza viral amino acid specificity at various locations in the virion also confers species tropism as well. Additionally, the specific HA glycosylation pattern also has a role in host tropism due in part to how the sugar moieties interact with host cell receptors upon binding.

Host cell receptors also have a role in virus tropism. Avian species generally exhibit a higher concentration of alpha-2,3 sialic acids vs. alpha-2,6 found more commonly in mammals [216]. However, the distribution of the two sialic residues not only varies by species, but also within a given species depending on which tissue is being examined. Certain species such as swine and humans have both receptors present, and as such this may explain why both are routinely infected with new influenza variants from multiple species to include birds [217]. This may also explain why birds, dogs, horses, are not infected with certain influenza subtypes.

Also related to the sialic acid residues is how the sialic acid is linked to the glycoprotein chain in that the conformation of this sugar linkage confers additional host specificity [218]. Also with respect to the host receptor is the conformation of the receptor both internally and externally as highly pathogenic avian species often preferentially bind a cone shaped receptor whereas lower
pathogenicity isolates often attach preferentially to a mound-shaped receptor [216].

**Influenza Virus Modifies Host Responses**

Studies have shown that IAV modifies the host immune response to infection (reviewed in [219, 220]) and may contribute to immune evasion and viral persistence in addition to undergoing constant evolution. The 2009 pandemic virus, A/California/04/2009 (H1N1) has been shown to upregulate 69 miRs and downregulate 13 miRs in human A549 cells based on one microarray analysis [221]. Differential miR expression has also been demonstrated in mice infected with the 2009 pandemic virus [222, 223], and differential miR expression has also been shown in A549 human lung epithelial cells and human serum samples infected with different Influenza A viruses [121, 221, 224]. Additionally, altered miR expression has been seen in multiple porcine tissues including lung tissue from pigs infected with an H1N2 virus, as well as during respiratory bacterial infections [118, 119, 225]. Differential miR expression patterns have also been demonstrated between IAVs [226]. Additionally, over 50 human and swine host-encoded miRs that specifically interact with influenza virus have been identified [227-229]. These findings suggest miRs can be used as biomarkers of disease, and host response to viral infection. As miRs can contribute to pro-viral or anti-viral responses, their potential use as therapeutics is not withstanding.
**Innate Immune Responses**

Innate immune responses are critical in preventing virus infection and minimizing virus replication. Innate immunity encompasses passive and active systems designed to mount an early, non-specific response against pathogens. The primary barrier against all pathogens is the epithelial cell layer which serves as the first line of defense by preventing direct virus access into the host. As seen with HIV, compromise of this barrier can lead to direct virus inoculation in the host [230].

In addition to acting as a physical barrier against virus infection, epithelial cells also have several anti-pathogen defenses. In mucosal areas, epithelial cells secrete mucous which can not only prevent virus binding to epithelial cells, but can be used as a clearance mechanism. Epithelial defenses can include direct pathogen countermeasures via a system such as dual oxidase whereby contact with virus mediates the release of reactive oxygen species on the apical side of the cell which can inactivate the virus before it can infect the cell [60]. It can also include endocytosis of the virus once attached to a surface receptor for eventual destruction in a lysosome, although some viruses use this to their advantage in terms of using pH changes in the lysosome to initiate structural modifications that lead to infection as opposed to virus destruction.

Epithelial cells also display pattern recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMPS) resulting in the release of other molecular mediators such as cytokines. These cytokines include type I and III IFNs [231], which can mediate antiviral states in adjacent cells.
reducing infection. Pathogens can be detected by the type of RNA/DNA the pathogens display. For example TLR3 recognizes double-stranded RNA [232] and defective interfering particles while TLR7/8 recognize ssRNA [233]. If the virus successfully evades these countermeasures, additional anti-viral mechanisms come into play.

The complement system produces antimicrobial peptides which can prevent virus infection. Phagocytic cells including neutrophils, macrophages, and dendritic cells are often found in the tissues or recruited there post-infection [234, 235] and can engulf the virus itself as is the case with alveolar macrophages. Natural killer cells can also control virus-infected cells to directly limit viral replication, often by expressing IFNs, although some viruses can circumvent this process [236]. Additionally, B cells can respond to infection and release antibodies, which are polyspecific meaning they can bind multiple antigens. As such, they can not only bind virus directly pre-infection, but also post-replication as the virus egresses from the cell. NK cell-mediated killing of infected cells, and apoptosis of infected cells can prevent viral spread throughout the tissue.

Many cells have internal signals identifying virus hijacking of cellular machinery that can initiate cellular apoptosis. This process may occur in conjunction with RNA-induced silencing via Dicer/RISC complex whereby short RNAs in the form of miRNAs are generated that block virus replication and translation. However, this same system can also be co-opted by the virus itself to induce pro-viral miRNAs and thus its effect on replication may not be consistent depending at what point it is initiated, or how quickly the virus is able to take over
host cell machinery. All of the mentioned innate responses help prevent infection or minimize/stop virus replication in an attempt not only to modulate the infection, but to give the adaptive immune system time to respond and generate highly specific immune responses to the invading virus.

The Interface Between Influenza, Exosomes, and miRs

The relationship between exosomes, miRs they carry, and host responses to virus infection has not been defined. Hepatitis C virus (HCV) is somewhat better understood as it has been shown that IFN-mediated responses in human liver cells are critical, and this response induces the formation of exosomes with antiviral activity against HCV [50]. This pathogen/exosome connection is not limited to viruses in that *Mycobacterium tuberculosis* (*M.tb*) has also been shown to generate exosomes containing pattern associated molecular patterns once it infects a cell, which may allow it to actually suppress the host immune response to infection [237].

Similar phenomena have been observed with dendritic cell recognition of TLR ligands leading to the increased production and secretion of IFNγ [238]. However, others have shown that virions can also use exosome-independent mechanisms such as the transfer of cyclic GMP-AMP to infected cells to trigger a STING-dependent antiviral response, exclusive of exosomes or other nucleic acid signaling [239]. It remains unproven that exosomes transfer influenza virions from one cell to another, as the internal cargo size is most likely a mismatch. However, there are amorphous (spherical) and linear forms of influenza virus
[240], and exosome transfer of viruses has been reported for both Enterovirus (EV) and Hepatitis C Virus (HCV) [78, 79].

The influenza virion has a diameter between 100-150 nm while exosomes have diameters between 30-150 nm on average [78, 241]. It really does not matter if the virus is being transported via internalization or sticking to the outside of the exosome, as the end result is likely the same. Since exosomes display common cell surface markers including various mucins, CD10, CD63, and alpha-2,6- and alpha-2,3-linked sialic acids, it is expected that exosomes can bind viruses externally and potentially act as infectious delivery systems to adjoining cells, a phenomenon that has also been observed with HCV [77].

It is likely that exosomes traffic key portions of the influenza genome to adjacent cells such as the vRNP complex, which is approximately 10-20nm in diameter making it well within the size constraints to be considered as a possible exosome cargo [242]. As such, the working hypothesis remains that substantial portion of the influenza vRNP is being moved via exosomes or larger vesicles to neighboring cells, not just through filopodia and actin filaments [21, 23, 205, 243, 244].

Examining the relevance of exosome trafficking is understudied. For example, the current method has been to block exosome egress from the cell. The gap junctional protein Connexin 43 (CX43) has been shown to modulate the transfer of exosomal material to adjacent cells via CX43 containing channels [245]. CX43 is found not only in pores at the gap junction, but also as a hexameric structure on the exosome membrane itself [245]. This affords two
possibilities for affecting the transfer of material from exosomes via the use of anti-CX43 antibodies which could be used to bind not only the CX43 on the exosomes, but also in the gap junction channels. Additionally, gap junctional CX43 can be upregulated (as shown experimentally in rats) via the use of fluoxetine and amitriptyline and as such would be a way to further enhance trafficking of influenza components between cells potentially showing the opposite phenomenon is also possible [246].

Short hairpin RNAs (shRNAs) can also modulate genes involved in exosome biogenesis. It has been shown that shRNAs targeting the HRS, TSG101, ALIX, and VPS4 genes involved in the endosomal sorting complex required for transport (ESCRT) can modulate exosome excretion, the first three genes decrease exosome production while the last increases vesicle production [47]. shRNAs delivered via liposomes directly into the lung provided a second level mechanism to regulate exosome production while leaving pores unaffected. The two methods would allow differentiation between whether the CX43 pore is critical or whether the virus has to be encased inside the exosome. While using siRNAs or shRNAs encased in liposomes would likely have higher transfection efficiency. This approach has been shown to be efficacious with poorly soluble antibiotics as was shown when rifampin was delivered in liposomes via nebulization achieved a higher dose in the lung tissue for an extended duration compared to rifampin given orally [247]. As such the inclusion of small RNAs in liposomes delivered via inhalation would appear to have the most promise for direct application of exosome trafficking studies in vivo.
Finding miRs that affect terminal differentiation of epithelial progenitor cells on the basolateral side of tracheobronchial epithelial cells (TECs) has several implications relative to \textit{in vivo} influenza infection. This would imply that the infected cell(s) or their neighbors, are undergoing differentiation to prevent further spread of the virus via a differentiated cell type which is typically more immunocompetent than the undifferentiated progenitor cells [59]. Thus, virus reassortment may be more likely to occur in undifferentiated cells due to enhanced permissivity to infection with multiple virus and/or viral components like vRNP complexes. Experimentally, we have also observed enhanced cell death using undifferentiated cells compared to fully differentiated cells where virtually no cells die within the first 24 hours of infection (unpublished data). While some of this may be explained by less virus penetration due to mucous on the apical surface causing less virus binding in the fully differentiated cells, increasing the viral dose used for the infection by a factor of 10-100 (from 0.01 to 0.1 to 1 MOI) did not yield enhanced cell death in the differentiated cells. This is circumstantial evidence for the enhanced immunocompetency of the fully differentiated cells and may also relate to their ability to use other virus killing mechanisms like dual oxidases [60, 248] which have been shown to have altered expression depending on cell differentiation status.

Others have shown with H7N7 influenza that knocking down Dicer processing of miRNA via siRNA led to increased viral replication [103]. However, knocking down miRNAs via RNA-silencing of Argonaute (1, 2, or 3), Dicer, or Drosha yielded no change in influenza replication \textit{in vitro} (unpublished data).
although the same process was utilized for RSV where we observed substantial decreases in viral titer. These results indicate that attempting pan-miRNA knockdown is not likely to be successful with H1N1 influenza in vivo, unless the observed results were due in part to knocking down a miR essential to a differentiation trait that is protective against influenza even in undifferentiated cells in vitro which seems unlikely.

**Interferons and Influenza**

IFNs do not appear to be limited to specific bacteria or viruses. However, relative to influenza virus, type I and III IFNs have been shown to be the first line of defense at the point of infection in the respiratory epithelium [56, 231, 249]. This is interesting since both type I and III IFNs share many functional similarities [250]. Also of interest are species differences seen with regards to anti-viral responses specifically related to IFN. Our laboratory has previously shown much higher levels of Type III IFNs and IFN-stimulating genes (ISGs) in human bronchoepithelial cells compared to swine bronchoepithelial cells [56]. This delayed response to influenza infection is perhaps a reason swine replicate IAV so well in vivo.

Type III IFNs are not only important to epithelial cells, but also as immune modulators. IL-28B has previously been shown to be an important regulator of Th1 versus Th2 response during influenza vaccination and infection [251]. Additionally, type III IFNs have shown robust activity against encephalomyocarditis virus and vesicular stomatitis virus delivered as part of a
vaccine vector system, yet have shown very limited activity against herpes simplex virus-type 2 (HSV-2) even though IFN inhibited both EMCV and HSV-2 [231, 252]. Additionally, IFN-induced transmembrane protein 3 (IFITM3) has also been shown to limit influenza infections [253]. This appears to occur via inhibition of hemifusion via the alteration of cellular membranes, or possibly by redirecting the infectious virion into a multivesicular body for release inside an exosome [4]. This is important since IFITM subtypes and gene expression levels vary across species with influenza infection upregulating IFITM1 levels in swine rather than IFITM3 including humans [254]. Others have shown that IFITM3 may actually be transferred via exosomes and could explain some of the exosome mediated antiviral effects discussed previously [255]. Given that IFITM3 alone has been shown to prevent the emergence of influenza virus in the cell in the presence of IFN, it would seem that low levels of IFITM3 in swine may predispose them to influenza infection [256].

**miR 29**

MiRs are short, non-coding RNA molecules that exhibit a high degree of sequence and function conservation across multiple eukaryotic species [85, 86]. MiRs bind to a “seed” site (2-8nt, Figure 1) that is a complementary miR recognition element (MRE) in the 3’ untranslated (3’-UTR) of its target RNAs causing messenger RNA decay, or blocked translation. Individual miRs can potentially regulate multiple genes and are global rheostats of gene expression [88]. The theater of miR activity and function is extremely diverse; miRs have
roles in stem cell differentiation \textit{in vivo} [87], development and metastasis of cancer. The current version of mirBase [257] shows that the canine genome also encodes 453 miRs, and most of these show a high degree of sequence identity with human miRs. In addition to their intra-cellular roles, miRs are also exported out of cells in exosomes. These exosomes protect miRs from degradation enabling their detection in biofluids such as serum and enable use of the miRs as disease biomarkers. For example, the expression of miR-4276, as well as several other miRs, has been shown modulate the expression of cytochrome c oxidase which can help respiratory epithelial cells resist IAV infection [109, 110]. Other miRs have been shown to induce or interfere with IFN production, modulate NF-kappa B signaling, modulate immune responses via antigen binding to Toll-like receptors [111-116]. The immune altering effects of miRs have been observed in multiple species indicating this is a genetically conserved messaging system that has been retained across many species [118-124].

