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Review

Respiratory syncytial virus receptor expression in the mouse and viral tropism

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Summary. Human respiratory syncytial virus (RSV) infects airway epithelium and can cause serious illnesses such as bronchiolitis and pneumonia. With the discovery of cell-surface nucleolin as a fusion receptor for RSV, the question arose as to whether nucleolin could explain RSV tropism *in vivo*. Here, we report the distribution of cell-surface nucleolin expression in tissues of normal mice and how this distribution of expression relates to what is known about RSV tropism and its clinical manifestations. Our results show evidence of cell-surface nucleolin expression in the respiratory tract. In addition, cell-surface nucleolin is expressed in tissues outside of the respiratory tract, many of which correspond to previous reports of tissue-specific RSV infection, and others that may allude to additional potential sites for RSV infection *in vivo*. Furthermore, our work provides a foundation for the investigation of nucleolin's physiological function in various healthy mammalian tissues.

Key words: Respiratory syncytial virus, Receptor, Histological mapping, Nucleolin, Mouse

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Introduction

Respiratory syncytial virus (RSV) is the major viral agent causing acute lower respiratory infections in young children (Nair et al., 2010) and is the most common cause of infant respiratory failure leading to admission into the hospital intensive care unit (Pilar Orive et al., 1998). RSV also causes significant disease in adults, especially in immunocompromised individuals and in the elderly with congestive heart failure or chronic obstructive pulmonary disease (Walsh, 2011). Despite many decades of intensive research dating from the discovery of RSV in 1956, there are currently only a few pharmacological agents available for therapy or prophylaxis (Tayyari and Hegele, 2012). Although RSV is considered as mainly a respiratory pathogen, extrapulmonary presentations of RSV infection can occur, particularly in cases of severe lower respiratory tract illness (Eisenhut, 2006). It remains unclear if these clinical manifestations reflect direct RSV infection of extrapulmonary sites or are related to a host response to an established respiratory infection. The determinants of RSV tropism in various tissues are poorly understood.

We have identified cell-surface nucleolin as a functional receptor of RSV *in vitro* and in a mouse model of experimental RSV lung infection (Tayyari et al., 2011). Ubiquitously expressed, nucleolin binds both RNA and proteins in eukaryotic cells. Nucelolin is associated with several cellular compartments: in the nucleolus, where it is involved with DNA and RNA

metabolism; in the cytoplasm, where it controls protein shuttling into the nucleus and posttranscriptional mRNA regulation; and on the cell surface where it serves as an attachment protein for a variety of ligands (Storck et al., 2007). *In vitro*, nucleolin is expressed on the surface of a wide variety of continuous cell lines which are permissive to RSV infection (Tayyari et al., 2011). In this context, cell-surface nucleolin expression is considered to be an attribute of proliferating or transformed cells (Joo et al., 2005). Whether the *in vivo* counterparts of these cultured cells also express nucleolin on the cell surface is not well characterized.

Considering reports of extrapulmonary RSV infection and the lack of knowledge regarding normal expression of cell-surface nucleolin in healthy tissues, we embarked upon a histological mapping of cell-surface nucleolin expression in mouse tissues using immunohistochemical staining with nucleolin-specific antibody. Our findings confirm cell-surface nucleolin expression in expected target cells in the lung and reveal expression in a number of other tissues that may be relevant to RSV tropism and potential sites of RSV infection *in vivo*.

Expression of cell-surface nucleolin in mouse tissues

Methods

Healthy adult (8-week old) male and female BALB/c and C57BL/6 mice (n=4) were euthanized, organs were dissected and tissue sections fixed in formalin and embedded in paraffin. Four micron-thick sections were cut, de-paraffinized, re-hydrated and processed for nucleolin immunostaining as described (Tayyari et al., 2011). Briefly, the primary nucleolin-specific antibody (goat polyclonal antibody C23: F-18; Santa Cruz Biotechnology, Dallas, TX; Catalogue # SC-9893) was used at 1:25 dilution at 4°C, and overnight incubation. The secondary antibody (biotinylated horse anti-goat IgG: Vector Labs, Burlingame, CA; Catalogue # BA9500) was used at 1:200 dilution, at room temperature for 30 min. The "ABC kit" (Vector Labs; PK=6100) with DAB chromogen was used for detection as per manufacturer's instructions. Immunostaining was carried out by the Histology Laboratory at the Toronto Centre for Phenogenomics (Toronto, Ontario, Canada).