MiR-29b is part of the miR-29 family cluster located on chromosome 7q32.3 and 1q32.2 in humans (miRbase.org). Mature miR-29b is 100% identical in sequence among several mammalian genomes, and thus is predicted to regulate similar patterns of genes post-transcriptionally. Analysis of miR-29b pathways shows that miR-29b is predicted to and shown to regulate genes such as vascular endothelial growth factor (VEGF) that are critical for cell growth [186]. Others have shown that miR-29b also impairs cancer stem cells (CSCs) involved in OSA growth and chemotherapy resistance via the suppression of their stem cell-like properties [185]. MiR-29b expression is thought to be aberrant in the
majority of human cancers in that its primary function is tumor suppression and
subsequent to its reduction during cancer development, tumor suppression is
decreased throughout the host [258]. Equally as important, the miR-29 family
[258, 259] has been shown to revert improper host DNA methylation events
induced by many cancers and other prolonged stress events which is critical as
many of the genes methylated by the cancerous lesion are directly involved in
tumor suppression thereby enabling the cancer to escape traditional host cancer
detection and elimination mechanisms [260]. This may be due in part to the
complementarity of miR-29b with the 3'-UTRs of DNA methyl transferase 3A and
3B, both of which are thought to be involved in increased methylation patterns
seen with certain cancer types, including lung cancer [260]. MiR-29 could be
used as an adjuvant for those with a decreased ability to differentiate T cells, and
who instead possess populations of highly differentiated T cells [261]. Pre-clinical
studies need to be performed. Use of miR-29 as an adjuvant in these individuals
may promote more normal T cell maturation especially in conjunction with a cell
cycle promoter such as miR-17-92 [84].

Suppressing miR-29 is likely to negatively affect vaccine efficacy, and lead
to an increase cancer development to a loss of inhibition of DNA methylation
[259, 260]. Increasing miR-29 expression in the general population would
improve the efficacy of a killed vaccine via increased T cell differentiation with the
exception of the elderly where the opposite effect would be seen. Increasing
expression with a live vaccine would most likely reduce immunity via the
production of increased amounts of IFNs leading to an enhanced non-specific immune response prior to the development of a solid adaptive immune response.
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CHAPTER 3
THE ROLE OF MICRORNA AS MESSENGERS IN BRONCHOEPITHELIAL
CELL-DERIVED EXOSOMES DURING INFLUENZA A VIRUS (IAV)
INFECTION

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Abstract

The role of exosomes as cellular messengers and in cellular trafficking has become better understood from that of a generalized cellular waste removal apparatus to that of a more specific intercellular signaling mechanism. To determine the role of exosomes and microRNA (miR) signaling during IAV infection, a miR panel was used to screen, identify, and evaluate exosome-associated miR expression patterns. Expression of total miRs was evaluated via the use of a novel P19 protein-based staining technique. Additionally, basally-derived miRs were assessed for their potential to act as signaling agents via their addition to undifferentiated cells, with changes in miR content evaluated in recipient cells as well as basally secreted exosomes from the recipient cell population using PCR and next generation sequencing. Our studies indicate that exosomes act as carriers for the miRs, which are vital in cellular communication.
Introduction

Several studies have evaluated miRs during IAV infection both in vitro and in vivo. Many studies revealed different findings relative to overall miR expression. The pattern and tempo of exosome-associated miR expression during IAV infection remains to be explored, as does the effect of exogenous miRs on endogenous miR expression during viral infection. Importantly, the role of miR-containing exosomes from IAV-infected cells needs to be explored to determine if these affect adjacent cells via a miR signaling pathway.

Exosomes are small (30-150 nm) vesicles derived from the inward budding of cellular compartments producing multi-vesicular bodies (MVBs). These MVBs fuse with the plasma membrane causing the vesicles inside the MVB to be released as free exosomes [1, 2]. Once released from the plasma membrane, the exosomes can travel freely throughout the host system affecting a multitude of host physiological, signal transduction, and cellular responses [3, 4]. Exosomes can carry a variety of contents taken up from inside the cell such as nucleic acids, proteins, lipids, etc. and have also been found to mediate immune effects such as the inducement of interferon (IFN) α activity [5] via a signaling role once released from the host cell [1, 3, 5, 6].

Interestingly, serum miRs have been found in exosomes further indicating their biological significance [7]. For example, miRs contained in exosomes can have transcription level effects at multiple locations in the genome. As such, exosomes serve not only as a signaling system, but also as important effectors in
maintaining overall cellular fitness during stress, viral infection, and other cellular destabilizing events [8].

The ability of exosomes to pass from one cell to another appears to be one mechanism by which they are able to transport their cargos intracellularly. Exosomes have been shown to transduce cells and do so in minutes versus the traditional laboratory transfection procedures that typically takes hours [9]. This rapid movement into recipient cells indicates a key role in cell-to-cell signaling, although the mechanism by which this signaling occurs remains unclear.

miRs are small, non-coding single-stranded RNA molecules 18-23 nucleotides in length which function as regulators of host gene expression in eukaryotic organisms. They are one of the most abundant gene families and are evolutionarily conserved [10, 11]. Additionally, miRs exist across multiple phyla in the animal and plant kingdoms showing evolutionary conservation as a gene expression regulatory system [11]. miRs have also been found to have an important role in cell differentiation [12]. miR dysregulation can occur via up- or down-regulation of miR expression with only small changes in relative expression leading to large changes in protein expression. Also, miRs have a role in cellular homeostasis [13].

miRs are detectable in serum, plasma, tissues, and other clinical samples, and have been shown to directly modulate the immune system, as well as have a role in regulating the host response during viral infections. For example, the expression of miR-4276 has been shown to modulate the expression of cytochrome c oxidase which can help respiratory epithelial cells resist IAV infection [14, 15].
Other miRs have been shown to induce or interfere with IFN expression, modulate NF-kappa B signaling, and modulate immune responses via antigen binding to Toll-like receptors. [16-21]. The immune regulatory effects of miRs have been observed in multiple species [22-28]. Others have shown that with enteroviral infection, inhibiting miRs can restore IFN production, and limit Enterovirus-induced cell death [17]. Additionally, herpes simplex virus 1 has been shown to be associated with exosome export and miRs to neighboring uninfected cells [29].

IAV is a single-stranded, negative-sense, segmented RNA virus in the Orthomyxovirus family. Three of the six genera in the Orthomyxoviridae family include the Influenza A, B, and C variants. IAV is further subdivided based on hemagglutinin (HA) and neuraminidase (NA) receptor types with 18 HA and 11 NA variants that can be combined to yield unique viruses. Evidence suggests that the IAV can modify the host immune response to infection which may contribute to immune evasion and viral persistence. Studies with several IAV variants have shown host miR expression following infection can affect immune outcomes.

The 2009 IAV pandemic virus A/California/04/2009 (H1N1) is a well-studied strain and much is known regarding miR expression induced by both the host and virus. In one study it was shown that following infection in A549 cells, the upregulation of 69 miRs and downregulation of another 13 miRs was observed in a microarray analysis [30]. Differential miR expression has also been demonstrated in mice infected with the 2009 pandemic virus [31, 32], as well as
in A549 human lung epithelial cells and human serum samples infected with different IAV strains [25, 30, 33].

Additionally, altered miR expression occurs in swine infected with an H1N2 IAV as well as during respiratory bacterial infections [22, 23, 34]. Differential miR expression patterns in swine have also been observed with other IAV strains [35]. Additionally, over 50 human and swine host-encoded miRs, as well as over 200 other host cofactors, induced during IAV infection have been identified [36-38]. Together, these findings suggest miR expression can be used as biomarkers of influenza.

miRs are useful in determining how viruses may interact with host cells during and after infection, and if the viral infection induces pro- and/or anti-viral miR responses. miRs may also be useful in therapeutic approaches for improving multiple outcomes such as modulation of host immune responses during vaccination.

miRs also have the potential for use as therapeutic agents. miR-29c is induced by IAV infection and has an important role in regulating apoptosis, and thus could serve as a drug target [39]. Related to the use of miRs as therapeutic agents is P19 binding protein, a tombusvirus-derived protein [40] that is able to suppress RNA silencing, as well as bind miRs [40, 41]. Since P19 binds specifically to short 21-23 nucleotide double-stranded RNA sequences, its use to block all miRs if transfected into cells and/or viruses has interesting implications [42]. Additionally, P19 has also been shown to increase virus and miR production
in viral culture systems via complete miR silencing limiting the potential effects of any anti-viral effects induced by miRs generated by the infected host [40, 41, 43].

The relationship between exosomes, miRs, and host responses has not been fully defined for IAV. Hepatitis C virus (HCV) has been shown to initiate an IFN-mediated response in human liver cells and induce the formation of exosomes with antiviral activity against HCV by those same cells [44]. Others have shown that this pathogen/exosome relationship is not limited to viruses in that *Mycobacterium tuberculosis* (*M.tb*) has also been shown to generate exosomes which may allow it to suppress the host immune response [45].

Given the unique abilities of exosomes to capture cellular mediators and transport them outside the cell, they represent an opportunistic target that can be used to better understand host-virus interactions affecting pro- or anti-viral signals affecting neighboring cells. This study sought to elucidate key features of the exosome-miR relationship in cell lines infected with IAV, as well as determine if exosomal miRs could regulate IAV-infected cells or non-infected cells. It also sought to demonstrate species differences in exosomal miR expression patterns that may determine the limited immune response to IAV infection seen in swine compared to the robust response typically seen in humans using an *in vitro* model.
Materials and Methods

Cell culture and viruses

Calu-3 cells (ATCC HTB-55) were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose supplemented with 10% heat-inactivated fetal bovine serum for growth at an air-liquid interface (ALI) using 1 mM l-glutamine, 1 mM HEPES, and 1× nonessential amino acids, and were cultured at 37°C with 5% CO₂. Differentiated Calu-3 and NHBE cells were cultured at ALI for four weeks once confluent. For all respiratory epithelial cell lines utilizing FBS, cells were switched to exosome-free FBS (System Biosciences) 24 hours prior to infection with IAV. Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34) for plaque assays were cultured in DMEM containing high glucose (HyClone) supplemented with 5% HI-FBS at 37°C with 5% CO₂.

Culture of NHBE cells in an ALI system was performed as previously described [46]. Normal swine bronchoepithelial (NSBE) cells were grown in DMEM with 5% FBS using epithelial cells harvested from lung tissue from transplant-approved swine (Springpoint Project). NHBE cells (Clonetics) were seeded into T75 tissue culture flasks at a density of 500 cells/cm² and incubated at 37°C with 5% CO₂ until 80% confluency was reached. Cultures were then dissociated with trypsin and moved to an ALI transwell system via the seeding of NHBE cells (passage 2) at a density of 2x10⁴ cells/cm² in each well. The transwell membranes were coated with rat-tail collagen, type I prior to seeding with cells. Cells were grown under liquid culture media for 2-3 days until they were visually confluent under a light microscope. Subsequent to confluency, cells
were used for infections in an ALI monolayer was established via the removal of the apical culture media, and the addition of basal culture media only from that point forward. The apical surface of the cells was exposed to a humidified 95% air, 5% CO2 environment. Media in the basal chamber was changed at 2-3 day intervals, and apical layers were washed with Hank’s balanced salt solution.

Undifferentiated cells were cultured until 100% confluency regardless of cell-type. 24 hours prior to initiation of IAV infection both the apical and basal wells were washed twice with PBS. The basal medium was replaced with 10% exosome-free FBS/DMEM, 5% exosome-free FBS/DMEM, and NHBE-specific media used for Calu-3, NSBE, and NHBE, respectively. Regardless of cell type, exosome-free media was used starting 24 hours prior to IAV infection through the duration of the infection with basal medium collected at 24 hour intervals for exosome collection and replaced with new exosome-free media.