For analysis and documentation, immunostained slides were scanned at the Advanced Optical Microscope Facility (University Health Network, Toronto, Ontario, Canada) and then viewed using ImageScope software (Version 11.1.2.760; Aperio Technologies, Leica Microsystem, Concord, Ontario, Canada) on a Dell computer workstation. A reproducible work setting was created and all images were viewed using the same brightness and magnification parameters and overseen by a credentialed and experienced anatomical pathologist (RGH).

Results

Figure 1 shows photomicrographs of sections of mouse tissue taken from the upper and lower respiratory tract and myocardium. In the respiratory tract, as expected, ciliated columnar epithelial cells (Fig. 1B,C) showed positive immunostaining for cell-surface nucleolin. In addition, alveolar lining epithelial cells (Fig. 1A), alveolar macrophages (Fig. 4C), and cardiomyocytes (Fig. 1E) were also immunopositive for cell-surface nucleolin. In the gastrointestinal tract (Fig. 2), gastric foveolar cells (Fig. 2A), enterocytes within the small bowel (Fig. 2B-D), colonic epithelial cells (Fig. 2E), and gallbladder (Fig. 2F) showed positive immunostaining for cell-surface nucleolin. The stratified, squamous non-keratinizing epithelium of the esophagus was negative. Cell-surface nucleolin immunoreactivity varied within the liver: hepatocytes were negative, while the luminal aspect of epithelial cells of bile ductules within portal triads (Fig. 2G), and extrahepatic bile ducts were positive. In the genitourinary tract (Fig. 3), positive immunostaining for cell-surface nucleolin was observed in renal tubular epithelial cells (Fig. 3A); prostate (Fig. 3B), seminal

Table 1. Mouse body sites and cell types showing positive surface immunostaining for nucleolin.

System	Organ	Cell type
Respiratory system	Lung	Alveolar lining epithelium
	Bronchi	Columnar epithelium
	Larynx	Columnar epithelium
	Turbinate	Columnar epithelium
Cardiovascular system	Heart	Cardiomyocytes
Gastrointestinal system	Stomach	Gastric foveolar cells
	Small intestine	Duodenal enterocytes
		Jejunal enterocytes
		Ileal enterocytes
	Colon	Colonic epithelium
Hepatobiliary system	Gallbladder	Gallbladder epithelium
	Bile ductule	Bile ductule epithelium
Genitourinary system	Kidney	Collecting duct epithelium
	Prostate	Prostatic epithelium
	Epididymis	Epididymal epithelium
	Seminal Vesicle	Seminal Vesicle epithelium
	Uterus	Endometrial cells
	Oviduct	Oviduct epithelium
	Ovary	Germinal epithelium
Hematopoietic and Lymphoid systems	Bone marrow	Bone marrow reticular cells
	Lymph node	Medullary sinus
	Lung	Alveolar macrophages
Nervous system	Conjunctiva	Conjunctival epithelium
	Spinal cord	Central duct lining cells
	Choroid Plexus	Secretory epithelium
	Olfactory bulb	Marginal cells
	Peripheral Nerve	Fascicle cells