A/California/04/09 (huH1N1) was cultured in embryonated chicken eggs and harvested as previously described [47]. Allantoic fluid was then used to infect MDCK cells to generate virus stocks as previously described [48]. The MDCK cells used for virus passaging were grown DMEM containing high glucose supplemented with 1 mM l-glutamine with 1-μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin) for both passages. Viral stocks were centrifuged for 4 minutes at 1200rpm to clarify virus stocks. Viral stocks were quantified using plaque assays as previously described [49].
**IAV infections and virus plaque assays**

IAV infections used cell lysate prepared via sonication using a scaled down version of a previously described method [50] after removal of the cells from the flask with a cell scraper. Additionally, 3% BSA Fraction V (Gibco) was added to all undifferentiated cell infections to help preserve monolayer integrity. IAV infections were performed at a multiplicity of infection (MOI) = 0.1 or 1.0 using A/California/04/09 (huH1N1). At 24 hrs. post-infection (hpi), basal supernatants were harvested and stored at −80°C for plaque assays and exosome isolation. For differentiated cells the apical layer was washed twice with 500uL of PBS, pooled, and frozen at -80C for plaque assays. Apical and basal supernatants (or apical PBS wash for differentiated cells) were serially diluted from $10^{-1}$ to $10^{-6}$ on MDCK cells. The supernatants were allowed to adsorb to the cell monolayer for 1 h. Cells were rinsed three times with phosphate-buffered saline (PBS) and overlaid with a 1:1 mixture of 2% agarose and 2× overlay medium: 2× minimum essential medium (MEM) (Invitrogen) supplemented with 0.3% NaHCO₃, 4 mM l-glutamine, and 0.4 M HEPES. The plates were incubated for 72 h at 37°C with 5% CO₂. After 72 hpi, the cells were washed twice with PBS, fixed with 80:20 methanol:acetone, and stained with crystal violet in order to quantify plaques.

**Exosome isolation and transduction**

Exosomes were isolated from basal media using ExoQuick TC (System Biosciences) for tissue culture media and for swine sera samples per the
manufacturer’s instructions. Briefly, samples were centrifuged at 1200xg for 5 minutes to remove debris, then Exoquick TC™ was added to each of the samples in a 1:5 volume:volume ratio for each experiment. Once the Exoquick TC was added, the samples were mixed by inversion 5-6 times and the samples were refrigerated overnight at 4°C. Samples were then centrifuged at 3000xg for 30 minutes at 4°C. For experiments involving only RNA isolation, the exosome pellet was stored at -80°C until RNA isolation was performed.

**Exosome transfection/Interferon stimulation**

For transfer experiments, the exosome pellets were immediately resuspended after isolation in DMEM containing 0.001% of a mixture of 4 monoclonal A/California/04/2009 (H1N1) HA antibodies (BEI Resources) to bind any free virus, and allowed to incubate for one hour at room temperature. The exosomes were then added to recipient cells after removal of the apical media, and two PBS washes of the cell monolayer to remove any residual exosome-free FBS. The exosomes were allowed to naturally transfect into the recipient cells by addition to the apical well (of undifferentiated cells) and allowed to sit for 24 hrs. Additionally, parallel recipient sample wells were treated with 100 ng (100-500IU) per well of human recombinant interferon λ (R&D Biosystems).

**P19 staining of miRs**

The transwells were washed twice with PBS, then fixed overnight with 10% formalin by adding to the apical and basal chambers, permeabilized with
Triton-X 100 for 15 minutes, and washed three times with PBS. The permeabilized cells were then blocked with blocking buffer (PBS with 3% BSA/0.5% Tween 20) for one hour. P19 with a chitin-binding domain (New England Biolabs) was then added to each well (100uL per well) after adding it to PBS with 0.5% Tween 20. After washing, 100uL of polyclonal IgG antibody against the chitin-binding domain (MBL International) was added to each well. Subsequently, 200uL of 0.001% red fluorescently labeled anti-goat IgG antibody (Thermofisher) diluted in blocking buffer was added to each well. This combination fluorescently labeled miR fixed within the cells. NP fluorescent staining for the influenza NP protein was then performed with an anti-IgG utilizing a red fluorophore conjugated to an anti-murine IgG antibody (Life Technologies). Finally, the cells were stained with DAPI to identify individual cells. Due to the variable mucous coverage on the cells (especially the differentiated cells) the fields had to be scanned for mucous-free areas for observation of the underlying cell layers.

RNA isolation

RNA was isolated from exosome samples and transwell cell monolayers using RNAzol®RT (Molecular Research Center) following manufacturer’s instructions. Briefly, 1 mL of RNAzol®RT was added per exosome pellet (2mL per cell monolayer) with each exosome pellet isolated from 0.5 mL of basal media sample, and allowed to sit at room temperature for 15 minutes. The pellet or monolayer was further homogenized via pipetting up/down several times to
dissociate any large particulates. 0.4 mL of water per 1 mL of RNAzol® RT was added to the resulting homogenate. The homogenate/water mixture was briefly vortexed to mix, and then held at room temperature for 15 minutes. Samples were then centrifuged at 12,000g for 15 min at 4°C. Centrifugation pellets DNA, protein, and water-soluble polysaccharides, at the bottom of the tube in the aqueous phase while the RNA remains suspended in the organic phase at the top of the sample.

One milliliter of the organic supernatant was then transferred to a 2 mL microcentrifuge tube. RNA was precipitated by mixing 1 mL of the supernatant with 1 mL of 100% isopropanol and 1 µl of polyacryl carrier (Molecular Research Center, City). Samples allowed to sit at ambient temperature for 10 minutes and subsequently centrifuged at 12,000g for 10 min at 4°C. The resulting RNA pellet was washed twice by resuspending the pellet in 75% (v/v) ethanol, and then centrifuged at 3,000g for 2 minutes. The remaining alcohol solution after each wash was removed using an aspirator, and the RNA pellets further dried using a vacuum centrifuge concentrator (Eppendorf). The resulting RNA pellet was resolubilized in 20 µl of RNase-free water, with total RNA concentration measured using a Nanodrop spectrophotometer. Samples were then frozen at -20°C until used for cDNA synthesis.

**miR primers for miR screen**

Human-specific miR sequences were obtained from miRBase for the miRNAs of interest with the panel focused on miRs that have a potential
biological role affecting aspects of the respiratory tract or viral replication. Forward primers were designed based on the mature-miR sequence, and synthesized by Integrated DNA Technologies (IDT). The human-specific miR primer sequences are shown in Table 3.1.

**Quantitative RT-PCR for miRs**

miRs were first converted to cDNA via the 1st-Strand cDNA Synthesis Kit (Agilent Technologies) to polyadenylate the miRs at the 3’ end of the total RNA samples. Reverse transcription converted the polyadenylated miR to cDNA using the same kit. PCR reactions were carried out using 0.5 μL of forward and reverse primer for each reaction. Reaction parameters included an initial denaturation step at 95°C for 5 minutes, followed by 40 amplification cycles each of which included denaturation at 95°C for 15 s and annealing of 20°C for 20 s. Subsequently, cDNA was detected using Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies) with a Stratagene MX3005P real-time PCR instrument to quantify the expression levels of each of the 94 forward miR primers indicated in Table S1. Universal reverse primer (Agilent Technologies) was used for all reactions. All miR levels were normalized to human-specific U6 miRNA cDNA (Agilent Technologies) expression. Values are represented as fold change in expression relative to mock treatment unless noted.
Swine sera

Swine sera were obtained from a swine vaccination study where swine were vaccinated with a single dose of commercial swine CA 04/09 pandemic vaccine or a PBS sham vaccination 21 days prior to challenge with homologous virus. Blood was collected at the time of infection as well as 24 hrs. post-infection via jugular venipuncture. Samples were allowed to clot, then spun at 1,500xg for 15 min. to separate sera from the clot. Sera samples were then aliquoted into 1mL volumes and frozen at -80°C to preserve exosome integrity. Exosomes were isolated from serum using Exoquick (System Bio) with the pellet then used for RNA isolation using RNAzol RT as was done for other exosome samples.

Next-Generation sequencing

Total RNA from sample will be isolated using RNAzol reagent (MRC gene) as noted previously and fractionated using manufacturer’s protocol. Exosomal RNA samples from the donor cells or cellular RNA from the recipient cells were pooled with 2-3 related samples to maximize RNA input. Small RNA collected from both cell types was quantified using a QuantiT miRNA quantitation assay, a sensitive fluorometric assay for RNA quantitation and validated for size distribution using an Agilent Tapestation 2200 analyzer. Quantified RNA was ligated to 3’ and 5’ adaptors using a deletion mutant and wild type T4 RNA Ligase (Epicenter/ Illumina respectively). Ligated RNA was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen) per the manufacturer’s protocol.
First strand cDNA obtained in the previous PCR was subsequently bar-coded using sample specific indices that enable later read quantitation. Indexed PCR products were pooled resolved on 6% polyacrylamide gels along with custom RNA ladders containing 147 and 167 nt long sncRNA specific bands and products corresponding to these sizes were cut out and eluted from the gel. These products constitute the cDNA libraries for sequencing. Products were precipitated, reconstituted and denatured into single stranded pools before sequencing by synthesis on a small RNA chip on an Illumina Miseq instrument. Data files were exported as FASTQ reads and analyzed on a Linux computing cluster with a total computing power of 25.6 peta flops using available open source software and custom pipelines. Reads were finally compared to custom human databases of miRs, tRFs, snoRNAs, and piRNAs.

**Animal use statement**

All animal studies were conducted under approval by the Animal Care and Use Committee of the University of Georgia, an institution accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC).

**Statistical analysis**

Fold-change in miR expression over mock-infected controls was calculated using the standard $2^{\Delta\Delta Ct}$ equation. Fold-change values were compared to one another using one-way ANOVA or Student’s T test (in vivo
swine experiments) in GraphPad Prism 5.01 (La Jolla, CA). Results were considered significant when the P value was less than 0.05. Data are shown as mean±SEM of two or more experiments done in triplicate unless otherwise noted. NGS data consisted of pools of 3 RNA samples per group and were analyzed by both miR reads and fold change in normalized miR reads.

Results

Calu-3 cells infected with A/California/04/2009 (H1N1) at an MOI of 0.1 or 1.0 were examined polarized, and differentiated over a 72 hour period to determine if there was any loss of cell monolayer integrity that would lead to leakage of the basal well onto the apical surface which could allow apically-derived exosomes, virus, to enter the basal compartment of the transwell. Monolayer integrity was verified by minimal change (-25 Ω/cm² or less in all cases) in transepithelial electrical resistance (TEER) readings as well as visible leakage into the apical well.

Initial experiments with differentiated Calu-3 cells at ALI 24 hrs. post-infection with A/California/04/2009 (H1N1) at 0.1 MOI (Fig. 3.1A) revealed miR patterns induced by IAV infection. Several differences in miR expression were observed, specifically (Fig. 3.1). Subsequent experiments at MOIs of 1.0 and 0.1 (Fig. 3.1B) showed 24 hours pi as optimal for exosome collection (Fig. 3.1) as it consistently showed miR dysregulation points. Fully differentiated Calu-3 cells (Fig. 3.1C) compared to differentiated NHBE cells (Fig. 3.1C) showed that Calu-3
cells could serve as a proxy to NHBE cells. However, some differences in miR expression were observed.

miR expression in mock-vaccinated and vaccinated swine infected with a homologous A/California/04/2009 (H1N1) virus at 24 hrs. post-infection was also observed (Fig. 3.2A, B, C). miRs -29a, -let-7g-5p, and -150-5p were all upregulated in vaccinated swine, while miR-154-5p (Fig. 3.2D) was upregulated in mock-vaccinated control animals (2 pigs per group). While the results for the first three miRs were not statistically significant due to the minimal number of swine available, these results correlated with miRs expression patterns observed in vitro for NHBE cells proposing their potential use as biomarkers for vaccination.

To elucidate the total miR expression occurring, P19 was used to identify miRs in IAV-infected cells. A549 cells (Fig. 3.3A) were used because of the heavy mucous layer present on undifferentiated Calu-3 cells which precluded effective washing of the cell monolayer during staining, and subsequently produced large areas of fluorophore aggregation in the mucous preventing observation of the underlying cell layer. Relatively little miR staining was observed in the mock-infected cells (Fig. 3.3). However, for IAV-infected cells, the influenza infection does cause miR expression (green).

The studies were repeated in NHBE cells to confirm whether the sequestration of miRs occurred in fully differentiated cells. While baseline miR expression was higher in the mock-infected cells than A549 cells, there was an increase in fluorescence in both IAV-infected samples. The miRs were broadly
distributed throughout the cells; however, miR expression was not detectable in every cell, nor was it limited to virus- or non- virus infected cells. This indicates that viral infection induces miR production in virus-infected cells and neighboring cells.

With the differences observed in miR distribution, we examined IFN signaling as mimicked via exosome transduction in recipient cells (Fig. 3.4A). Cells were infected with IAV, UV-inactivated IAV, or mock infected with cell lysate. Exosomes were collected and combined with polyclonal anti-IAV antibody to bind virus that may have entered the basal well. The exosomes were then added apically to uninfected cells of the same cell type, as well as an IFN-lambda control well to determine if exosomes could mediate the same effect on recipient cells with respect to selected miR levels. miR-154-5p and miR-29a-3p (Fig. 3.4B). The results suggest there is a poor miR response in NSBE cells compared to the other cells types.

Studies were also performed with differentiated Calu-3 cells with the basal exosomes transferred to undifferentiated Calu-3 cells to determine if the results mimicked undifferentiated cell-to-cell transfer. As shown in Fig. 3.4, miR-154-5p and miR-29a-3p levels were higher in the donor cells, were decreased for the donor exosomes, and are not observed in the recipient cells. This is contrary to that observed in undifferentiated cells suggesting cell differentiation state may have a role in exosome cargo and transduction.

Next generation sequencing (NGS) was used to analyze the donor exosome and recipient cell RNA from several pooled RNA samples as shown in
Fig. 3.4. Pools were used to maximize the number of samples that could be sequenced and generate higher numbers of reads due to the limited amount of RNA present in the exosomal RNA samples compared to the cellular RNA samples. Interestingly, the number of total reads (Fig. 3.6A) was remarkably similar to Calu-3 (C), NHBE (H), and NSBE (S) samples (I=infected, M=Mock, D=donor exosomes, R=recipient cells, λ=IFN recipient cells). Of particular interest is that the infected recipient, mock donor, and IFN-recipient cells are all nearly equal for each cell type while the infected donor exosome and mock recipient cell RNA samples are lower, but also very similar in total reads. Unique miRs per sample were also evaluated (Fig. 3.6B) with the infected donor exosomes and mock donor exosomes. The miR read data was normalized to eliminate any biases in miR ratios due to RNA amounts in the samples, results summarized in Figure 3.7. Based on this data, fold-change data based on miRs absent/present in the samples and the likelihood they could induce IFN were evaluated based on the IFN-treated cell miRs.

A heat map showing the miR diversity among the cell lines treated with IFN λ was constructed in order to identify miRs that were specifically induced by IFN and to compare to miRs in the IAV-infected exosomes and their recipient cells (Fig. 3.8). The heat map reveals many of miRs absent in the swine cells (gray color). Figure 3.9 shows miRs specifically upregulated in IAV-infected NHBE exosomes; however, only miR-3168 was upregulated in both the donor and recipient exosomes for NHBE cells.
Several miRs were downregulated in IFN λ-treated NSBE cells with miR-30-e-5p (Fig. 3.10A) and miR-183-5p (Fig. 3.10B) downregulated in both exosome-treated recipient cells, and IFNλ-treated cells. Additionally, miR-487a-5p and miR-487b-5p were downregulation in IFN λ-treated cells, while NSBE and Calu-3 cells exhibited upregulation of the same miRs (Fig. 3.8C and 3.8D). Interestingly, miR-130b-3p (Fig. 3.11A), miR-301b-3p (Fig. 3.11B), and miR-6073 (Fig. 3.11C) were highly upregulated in NHBE cells treated with exosomes from IAV-infected cells or IFN λ implying that these may be critical for IFN responses in the cell. miR-6073 is of particular interest as swine cells downregulated this miR when treated with IFN λ.

Furthermore, miR-423-5p (Fig. 3.12A), miR-3184-3p (Fig. 3.12B), and miR-7161-5p (Fig. 3.12C) were upregulated in NSBE cells that received exosomes from IAV-infected donor cells. This may imply these miRs were specific to viral infection in swine cells, since they were absent or far less upregulated in the other cell lines. Other anomalies with regard to miR expression in NSBE cells that were observed were miR-7157-5p (Fig. 3.13B) and miR-16-5p (Fig. 3.13A) were both exhibited modest increases in IFN λ treated cells compared to the other two cell lines. However, miR-29a-3p (Fig. 3.13C), the miR evaluated in the PCR experiments, was conspicuously absent in Calu-3 cells. Also of interest was that miR-362-5p (Fig. 3.14A) and miR-6809-3p (Fig. 3.14B) both exhibited upregulation in IFN λ treated NSBE cells, but downregulation in NHBE cells.
Discussion

Our data showed wide variation in the pattern and tempo of exosomal miR expression based not only on experimental conditions, but also on cell type. This highlights the difficulties of assessing significant miRs. It also highlights the need for miR studies to include as many relevant cell lines as possible, especially when evaluating exosomal miRs as differentiated cells express different exosomal miRs than non-differentiated cells.

While the number of swine serum samples examined with the screening panel was limited, the data indicated several miRs of interest for both vaccinated and non-vaccinated swine as potential biomarkers. Additional swine studies to increase numbers and evaluation of a more diverse genetic background in the swine would help with interpretation of the findings is need as the swine used were highly inbred. Also, identifying the host cells expressing the miRs following IAV infection is important. The P19 method is a useful and novel mechanism to identify total miR expression in both infected and uninfected cells. While not suited to identifying a particular miR, it does allow one to establish which cells are expressing the majority of the miRs which is key in understanding differences between pro-virus and pro-host/anti-viral miRs.

We have demonstrated cellular signaling occurs following IAV infection via cellular transfer of exosomes. Interestingly, miR-154-5p and miR-29a-3p for the PCR-based signaling experiments were not evident in the NGS fold-change evaluation which is likely due to the promiscuity of the PCR primers versus the
NGS primers which are highly-specific for only mature miRs. It could also imply the movement of immature miRs via exosomes.

Of interest is the NGS data which revealed the relationship between cellular miRs induced when cells were treated with IFN λ versus when cells were treated with exosomes derived from IAV-infected cells. The miR patterns observed in IFN-treated NHBE and NSBE cells is consistent with previous findings from our lab that swine cells have an aberrant immune response to type III IFN (ref), which appears to be miR-driven in some part.

Exosomal miR expression patterns can differ greatly from the host cell. miR-183-5p may represent an interesting anti-viral target as it can promote cell growth via Fox01 inhibition, it is under-expressed in swine cells treated with exosomes or IFN I. However, this may be related to the dose as well as the type of IFN used (recombinant human) as well differential expression of IFN receptors on the three cell lines (which was not evaluated as part of this study). More work is necessary to elucidate how miRs signal via exosomes, and whether signaling is via homologous miRs only, or can heterologous miRs induce one another.

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Author contributions

Conceived and designed experiments: JH AB RAT. Performed experiments: JH AB LA CK. Analyzed the data: JH AB LA RAT. Contributed reagents/materials/analysis tools: RAT. Wrote the paper: JH AB RAT.
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Figures and legends

A

B

C

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Figure 3.1. Heat maps of exosomes from differentiated Calu-3 and/or NHBE cells show miRNA dysregulation in response to IAV infection. Fully differentiated Calu-3 cells were infected with Influenza A/California/04/2009 (H1N1) at 2 MOIs using either cell lysate or infection media (1A) with exosomes collected at 24HPI and screened for 94 miRs. Differentiated Calu-3s were also infected with exosomes collected at 24, 48, and 72HPI. using MOIs of 1.0 and 0.1 (1B). Differentiated Calu-3 cells were also compared to differentiated NHBE cells (1C) at 24HPI. RNA was extracted from the isolated exosomes and screened via RT-qPCR. miRNA values are shown as fold change in the infected versus the mock infected cells. Data are shown as mean fold change.
Figure 3.2. Dysregulation of swine serum miRs in response to challenge with A/California/04/2009 (H1N1) virus. Two pigs were vaccinated with a single dose of a commercial 2009 pandemic virus vaccine while two others were mock vaccinated with PBS. All four pigs were challenged intranasally and intratracheally with A/California/04/2009 (H1N1). The same panel of miRs was used to evaluate serum exosome samples at the time of infection (T=0 hrs.) and again 24 hrs. post-infection. Several serum miRNAs were identified as potential biomarkers of infection and/or protection with miR 154-5p statistically different (p=0.04) and miR 29-3p trending towards significance (0.08). Data shown are the mean±SEM of the fold change in miR levels for 2 pigs for both the vaccinated and mock vaccinated groups.
Figure 3.3. miRNA expression was induced by IAV infection in A549 and NHBE cells. Images shown at 20x magnification. miRNA expression via P19 labeling varies by MOI in both A549 (A) and NHBE (B) cells (green). A/California/04/2009 (H1N1) virus stained with NP antibody (red) is shown in second column. DAPI staining of cell nuclei (blue) shows cell density. Overlaid images in 4th column depict miR expression in both infected and uninfected cells, with greater expression in NHBE cells.
Figure 3.4. miR 154-5p and miR 29a-3p expression are induced by IAV infection in undifferentiated Calu-3, NHBE, and NSBE cells. Donor cells were infected with A/California/04/2009 (H1N1) with cell RNA and basal exosomes collected 24hrs post-infection (workflow shown in A). Donor exosomes were then allowed to transfect undifferentiated cells for 24hrs, with recipient cell RNA and basal exosomes again collected. RNA was isolated from all cell and exosome populations. Expression levels of miR 154 and miR 29 in both A/California/04/2009 (H1N1) infected, and UV-inactivated treated wells were compared to mock-infected wells.
Figure 3.5. miR 154-5p and miR 29a-3p expression were induced by IAV infection in differentiated Calu-3 cells, but not their exosomes. RNA was collected from infected donor cells, donor cell exosomes, uninfected recipient cells treated with donor exosomes, and recipient exosomes. Expression levels of miR 154 and miR 29 in both A/California/04/2009 (H1N1) infected, and UV-inactivated treated wells were compared to mock-infected wells.
**Figure 3.6. NGS total and unique number of miR reads per pooled RNA sample varied by source.** RNA was collected from donor cell exosomes and uninfected recipient cells treated with either donor exosomes or interferon. miR reads via NGS are expressed as total miR reads per pooled sample (A) as well as total unique miRs per sample (B). Acronyms are as follows: Calu-3 (C), NHBE (H), and NSBE (S) samples (I=infected, M=Mock, D=donor exosomes, R=recipient cells, λ=IFN recipient cells).
Figure 3.7. Venn diagrams show the disparity in response to exosome and IFN treatment relative to mock-infected donors. RNA was collected from infected donor cells, donor cell exosomes, uninfected recipient cells treated with donor exosomes, and recipient exosomes. Total reads were normalized for each miR and then compared for expression among groups differing only by cell type.
Figure 3.8. IFN-induced miRs were less numerous in NSBE cells than in Calu-3 or NHBE cells. RNA was collected from uninfected recipient cells treated with IFN lambda. Significant miR diversity existed between Calu-3, NHBE, and NSBE IFN-treated cells (A) while comparing just the primary cells (B) showed the absence of many miRs in the NSBE cells.
Figure 3.9. Several miRs were enriched in donor exosomes from NHBE cells. RNA was collected from infected donor cells, donor cell exosomes, uninfected recipient cells treated with donor exosomes, and recipient exosomes. Total reads were normalized for each miR and then compared for expression among groups differing only by cell type. All 5 miRs were present in the donor exosome samples while miR-3168 was increased in both the donor exosomes and recipient cells for NHBEs only. Each sample represents a pool of 3 experimental samples.
Figure 3.10. Several miRs were downregulated in NSBE IFN λ-treated cells.

RNA was collected from infected donor cells, donor cell exosomes, uninfected recipient cells treated with donor exosomes, and recipient exosomes. Total reads were normalized for each miR and then compared for expression among groups differing only by cell type. All 4 miRs were present in the donor exosome samples for both infected and mock exosome samples except in the NSBE samples. Each sample represents a pool of 3 experimental samples.
Several miRs were enriched in NHBE cells treated with exosomes from IAV infected cells or IFN λ. RNA was collected from infected donor cells, donor cell exosomes, uninfected recipient cells treated with donor exosomes, and recipient exosomes. Total reads were normalized for each miR and then compared for expression among groups differing only by cell type. All 3 miRs were present in the donor exosome samples in the infected NSBE exosome sample, but not in the Calu-3 or NHBE samples. Each sample represents a pool of 3 samples from the same experimental group.
Figure 3.12. Several miRs were enriched in NSBE cells receiving donor exosomes from infected cells, but not in NHBE cells. RNA was collected from infected donor cells, donor cell exosomes, uninfected recipient cells treated with donor exosomes, and recipient exosomes. Total reads were normalized for each miR and then compared for expression among groups differing only by cell type. All 3 miRs were present in the donor exosome samples in the infected NSBE exosome sample, but not in the Calu-3 or NHBE samples. Each sample represents a pool of 3 experimental samples.
Figure 3.13. miRs were enriched in NSBE cells treated with IFN λ. RNA was collected from infected donor cells, donor cell exosomes, uninfected recipient cells treated with donor exosomes, and recipient exosomes. Total reads were normalized for each miR and then compared for expression among groups differing only by cell type. All 3 miRs were present in the donor exosome samples in the infected NSBE exosome sample, but not in the Calu-3 or NHBE samples. Each sample represents a pool of 3 experimental samples.
Figure 3.14. Two miRs were enriched in NSBE cells treated with IFN λ, but downregulated in NSBE cells treated with exosomes from infected cells. RNA was collected from infected donor cells, donor cell exosomes, uninfected recipient cells treated with donor exosomes, and recipient exosomes. Total reads were normalized for each miR and then compared for expression among groups differing only by cell type. All 3 miRs were present in the donor exosome samples in the infected NSBE exosome sample, but not in the Calu-3 or NHBE samples. Each sample represents a pool of 3 experimental samples.
### Supplemental Information

#### Tables:

**Table S3.1. MiR Human Forward Primer Sequences**

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CHAPTER 4

THE ROLE OF FETAL BOVINE SERUM-DERIVED, EXOSOME-ASSOCIATED MICRORNAS ON MADIN-DARBY CANINE KIDNEY CELL IN VITRO GROWTH\textsuperscript{1}

\textsuperscript{1}Hanson, J, Gilreath, N, Orr-Burks, N, Bakre, A, and R.A. Tripp. Submitted to Journal of Extracellular Vesicles. 6/1/2016.
Abstract

Fetal bovine serum (FBS) is an essential component of cell culture systems. FBS is the most commonly used source of serum, but has several drawbacks including cost, lot-to-lot variation, and ethical concerns related to its procurement. Various types of chemically-defined media are available, but are more expensive and in some cases unable to support cell growth as efficiently as FBS. This study examines whether variations in the pattern of exosomal microRNAs (miRs) found in FBS influence the growth rate of Madin-Darby Canine Kidney (MDCK) cells. We hypothesized that FBS samples contain specific exosome-associated miRs that impact cell growth parameters. Serum samples from several commercial sources were assessed for their ability to alter the growth rate of MDCK cells. The serum samples were evaluated for exosomal miRNA (miR) via RT-qPCR. Several distinct miRs were identified in both the high and low growth serum implying a potential role relative to cell growth characteristics. *In silico* analysis showed several of the identified exosomal miRs are critical regulators of various cell cycle pathways implying the potential to affect cell growth rates beyond other intrinsic growth factors in the serum.
Introduction

Modern cell culture techniques often include the use of animal-derived serum as an undefined media component to provide protein, growth factors, and other molecules necessary for cell growth. Serum, which begins as whole blood from one of several different animal species, has proven to be a reliable, but somewhat inconsistent, cell culture growth component exhibiting lot-to-lot variation in supporting cell growth. Serum is commonly used for *in vitro* cell culture systems and has been derived from a variety of species including human [1], caprine [2], and porcine [3, 4], and bovine sources with fetal bovine serum (FBS) the most commonly utilized supplement.

Numerous ethical issues surround the use of animal-origin serum products which has led to a movement to reduce overall serum usage, or replace it with alternatives [5, 6]. Chemically-defined media that does not utilize serum is among the most common serum free media (SFM) alternatives, and has been used for several cell lines with varying success compared to serum-based media [7-10]. Non-serum based alternatives, such as royal jelly from honey bees [11], and platelet lysates from human [1, 12], equine [13], porcine [14], and canine [15] sources, have also been investigated as alternatives to FBS, but have not been widely adopted due to lot-to-lot variability, and less than optimal cell growth rates. Commercial SFM variants are also not without problems as at least one commercial variety has shown considerably altered phenotypes in both equine and canine stem cell lines compared to traditional FBS-based media [16].
Given the pervasiveness of its usage, a basic understanding of FBS is needed to reduce variability and to increase the robustness of cell culture. Batches of FBS are first collected and processed, then tested for endotoxins prior to undergoing a series of filtering to yield a final product [4]. Historically, endotoxin levels reportedly varied quite widely by manufacturer [17], but little data exists on endotoxin levels in FBS today in part because many manufacturers screen out lots with endotoxin levels that inhibit cell growth. Suppliers may test individual lots of FBS for a variety of features, including chemical/physical composition, microbiological tests, and cell growth performance as part of their quality control process to try to ensure product consistency and minimize lot-to-lot variation [4]. Additional treatments may be applied to the serum, such as heat inactivation (HI), gamma irradiation, dialyzation, charcoal stripping, etc. [4] to further define or enhance the performance characteristics of the FBS relative to cell growth, contaminating virus content, virus viability, immune interference potential, or other potential features that may impact performance.

In addition to essential biomolecules and growth factors necessary for *in vitro* cell culture, serum also contains extracellular vesicles (exosomes) involved in cellular communication, cell differentiation, and cell programming [18]. Exosomes are found in nearly all bodily fluids and have significant regulatory roles on a cellular and system-wide level [19]. As endocytotic vesicles, exosomes are known to carry a variety of proteins, lipids, and RNAs, the latter of which is believed to have an appreciable role in cell-to-cell communication. Exosomes
have been shown to alter cell growth phenotypes [18, 20], impact cell growth [21], affect differentiation of skeletal muscle cells in vitro [22], and inhibit the macrophage response to lipopolysaccharide (LPS) [23]. Others have shown that FBS can change the efficiency by which stem cells can be reprogrammed as well as their proliferation rate [24].

MicroRNAs (miRs) are evolutionarily conserved small non-coding RNA molecules 21-23 nucleotides in length that function in RNA silencing and post-transcriptional regulation of gene expression, and are commonly transported in exosomes. In addition to a transport function, exosomes also protect the encapsulated miRs (and other RNA) from degradation [20]. A recent study has shown that exosomes contain higher concentrations of miRs as compared to extracellular fluid [25], although others have shown that exosomes may contain on average less than one copy of a selected miR per exosome, which makes it less likely that these singlet miRs are physiologically relevant [26]. However, several studies have demonstrated that the presence/absence of exosomes in serum has a substantial influence on cell culture outcomes [20-22]. Additionally, exosomes have been shown to be highly efficient transduction mechanisms, where cell entry occurs in a matter of minutes, surpassing the ability of traditional transfection systems to transmit lipid vesicle contents into the interior of a target cell, which can take hours and occurs at a lower efficiency than that of exosomes [27].

While human platelet lysates have been suggested as the new gold standard over FBS for human-origin cell culture [28], this strategy is not without
similar caveats to FBS use. Platelet concentrates, the source of platelet lysates, have been shown to contain not only exosomes, but also low-density lipoprotein (LDL), which can mimic exosomes and purify with exosomes using commonly employed purification techniques [29]. This co-purification of LDL with platelets can lead to false ratios of exosomal miRs as the LDL molecules will not contain any miRs. Further, platelet lysates contain platelet microparticles, a crude mixture of both exosomes and microvesicles. Others have shown that platelets contain large amounts of miRs as well as miR processing machinery (reviewed in [30]), all of which move forward during the platelet lysate preparation process.

Platelet microparticles have been shown to positively impact angiogenesis [31, 32], as well as induce gene expression in monocytes [33]. Techniques such as activated charcoal sponging [31], heat inactivation [31, 34], and/or addition of antibodies [32], designed to remove growth factors or other molecules contributing to these effects are not completely effective in removing the genotype or phenotype changes implying the existence of a protective mechanism preserving functionality of growth factors, miRs, or other biomolecules capable of inducing both local and regional changes. Given that exosomes use host cell membranes to comprise their exterior surfaces, they make the most logical choice for the vehicle while miRs seem the most likely phenotypic change-inducer given their post-transcriptional effects.

Of interest is the relatively high level of transduction efficiency of exosomes, and consequently the potential for their miR cargo to regulate cell growth characteristics or differentiation status. As miRs are non-coding RNA that
are involved in regulating upwards of 60% of all protein-encoding genes post-
transcriptionally [35], the ability of exosomes to serve as miR transports that then
affect cellular growth, protein synthesis, etc. is extremely relevant to cell culture
given the relative abundance of both exosomes and miRs in FBS as well as other
serum-based media supplements. Furthermore, several miRs in particular have
already been shown to have impacts on cell growth and differentiation including
miR-19a, miR-19b, and miR-26b affecting fibroblast differentiation [36] while miR-
29b has been shown to inhibit osteosarcoma cell proliferation both in vitro and in
vivo [37, 38]. Other studies have tied low in vivo expression of miR-26A and high
expression of miR-27a with enhanced osteosarcoma development in human
patients [39] which is of interest since many cell lines utilized for in vitro
experiments are derived from cancerous tumors, and thus potentially under
similar miR regulatory mechanisms. Although platelet lysates have gained favor
over serum for some cell culture procedures, it is important to understand
intrinsic factors (beyond growth factors) in cell culture media that may impact cell
growth rate and/or phenotype/differentiation. This study aimed to evaluate
commercially available serum products for their protein content and relative
exosome-associated miR profile as well as to determine whether any disparities
therein may contribute to observable differences in MDCK cell growth rates.
Materials and Methods

Initial characterization of serum samples

Materials

Seven serum aliquots were initially obtained (six fetal bovine serum samples and one porcine serum sample) from different commercial sources (see Table 4.1 for manufacturer information). Dulbecco’s Modified Eagles Medium (DMEM) was purchased from Thermo-Fisher Scientific Inc. No antibiotics were used in any of the experiments. All serum samples were thawed, heat-inactivated, and immediately aliquoted into 5-10mL to minimize freeze/thaw and prevent degradation [40]. Individual aliquots were maintained at -20C until use. All FBS samples remained frozen (-20C) since acquired and three FBS specimens had been held in long-term storage.

Protein assay

Protein levels were assessed in each sample using a BCA Protein Assay (Thermo-Fisher Scientific Inc.) Briefly, the samples were serially diluted alongside reference samples following the manufacturer’s instructions. The samples and controls were then measured using the BCA Protein Assay protocol for a NanoDrop spectrophotometer (Thermo-Fisher Scientific Inc.) Sample dilutions were replicated twice for each sample. A standard curve was then prepared and the protein concentration of each sample determined using the curve (sample identification numbers correspond to those listed in Table 4.1).
Cell growth and transfection

Madin-Darby Canine Kidney (MDCK) epithelial cells (ATCC CCL-34) were initially cultured in DMEM growth media supplemented with 5% heat-inactivated FBS (Sigma-Aldrich-Sample #8) at 37°C/5% CO₂ incubator. Initial frozen stocks were passaged 2-3 times to ensure cells were healthy and rapidly dividing prior to use in any experiments. MDCK cells were grown for each experiment in a single T150 flask using the same 5% heat-inactivated FBS DMEM growth media (Sample #8) and allowed to grow to 80-90% confluency to ensure cells from the same flask were used for all plates in the experiment. 96-well flat bottom cell culture plates were used for all cell growth and transfection experiments.

Cell growth experiments

Automated cell count

Initial cell growth was evaluated using approximately 2x10⁴ MDCK cells/well suspended in 500uL 5% FBS (sample 8) in each well of a 24 well plate. After 24 hours, the original media was removed, the plate was washed three times with ~250uL of phosphate buffered saline (PBS), and the media was replaced using 500uL of media supplemented with each of the eight different serum samples (5% serum concentration). 24 hours after the new media was added, the media was removed from the plate, and the plate was washed three times with PBS. One mL of 80:20 methanol:acetone was used to fix each well for 15 minutes. The fixative was promptly removed, the wells dried, and the cells then stained using a DAPI stain solution. After staining, 1 mL of PBS was placed
back in each well. Each well was then counted using a Cellomics Arrayscan VTI HCS Reader with DAPI as the nuclear stain.

**Manual cell count**

A second validation method was used to verify the Arrayscan data, and to evaluate the use of smaller plates to reduce the amount of media required. Instead of using a single media for the initial cell plating as was done initially, MDCK cells were resuspended to a final concentration of 200 cells/mL in separate media containing 5% serum using each of the 8 serum samples, and aliquoted in a 96 well plate using 100uL cell suspension per well. To determine the doubling rate, a subset of wells for each serum (at 24 hour intervals) was washed 3 times with PBS and fixed with 80:20 methanol:acetone, then stained with 100uL of pre-mixed crystal violet stain. Each well was counted manually at 20x magnification using a light microscope, centering the field within the well. Cell totals per 20x field across the 72-hour period were used to determine changes in doubling rate for all eight different media samples.

**siRNA transfection**

siRNA mimics or inhibitors (Dharmacon, GE Life Sciences) of five selected miRs (see Fig. 4.5) were reverse transfected in MDCK cells (20nM in 0.3% Dharmafect1 (Dharmacon). Both a negative control and a non-targeting GFP-labeled siRNA positive control (to verify transfection) were also included. 0, 24h, and 48h time-points for each miR were included in duplicate on each 96-well
plate (experiment repeated 3 times). The 0 hour time point equaled 24 hours after transfection. All wells for each time-point were washed 3 times with PBS, and then fixed with 100µL of 80:20 methanol:acetone for 15 minutes. The fixed wells were then refilled with 100µL of PBS until the end of all experimental replicates on that plate. The cells within each well were counted using Cellomics ArrayScan with DAPI used to stain cellular nuclei.

**Exosome isolation**

Exosomes were isolated from all 8 serum samples in parallel using ExoQuickTM (System Biosciences) following the manufacturer’s protocol. Briefly, 500 µL of Exoquick solution was added to 2 mL of undiluted serum. Samples were gently mixed by inversion 5 to 6 times and refrigerated at 4°C for 30 minutes. The samples were then centrifuged at 1,500g for 30 minutes, and supernatant was removed via aspiration. The remaining pellet was centrifuged a second time at 1,500g for 5 minutes so that any remaining fluid could be aspirated leaving a small beige pellet in each tube.

**RNA isolation and RT-qPCR**

Total RNA was isolated from the exosome pellets using RNAzol RT® (Molecular Research Center, Inc.) following the manufacturer’s instructions. Briefly, 1 mL of RNAzol RT® was added to each tube containing the exosome pellet. 0.4 mL of water was then added to the 1 mL of RNAzol RT® resulting in the RNA homogenate. The homogenate/water mixture was mixed by inversion,
and held at room temperature for 15 minutes to ensure sample dissociation. Samples were then centrifuged at 12,000g for 15 minutes at 4°C, and 1mL of the supernatant was then transferred to a 2 mL microcentrifuge tube. RNA was then precipitated by adding 1 mL of 100% isopropanol to the supernatant along with 1µl of polyacryl carrier (Molecular Research Center, Inc.).

Samples were then held at room temperature for 10 minutes and subsequently centrifuged at 12,000g for 10 minutes at 4°C. The resulting RNA pellets were washed twice by re-suspending the pellet in 75% ethanol, and then centrifuged at 3,000g for 2 minutes. The remaining alcohol solution (after each wash) was removed using an aspirator, and the RNA pellets were then dried to remove any remaining alcohol or water using a vacuum centrifuge concentrator (Eppendorf). The resulting RNA pellets were then re-solubilized in 20µl of RNase-free water. The quantity of total RNA was determined using a Nanodrop™ spectrophotometer.

**cDNA synthesis**

Agilent Technologies miRNA 1st-Strand cDNA Synthesis Kit later used to synthesize cDNA from the total RNA fraction for the assessment of relative miR levels in selected serum samples (see below). To better detect short miR sequences during RT-qPCR, the Agilent protocol elongates miRs via polyadenylation. Following polyadenylation, cDNA can be synthesized by combining the polyadenylated miR and the cDNA synthesis reagents.
miR primers for miR screen

Human-specific miR sequences were obtained from miRBase for the 94 miRs (see Supplementary Table 1 for specific miR name and sequence) used in the initial screening process. Forward primers were designed based on the mature-miRNA sequence, and synthesized by Integrated DNA Technologies (IDT).

Quantitative RT-PCR for miRs

Total RNA was used to generate cDNA using the 1st-Strand cDNA Synthesis Kit (Agilent Technologies) to polyadenylate the total RNA (including the miRs) at the 3’ end. Reverse transcription was used to convert the polyadenylated RNA into cDNA using the same kit. All steps were done per manufacturer’s instructions in a standard 96-well thermocycler. Subsequently, cDNA was detected using the miR primers noted previously and Brilliant III Ultra-Fast SYBR Green qPCR Master Mix kits (Agilent Technologies) to quantify the expression levels of each of the 94 forward miR primers. Universal reverse primer (Agilent Technologies) was used for all reactions. All miR levels were normalized to human-specific U6 miR cDNA (Agilent Technologies). Fold-change values were calculated using the $2^{-\Delta\Delta Ct}$ method using the slowest or fastest growing sample as the control sample as noted in the results section.
**Statistical analysis**

Cell growth and siRNA mimic/inhibitor transfection experiments were performed in at least duplicate and repeated two or three times as noted. Data were analyzed using one-way ANOVA with a Dunnett post-test for individual comparisons (using sample 8 as the reference sample), and are shown as mean ± SEM. Screening PCRs were performed once on each of the selected samples with miRs of interest validated with technical duplicates. Changes in miRNA fold expression values over high/low growth controls were analyzed using one-way ANOVA with Dunnett post-test for individual differences using GraphPad Prism 5.01 (La Jolla, CA). Results were considered significant when the P value was less than 0.05.

**Results**

**Protein concentrations varied little across serum samples**

Protein concentrations were evaluated using the BCA method. As shown in Fig. 4.1, there was virtually no difference in crude protein concentrations among the FBS samples with the only exception being the porcine serum sample which had a markedly higher serum concentration than any of the FBS samples.

**Cell growth rates are affected by total protein concentration**

While protein concentrations were similar among all the FBS samples tested, the final concentration of serum in the media was also evaluated for the MDCK cells being used in the study to determine its impact on cell doubling.
times. Using sample #8 as the reference sample, 5%, 10%, and 20% FBS concentrations were evaluated relative to cell doubling times over a 24 hr. period. While the higher concentrations did reduce doubling time (Fig. 4.3B) over the 5% standard, the effect was not proportional to the serum concentration. Subsequently, cell growth in 24-well plates was evaluated with an Arrayscan to determine if this was suitable for evaluating growth rates using larger numbers of plates/wells. The initial iteration showed considerably more variability in cell nuclei number per high power field than expected. Upon examination of these plates, it was clear that several of the serum samples suffered from severe over-confluency in one or more of the sample wells at the latest time point which the Arrayscan software was unable to resolve into individual nuclei even at high power.

The experiment was repeated in 96-well plates using a lower cell concentration to ensure that cell density was not impacting cell doubling time. The disadvantage of low plating density is the inability of the Arrayscan to differentiate the background from that where there are just a few cells, especially at early time points. Thus, wells were manually counted using a light microscope with the cells stained with crystal violet instead of DAPI. Population doubling time was calculated using the formula: 

\[ DT = \frac{T \times \ln 2}{\ln (X_{\text{end}}/X_{\text{beginning}})} \]

where \( T \) equals elapsed growth time, \( X_{\text{beginning}} \) is the starting cell number at the beginning of the time period and \( X_{\text{end}} \) is the final cell number at the end of the incubation time period [41]. As shown in Fig. 4.3A this produced less error evaluating serum doubling time rates. The slight differences observed in the
doubling time of sample 8 between Fig. 4.3A and 4.3B were largely the result of a higher seeding density used in Fig. 4.3B to enable the plates to be read by the Arrayscan unit. Sample 3 demonstrated markedly higher doubling times relative to sample 8 than any other samples for unknown reason. Interestingly, this slower growth rate for sample 3 was also present in the initial growth experiment (Fig. 4.2). Samples 1 and 8 (high growth rates) were selected for further evaluation along with sample 3 (low growth rate). Interestingly, sample 1 was also noted by the manufacturer as having endotoxin contamination (concentration not provided) yet still yielded excellent cell growth with the MDCK cell line.

**Cell growth rates correlated with the presence or absence of miRs**

Exosomes were isolated from each serum sample with total RNA extracted from the exosome pellets for RT-qPCR. Relative miR ratios for samples 1, 3, and 8 were determined using sample 3 as the low growth standard and sample 8 as the high growth standard in fold-change calculations (allowing for the determination of the ratio of miR from one sample to the other) (Fig. S4.1). As shown in Fig. 4.4A and 4.4B, samples 1 and 8 had two miRs in common, 99a-5p and 148a-3p, and several unshared miRs at higher ratios than sample 3. In contrast, Sample 3 showed several distinct miRs at higher levels than present in sample 8 (Fig. 4.4C). Fig. 4.4D indicates increased, and Fig. 4.4E decreased miRs in the accompanying Venn diagrams. Thus, prospective studies focused on miRs that were increased in one or more of the three serum samples.
Several of the miRs identified in the screen were identified and validated using miR mimics and inhibitors. At 24 hours post-transfection, several of the mimic/inhibitor pairs appeared to be trending apart as expected, but by 48 hours these trends were less noticeable (Fig. 4.5). miR-148a-3p appeared to be of consequence at 24 hours, which may be due to the variation among the various mimic/inhibitor pairs. While inconclusive, the data support further investigation of these miRs relative to cell growth characteristics as the effects of miR transfection are affected by cell division at which point the concentration of the miR/cell is halved. Additionally, the transfection process requires media containing serum to be added 24 hours post-transfection, making it highly likely that any miRs present in the serum could impact the outcome of the transfection by either enhancing or countering the effect of the transfected miRs.

Discussion

The insignificant differences noted as they related to total protein concentration across the serum samples (other than for the porcine sample), suggests that perhaps differences in miR levels in serum may influence cellular doubling time. Others have previously shown that anti-growth factor antibodies can decrease cell growth rates, and that exosome depletion also negatively affects cellular growth rates [21]. These findings support the notion that growth rate is regulated potentially by miRs.

MDCK cells were utilized in this study due to their high growth rate, inclusion in many laboratory studies, and their current use as a cell line substrate
for influenza vaccines which offers the opportunity to study ways to enhance vaccine production. It is known that there is a high degree of conservation of miRs across species [42] including their passenger strand [43]. Interestingly, many of the miRs evaluated share identical seed region sequences (if not entire sequences) between humans and bovines, including the U6 control used in the PCR experiments.

Although the MDCK cell growth data indicated that there were detectable differences in cell growth rates for the serum samples, the results of the first two experiments did not fully concur due to the use of the Arrayscan to count cells that were not confluent compared to cells counted manually and plated at much lower cell densities initially. As revealed in Fig. 4.3A, the cell doubling time data showed minimal variation when manually counted.

Analysis of exosome-associated miRs from the serum samples that altered MDCK cell growth revealed variations in relative miR ratios across each serum sample. Of the five miRs selected for further examination (miRs 99a-5p, 200a-3p, 409-3p, 148a-3p, and 20a-5p), the effect on cell growth kinetics needs to be considered in the context of transfection and cell confluency. Using a transfection reagent in actively dividing cells can be problematic as the siRNA/miR concentration is effectively halved with every cell division. Given the already described transduction efficiency of exosomes, it is apparent that the miRs they carry have a physiological role in regulating the cells, a feature linked to levels present in sera.
Interestingly, the high relative ratios of several of the miRs identified have been shown previously to directly influence cell growth rates via the PI3-AKT pathway (Fig. 4.6-pathway generated using KEGG [44, 45]). miR-148a-3p (reviewed in [46]) is particularly interesting as it was found at increased ratios in both of the high growth FBS samples. PIK3IP1, ROCK1 [47] and p27 [48] are regulated by mir-148-3p which are involved in cell proliferation and/or differentiation events. The tumor suppressor PTEN is negatively regulated by miR-148a-3p thus having specific implications for the growth of cancerous cell lines relative to cell proliferation [49]. miR-200a-3p is also involved in cell proliferation via IL-6/STAT 3 signaling upstream of PI3K [50]. It is also involved in mesenchymal to epithelial cell transitions [51] and the phenotype of cancer cells [52]. miR-99a is implicated in tumor suppression and reduction of cardiac hypertrophy via PI3K/AKT/mTOR which has interesting implications given that many commonly utilized cell lines are derived from tumors. It also is involved in early differentiation of stem cells making the relatively high levels encountered in FBS of interest for primary cell culture. While miR-99a, miR-148, and miR-200a have regulatory roles in the cell cycle, their richness in sera need to be investigated with regard to their effects on cell growth as there is current evidence of potential deleterious effects on cell differentiation and/or phenotypic traits [47, 48, 51-54].

As there is a growing body of literature suggesting exosomal miRs regulating a variety of biological responses, it is imperative that those found in
serum (and platelet lysates) be further investigated to determine if they influence cell culture growth kinetics and/or phenotype.

**Acknowledgments**

The authors wish to thank Jackelyn Murray for her input and assistance on this project. We also wish to acknowledge the UGA Center for Undergraduate Research Opportunities (CURO), the Georgia Research Alliance, and the United States Army Long Term Health Education Training Program for their financial support. The authors declare no financial interest.

**Author contributions**

Conceived, designed, performed, and analyzed the experiments: JH, AB, NG, NOB, and RAT.
References


39. Taheriazam A, Bahador R, Karbasy SH, Jamshidi SM, Torkaman A, Yahaghi E, Shakeri M. Down-regulation of microRNA-26a and up-


# Tables and Figures

## Table 4.1. Serum Sample Product Information

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* USDA--approved sources
Figure 4.1. Mean protein concentration per serum sample. BCA protein assays were used to assess the total protein concentration of each sample as most manufacturers specified a range from 2--4g/mL. Only sample 7 (swine serum) was significantly different than the other products. Data are means from duplicates of each serum sample ± SEM. One-way ANOVA with Dunnett’s post-test was used to compare all means to sample 8 (the lab standard FBS) as it will be used as the reference sample for subsequent experiments. *p<0.05.
Figure 4.2. Average number of nuclei counted per high power magnification field after 24 hrs. incubation with media made using each serum sample. The number of machine validated fields per well varied from 12 to 444 with the variation due to the difficulty in differentiating sparse nuclei populations in some of the wells from the background. Data are the mean ± SEM of 3 experiments done in triplicate.
Figure 4.3. MDCK cell doubling time varied over 24 hr. growth period by serum source. MDCK cells were suspended and plated (20 cells/well) in the indicated serum sample from 0 to 24 hours. After 24 hours, a subset of wells was fixed for staining while the remaining wells were allowed to continue to grow. At 48 hours post--plating, all remaining wells were fixed, and stained with crystal violet. Each well was counted using 20x magnification with the well centered under the objective (A). Serum sample 8 was examined at different final concentrations (5% 10%, and 20% and the cells were fixed and DAPI--stained at 24 and 48 hours post--plating (Fig 3B). Wells were evaluated using Cellomics Arrayscan to determine nuclei number and calculate doubling time. Data are the mean ± SEM of 3 experiments done in at least triplicate. One-way ANOVA with Dunnett’s post-test was used to compare all doubling time to sample 8 *p<0.05.
Relative miR Content of Sample 1

A

Ratio of miR Content $(2^{-\Delta\Delta Ct})$ #1/#3

miR (Human primer)

Relative miR Content of Sample 8

B

Ratio of miR Content $(2^{-\Delta\Delta Ct})$ #8/#8

miR (Human primer)

Relative miR Content of Sample 3

C

Ratio of miR Content $(2^{-\Delta\Delta Ct})$ #3/#8

miR (Human primer)
Figure 4.4. Ratios of selected miRs among exosomes isolated from high and low growth serum samples. The exosomal miR content of the two high growth samples (#1 and #8) was compared to that of the low growth sample (#3). The 2^{−ΔΔCt} method was used with miR U6 as the internal control to compare miR ratios in both high growth sera, (#1(A) and #8 (B)) and low growth sera (sample #3) to the reference/high growth serum sample, #8. Only miRs that were present at 5x or higher ratios are shown (C). Venn diagrams were also constructed to identify shared miRs whether they were present at increased (D) or decreased (E) levels in the various sera. Shared miRs are indicated in the ratio graphs in black.
Figure 4.5. Doubling time of actively growing MDCK cells transfected with miR mimics and inhibitors indicated several of the identified miRs may affect cell growth. The doubling time of MDCK cells transfected with mimic and inhibitor siRNAs for the five selected miRs are shown above. There were detectable differences in growth rate some between mimic and inhibitor for the 24 hours, but that difference was not significant and was not present at 48 hours. The miR--148a--3p mimic and inhibitor trended towards significance. One-way ANOVA with Dunnett's post-test was used to compare all doubling time to the negative control. *p<0.05.
Figure 4.6. Proposed FBS miR target sites that positively influence cell growth rates via PI3–AKT Pathway. PIK3IP1, ROCK1, and p27 are regulated by mir--148a--3p and all are involved in cell proliferation and/or differentiation events. miR--200a--3p is also involved in cell proliferation via IL--6/STAT 3 signaling upstream of PI3K, miR--99a is implicated in tumor suppression and reduction of cardiac hypertrophy via PI3K/AKT/mTOR, and is involved in early differentiation of stem cells making the relatively high levels encountered in FBS of interest for primary cell culture. Image generated using KEGG [44, 45].
Supplemental Information

Supplemental Table S4.1: miR Human Forward Primer Sequences

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199
Supplemental Figure S4.1. Heat map showing relative ratios of individual miRs in PCR screening of samples 1, 3, and 8. Human miR primers were used to demonstrate the relative ratios of individual miRs using the $2^{-\Delta\Delta Ct}$ method in RNA samples isolated from exosomes. Sample 8 was used as the control for columns 1 and 2 while sample 3 was used as the control for columns 3 and 4. This allows for the determination of miRs present at higher or lower ratios relative to the growth rate of the specific FBS sample. Numerous miRs are present at elevated ratios in both fast growing FBS samples (columns 3 and 4) while several miRs are elevated in Sample 3 (column 1) that are not seen in the faster growing FBS samples. PCR screens were performed once on each sample, with miRs of interest further validated via additional PCRs as noted previously. Heat map generated using MeV [55].
CHAPTER 5

INFLUENZA A VIRUS VRNP COMPLEX TRANSPORT IN EXOSOMES

1Hanson, JM, Bakre, AA, Schubach, DA, and R.A. Tripp. To be submitted to Journal of Virology.
Abstract

The segmented genome of influenza A viruses (IAVs) allows for reassortment via the interchangeability of segments from one virus to another. The genome segments are transferred into the virion as part of virus ribonucleoprotein (vRNP) complex which contains the individual RNA segment along with the nucleoprotein and polymerase. As the vRNP complexes assemble in the nucleus and pass through the cytoplasm, it is possible that the newly formed vRNPs can be enveloped by invaginating multivesicular bodies thereby incorporating the vRNPs into exosomes. This would allow for individual vRNP complexes to leave the cell. As such, the potential for vRNPs to be transported within exosomes offers the possibility that this may be a mechanism for influenza virus reassortment. Here, we demonstrate the shuttling of vRNPs in exosomes from two different IAV strains indicating a potential path from an infected cell to another uninfected cell, as well as a potential mechanism for reassortment.
Introduction

Influenza virus reassortment is a major mechanism by which IAV escapes and evades the immune response. The segmented genome of IAVs allows for reassortment via the interchangeability of segments from one virus to another. This evasion allows for continued virus evolution ensuring that the virus stays one step ahead of the host immune system. With recent evidence supporting the movement of IAV vRNP complexes intercellularly via cellular connections, it is important to determine whether IAV vRNPs can traverse the host via exosomal transport of vRNPs to neighboring cells. Exosomes would provide a means to allow dispersion of the vRNP complexes, as well as protection from the immune system targeting the vRNP. As such, the potential for vRNP movement within exosomes presents a unique mechanism by which IAV may attempt to enhance genetic diversity and escape immune recognition.

Influenza structure

Structurally, IAV is composed of eight RNA segments including PB2, PB1, PA, HA, NA, NP, M(1/2), and MS1/NEP [1]. These segments are incorporated into a viral ribonucleoprotein complex (vRNP) that includes the nucleoprotein and polymerase. The eight vRNP complexes are then packaged into the virion at the host cell plasma membrane as the virion buds out from the host cell.

Hemagglutinin and neuraminidase are the two major glycoproteins comprising influenza virus’s exterior surface markers, and are involved in entry and egress from the target cell, respectively. The viral envelope encapsulates the
virus and RNA prior to their release into an infected cell after fusion with the host cell has occurred. Currently there are eighteen different hemagglutinin subtypes (H1-H18) and eleven different neuraminidase subtypes (N1-N11). Hemagglutinin is primarily involved in the initial attachment and binding of the target respiratory cells on the host via a glycoprotein receptor that expresses sialic acid and is found on the host cell’s surface. Subsequent to binding the host cell’s sialic acid receptors, hemagglutinin then begins the process of membrane fusion which perforates the host cell membrane. This allows the contents inside the viral envelope to be emptied into the host cell, effectively infecting that cell with influenza virus.

As was seen with the 2009 pandemic virus, the inclusion of NA and M segments from a Eurasian swine virus, as well as a minor change in the HA amino acid structure, allowed for efficient aerosol transmission [2]. Others have shown similarly altered phenotypes due to changes in PA and NA leading to enhanced respiratory disease and lung pathology [3]. The M segment has also been shown to be involved with contact transmissibility efficiency exchange between viruses, as well as morphological changes [4]. Interestingly, the route of infection also affects selective pressure in that direct transmission induces less selective pressure than aerosol spread [5].

Also of interest is that in swine, the 2009 pandemic virus NA and M genes cooperatively enhanced one another resulting in improve replication efficiency and transmissibility [6]. HA and NA segments from canine influenza virus (CIV) have also been shown experimentally to reassort with the 2009 pandemic virus
leading to enhanced replication and pathogenicity which is an on-going concern given the current outbreak of CIV that began in late 2014 and has continued to present [7]. Conversely, lack of a sufficient number of segments and/or defective segments can lead to the generation of defective virions known as defective interfering (DI) particles [8]. Since DI particles can often attach to host cells, it is possible they can also initiate cellular signaling, and as such their effect on miR genesis by the host cell must be considered.

In addition to enhanced transmissibility, the gene segments also confer other properties related to influenza virus assembly and pathogenesis. Specifically, virus assembly requires that the viral components be moved to the assembly location on the apical plasma membrane of polarized epithelial cells [9]. Neuraminidase not only helps with viral egress from cells, but is also integral to enhanced mucous penetration via the cleavage of sialic acids from glycans in the mucous as mentioned previously [10].

**Influenza viral ribonucleoproteins (vRNPs)**

Although the vRNP complex has been studied for over 50 years, only recently have several key features regarding its structure and transmission been elucidated. These discoveries give insight into how vRNPs may be involved in both influenza transmission and reassortment in host species leading to novel virus development. Several recent observations regarding the transport of vRNPs between cells yield key clues suggesting the potential for exosome involvement in vRNP trafficking as well.
Influenza vRNPs are comprised of one of the eight viral RNA segments along with a viral polymerase and several copies of the nucleoprotein (NP), all of which are packaged together into a rod-shaped vRNP complex (reviewed [11]). The vRNP contain an NP core, with the RNA wrapped around it in a helical arrangement [12]. However, if no viral RNA is present, M1 and NP are unable to self-assemble into the helical structure characteristic of the vRNP complex [13]. Live imaging techniques utilizing labeled vRNP complexes have elucidated key points in their trafficking from the nucleus out of cell [14] and vRNP purification protocols have allowed for the detailed studies on nuclear import and viral replication without other viral proteins contaminating the vRNP complexes [15].

Initial vRNP formation begins in the nucleus with polarized epithelial cells exhibiting unique asymmetric aggregation of either ribonucleoproteins (RNPs) or vRNPs at the inner nuclear membrane [16]. As the vRNP complexes emerge from the nucleus, they utilize Y-box binding protein (YB-1) as a carrier to move the vRNP to the microtubules where it subsequently enters the vesicular trafficking network to be carried to the plasma membrane of the cell for virus budding [17]. Additionally, Rab11 has also been shown to be involved with vRNP trafficking via its interaction with them at recycling endosomes [18]. The rod-like vRNPs are variable in length and position within the assembled virion, but the relative pattern of assembly implies the mechanism of assembly is quite specific [19].

Recent studies have looked at the specific assembly of the vRNP components. A live cell imaging study showed that the PB1/PA heterodimer
enters the nucleus via RanBP5 where it then joins with PB2, which is imported into the nucleus via a distinct importin alpha-linked mechanism [20]. The same group also observed the potential for abnormal formation of the polymerase trimer in the nucleus if nuclear import was blocked which may be a potential route for incorporation of one or more members of the vRNP complex to be incorporated into exosomes if they are aberrantly formed in the cytoplasm [20].

The potential for exosomes to mediate viral transport have been discussed previously for several viruses including Enterovirus and Hepatitis C virus. Exosomes have also shown the ability to utilize virus entry routes for the delivery of exosomal contents to the cell in order to avoid degradation prior to releasing their cargoes (reviewed in [21]). However, the potential for influenza subunits such as vRNPs to be transported via exosomes and then mixed with other viral subunits in the cytoplasm during virus assembly requires further exploration. Exosomes and influenza viruses are taken into the cell via several of the same pathways [21-23] and rely on much of the same cellular machinery for cellular uptake and egress.

**Exosome-vRNP complexes enable virus reassortment**

Exosomal transfer of influenza virions from one cell to another could theoretically occur, as it has been observed for both Enterovirus (EV) and Hepatitis C Virus (HCV) [24, 25]. There are amorphous (spherical) and linear forms of influenza virus [26] as well as defective, interfering particles, any of
which may not possess the same size constraints that would negatively affect a viable virion.

As noted previously, influenza virions typically have a diameter between 100-150nm while exosomes have diameters between 30-150 nm [24, 27]. Since exosomes display common cell surface markers including various mucins, CD10, CD63, and alpha-2,6- and alpha-2,3-linked sialic acids, it is expected that exosomes can bind viruses externally and potentially act as infectious delivery systems to adjoining cells, a phenomenon that has also been observed with HCV [28].

It is likely that exosomes traffic key portions of the influenza genome to adjacent cells such as the vRNP complex, which is approximately 10-20nm in diameter making it well within the size constraints to be considered as a possible exosome cargo [29]. As such, the working hypothesis remains that a substantial portion of the influenza vRNP complex is being moved via exosomes or larger vesicles to neighboring cells, not just through filopodia and actin filaments as others have demonstrated experimentally [14, 18, 30-32].

The relevance of exosome trafficking is understudied. The gap junctional protein Connexin 43 (CX43) has been shown to modulate the transfer of exosomal material to adjacent cells via CX43-containing channels [33]. CX43 is found not only in pores at the gap junction, but also as a hexameric structure on the exosome membrane itself [33]. This affords two possibilities for affecting the transfer of material from exosomes via the use of anti-CX43 antibodies which could be used to bind not only the CX43 on the exosomes, but also in the gap
junction channels. Additionally, gap junctional CX43 can be upregulated (as shown experimentally in rats) via the use of fluoxetine and amitriptyline, and as such would be a way to further enhance trafficking of influenza components between cells potentially showing the opposite phenomenon is also possible [34].

**Materials and Methods**

**Cells**

MDCK or MDCK-HA cells (a kind gift from Dr. Peter Palese) were plated in 24 well transwells at liquid:liquid, until confluent. Once confluent they were then taken to air:liquid interface (ALI) for 24 hrs. to verify tight junction formation via lack of media leakage to the apical surface (Fig. 5.1). Cells were then maintained with DMEM containing 5% FBS basally at air:liquid interface for 24 hours after confluent monolayer is obtained to polarize cells and establish tight junctions. Polarization of the cells ensures only apical budding the influenza viruses during replication thereby preventing contamination of the basal media with virus.

**Viruses**

WSN and ΔHA-WSN H1N1 viruses (the latter of which was kindly provided by Dr. Peter Palese) were used, with the ΔHA-WSN requiring the HA protein from MDCK-HA cells to be infective as the viron’s native HA protein was deleted. Both cell types were infected for 1 hr. with WSN, ΔHA-WSN, or mock at 1.0 MOI (no trypsin was added). The apical infection media was removed, the cells washed twice with PBS, and then apical chambers were left at ALI. 5%
exosome-free FBS in DMEM was used to replace the basal media. Plaques assays on MDCKs and MDCK-HAs for virus viability (48 hr. incubation) were conducted to determine virus viability. Plaques were visualized after fixing the wells in 10% formalin and permeabilized with 0.1% Triton-X for 15 minutes (wells were not crystal violet stained as with a normal plaque assay). NP staining was then conducted on the MDCK cells used for plaque assays using murine NP antibody which was then complexed to an anti-mouse Alexa fluor antibody.

**Exosomes**

To access exosomes, the basal media was collected 20 hrs. post-infection. The cells in the apical wells were washed with PBS, then freeze-thawed in PBS for disruption as cell lysate for virus plaque assays. Exosomes were isolated from PK1 cells using Exoquick-TC per the manufacturer’s recommendations with the final centrifugation at 4°C.

In order to generate highly concentrated exosome preparations from infected cells to improve visualization of any NP-stained exosomes, MDCK cells were then infected with WSN virus at an MOI of 1.0 after being polarized at ALI for 7 days. Basal supernatants were tested via plaque assay for live virus at 24 and 48 hrs. post-infection with no viable virus seen. The pelleted exosomes were resuspended in 20uL of PBS and placed into single chambers of 8 well chamber slides and allowed to dry. Once dry the chambers were filled with 10% formalin for 24 hrs, then washed twice with PBS and permeabilized with 0.05% Triton X for 15 min. Wells were washed three times with PBS and then blocked with 3%
BSA in PBS with 0.05% Tween 20. The chambers were then stained as before using NP antibodies, DiD to stain cell membranes, and anti-HA WSN antibodies to label HA on the virus surface. A green goat anti-mouse IgG and blue donkey anti-rabbit IgG fluorophore secondary antibody were used for NP and HA staining respectively (Life Technologies)

Results

Early studies propagating MDCK cells at ALI for 24 hrs. post-confluency produced no detectable virus in the basal media at standard plaque assay dilutions (Fig. 5.2). Basal media from ALI MDCKs contains virus below limit of detection (~50 PFU/mL). Basal media incubated on recipient MDCKs induced nuclear NP staining, primarily in cells displaying aberrant nuclear morphology including blebbing and nuclear disintegration (Fig. 5.3). In previous experiments NP staining of entire cells was visible in multiple wells indicating the presence of virus throughout the cell. However, virtually no staining outside of the nuclei was detected indicating the whole cell NP staining observed previously may have been leakage of intact virus. Cell lysate derived from two consecutive freeze/thaw cycles generated infective WSN virus. However, when tested with ΔHA-WSN, which must be grown in MDCK cells expressing HA proteins on their surfaces in order to be infectious, plaques are observed even when HA is not present on the recipient MDCK cells indicating the possible role for lateral spread of HA-deficient virions to adjacent cells via pores, or exosomes.
LLC PK1 cells, derived from the proximal tubule of swine kidneys, were for their potential for enhanced reassortment (Fig. 5.4). No live virus was observed in the basal media indicating the PK1 cells were polarized (Fig. 5.5). The exosomes from either the A/CA/04/09 or A/WSN infected wells were isolated from the basal media samples and incubated for 24 hours with uninfected cells, then infected with the ΔHA-WSN virus (MOI=10) to maximize the number of cells infected relative to cells that may have intact vRNP complexes delivered via the exosomes. However, no detectable virus was recovered from these wells (Fig. 5.6) as determined by plaque assay using MDCK cells suggesting no reassortment occurred between any HA-containing vRNPs and the ΔHA-WSN virus which cannot successfully escape normal MDCK cells due the lack of the HA protein.

Exosomes derived from WSN-infected, polarized MDCK cells concentrated onto chamber slides yielded by far the most interesting results. At 24 hours minimal co-localization is observed between NP and DiD staining (images not shown). However, at 48 hrs. post-infection (Fig. 5.7), significant co-localization between NP and DiD staining is observed indicating interactions between NP and exosomal membranes in the absence of HA-staining (which would be suggestive of virus particles as opposed to exosomes).

The previous experiment was repeated using A/California/04/09 (H1N1) virus as it is in active circulation compared to the more prototypic lab strain WSN. In addition to imaging via the EVOS microscope, additional images were
obtained with a Zeiss 510 confocal microscope at 1000x (Fig. 5.8). Both images revealed substantial overlap in red and green fluorescent (yellow).

Discussion

While these attempts at generating reassortment events were unsuccessful, the experiments indicate the presence of NP in cells treated with basal media or exosomes. The potential association of exosomes with vRNPs requires continued exploration as preliminary evidence supports the movement of vRNPs in exosomes, but the mechanism by which this process could generate reassortment remains elusive.

It may be that the vast majority of vRNP complexes transported via exosomes are degraded upon endocytosis into the recipient cell, a phenomenon which has also been documented for exosomal microRNA cargos. It is also possible that PK1 cells may also not support reassortment as expected. Given that possibility, subsequent experiments have been performed in MDCK cells as they have shown the most promise for vRNP transport in not only our experiments, but those examining vRNP movement between cells using the actin network and filopodia.

The concentrated MDCK derived exosomes show significant co-localization of NP and DiD staining in the absence of HA-staining indicating interactions between exosomes and NP complexes. While this is minimally seen at 24 hrs, there is strong evidence of co-localization at 48 hrs although this may be due to the absence of trypsin in the media forcing the virus to use exosomes
as a sort of escape hatch to try to ensure virus viability in the absence of a way to successfully bud virus from the cell. Additional confocal microscopy is still necessary to elucidate the exact nature of the observed NP/exosome interactions via Z-stacks as the basic images displayed here are highly indicative of co-localization, but not conclusive. It also must be confirmed as to whether the exosome aggregates are purely exosomes, or a more likely mixture of exosomes and larger microvesicles and apoptotic bodies in order to delineate which carrier is truly responsible for the large amount of NP-staining observed.

While these experiments have not conclusively shown vRNP movement in exosomes or reassortment, they have established a workflow by which MDCK cells can be successfully polarized at ALI to induce only apical virus budding as well as suggested that virus replication in the absence of budding may further induce exosomal NP interactions as a possible escape mechanism from the infected cells.
References


Figure 5.1. Experimental design to identify vRNP complexes in exosomes from influenza A virus infected MDCK cells. MDCK and MDCK-HA cells were used in conjunction with A/WSN and A/delta HA-WSN viruses to determine if vRNP complexes can be passed from the basal membrane of the cells via exosomes. Both cell lines were grown at liquid:liquid on transwells until confluent, then taken to air:liquid. After 24 hours at ALI to polarize the cells, they were infected with either virus at an MOI of 1.0 for 24 hours. Basal supernatants were collected at 24 hours and used for plaque assays to determine virus leakage through the basal membrane. Plaque assays (MDCK cells) were also fixed and NP stained in order to assess whether any NP staining could be observed in uninfected cells.
Influenza Infected SUPs Plaqued on MDCK Cells

Sample, Original Host Cell, and Virus Type

Influenza Infected SUPs Plaqued on MDCK-HA Cells

Sample, Original Host Cell, and Virus Type
Figure 5.2. Plaque assays verify infectious virus occurs only in cell lysate samples with no leakage of virus basally. MDCK and MDCK-HA cells sow plaque forming virus only in wells containing A/WSN and A/delta HA-WSN viruses. As expected, the delta-HA virus only produces plaques in MDCK cells expressing the appropriate HA protein on their surface, and no plaques are seen when grown in normal MDCK cells. No plaques are seen in any of the basal supernatant samples.
Figure 5.3. Basal supernatants from influenza-infected, but not mock-infected, cells show evidence of NP staining. Both virus types show evidence of NP staining in both the MDCK and MDCK-HA cells indicating NP movement basally out of the cell since no viable virus was identified via plaque assay.
Figure 5.4. Experimental design to identify vRNP complexes in exosomes from influenza A virus infected LLC PK1 cells, and determine if HA vRNPs are being passed and are capable of reconstituting an HA-deficient virus. Swine-origin PK1 cells were used in conjunction with A/WSN and A/CA/04/09 viruses to determine if vRNP complexes can be passed from the basal membrane of the cells via exosomes. PK1s were grown at liquid:liquid on transwells until confluent, then taken to air:liquid. After 7 days at ALI to polarize the cells, they were infected with either virus at an MOI of 1.0 for 24 hours. Basal supernatants were collected at 24 hours and the exosomes isolated. The exosomes were then combined with delta-HA WSN virus (MOI=10.0) on new MDCK cells to determine if HA-vRNPs could reconstitute the deletion virus if present.
Figure 5.5. PK1 basal media does not show evidence of basal virus budding. No basal budding of either virus was observed at 24 or 48 hrs. indicating the PK1 cells can be successfully polarized at ALI in the same fashion as MDCK cells.
Figure 5.6. Exosomes from IAV-infected cells do not enable ΔHA WSN IAV budding from PK1 Cells. Basal exosomes from IAV-infected cells at indicated time points incubated with PK1 cells for 24hrs, then cells infected with ΔHA WSN MOI=10 for 48 hrs. No viable virus was recovered from any of the wells for the 24 or 48 hour time points.
Figure 5.7. Exosomes from IAV-infected cells exhibit abundant NP staining 48 hours post-infection. Basal exosomes from IAV-infected cells 48 hrs post-infection with WSN IAV at MOI of 1.0 were pelleted and dried onto chamber slide wells for staining. NP (green), HA (blue) and membranes (red) show co-localization of NP and exosomal membranes in the absence of HA. No viable virus was recovered from the basal media used for exosome recovery. (Scale bar = 200uM).
Figure 5.8. Exosomes from IAV-infected cells exhibited abundant NP staining 48 hours post-infection via confocal microscopy. Basal exosomes from IAV-infected cells 48 hrs post-infection with A/California/04/09 IAV at MOI of 1.0 were pelleted and dried onto chamber slide wells for staining. NP (green), HA (blue) and membranes (red) show co-localization of NP and exosomal membranes in the absence of HA. No viable virus was recovered from the basal media used for exosome recovery. 1000x confocal images (Scale bar = 10uM).
CHAPTER 6
CONCLUSIONS

IAVs continue to adapt to ever-changing immune pressure. miRs have important roles in virus fitness as several miRs have been shown to aid virus fitness while others clearly induce host immunity. Exosomes are key mechanisms by which virus and host miRs are transported intercellularly as they prevent degradation of the miRs and allow for timely uptake of the miRs by recipient cells.

However, our understanding of how miRs and exosomes work in concert is still limited relative to the huge amount of data regarding intracellular miRs during influenza infection that is currently available. As such, continued exploration of how miRs and exosomes act in pro- and/or anti-viral fashion is desperately needed as they appear to play key roles in immune modulation during influenza virus infection.

Most data on miR dysregulation during IAV infection and replication was collected using cell-derived miRs which may not correlate with in vivo models of IAV infection. Continued focus on testing identified miRs in animal models is important to determine if miRs can be used as therapeutics or to enhance virus replication in the manufacture of vaccines, or during live virus vaccination, where higher virus yields during manufacture are critical to reduce costs.
Additional studies are also necessary to distinguish the role of exosomes during influenza infection, and determine their role as host cell-to-cell signaling agents particularly during infection. While many of the exact mechanisms by which miRs induce pro- or anti-viral effects remain unclear, they represent a new frontier in anti-viral research relative to targeting virus and host processes as they can be used therapeutically to modulate infection outcome in the host and perhaps reduce influenza complications such as acute respiratory distress syndrome (ARDS), by modulating the immune response. We have shown differential miR expression in exosomes from IAV-infected cells. Initial studies were conducted in differentiated immortalized and primary human bronchoepithelial cells to more fully mimic conditions seen in vivo by a host infected with IAV. We also showed miR transmission between undifferentiated cells via exosomes, a key facet of the host response to infection as this communication pathway can enable highly susceptible undifferentiated cells to adopt anti-viral states in order to minimize virus damage to the host.

Additionally, we have demonstrated that several of the miRs identified in our screens corresponded to miRs expressed during IAV infection in swine. This is important as we were able to identify key differences in miR profiles between vaccinated and unvaccinated swine at the time of IAV infection indication certain miRs appear to be induced upon infection in vaccinated animals that are not present in unvaccinated animals. The lack of expression of certain miRs in swine may help explain their enhanced susceptibility to virus infection and permissivity to viral replication, but currently that is unproven.
Finally, we have demonstrated that several exosomal miRs can be used to alter in vitro cell growth rates. The outcomes of several experiments including transfections, infection studies, and the like are modified, likely via exosomal miRs present in common media components and may not be adequately controlled by mock samples given the wide variety of effects possible as well as the durability of those effects on the cells being used.

Additionally, the potential for IAV vRNP complexes to be transported via exosomes needs further investigation. Preliminary evidence supports vRNP transport in exosomes, but more research on this phenomenon is necessary to prove not only that it is occurring, but also that it is a mechanism by which viral reassortment can occur in a host cell.

Taken together, we can establish that exosomes are key machinery not only to IAV, but also the host, during IAV infection. Exosomes can traffic miRs intracellularly as well as move IAV proteins which may also induce immunity in the host (even if the vRNP complexes cannot be used directly for reassortment by the virus). However, much work remains relative to determining the exact mechanisms by which IAV and the host use exosomes to modulate the attempts of the other to neutralize one another in vivo.