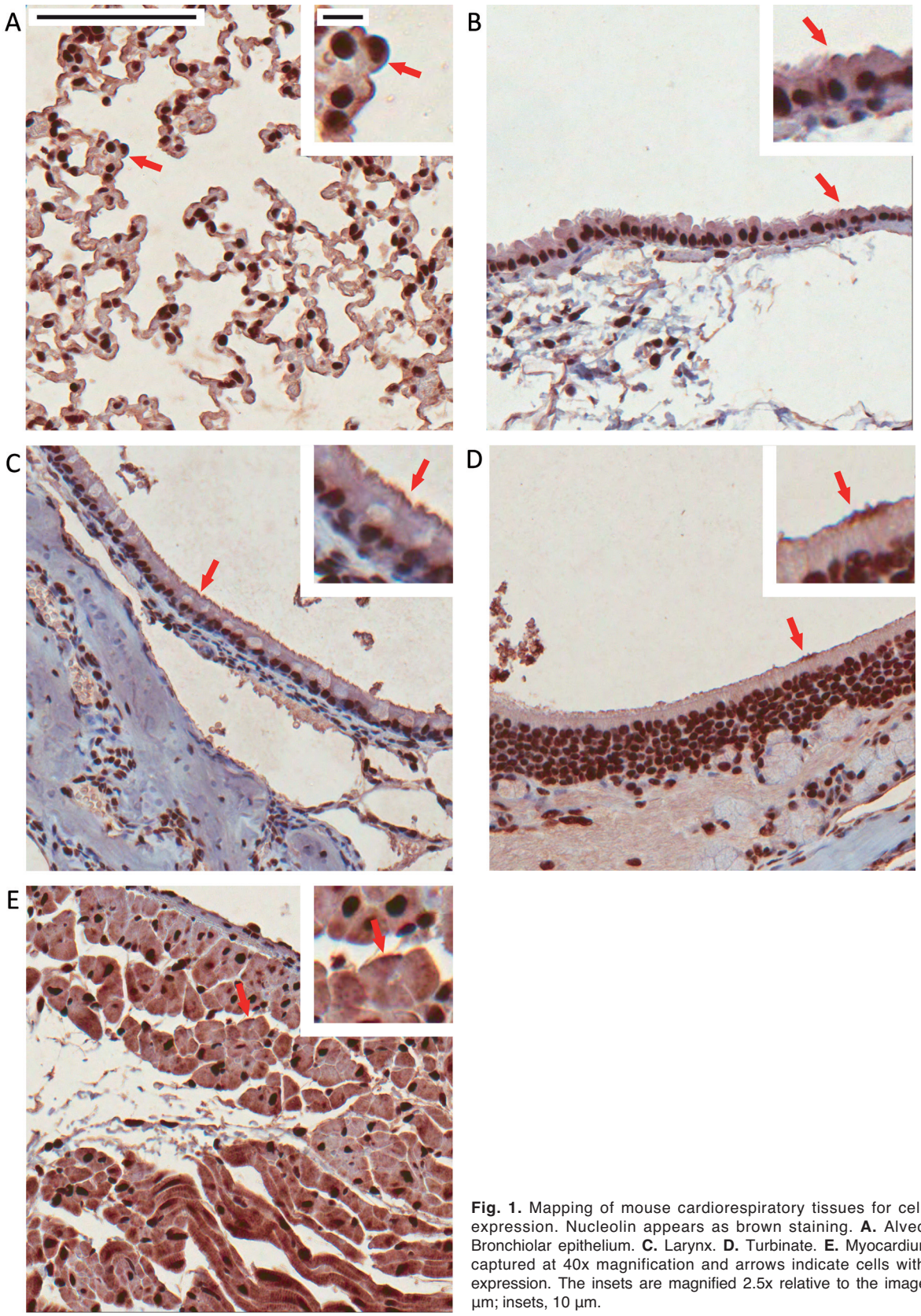

Fig. 1. Mapping of mouse cardiorespiratory tissues for cell-surface nucleolin expression. Nucleolin appears as brown staining. **A.** Alveolar epithelium. **B.** Bronchiolar epithelium. **C.** Larynx. **D.** Turbinate. **E.** Myocardium. All images were captured at 40x magnification and arrows indicate cells with surface nucleolin expression. The insets are magnified 2.5x relative to the images. Scale bars: 100 µm; insets, 10 µm.

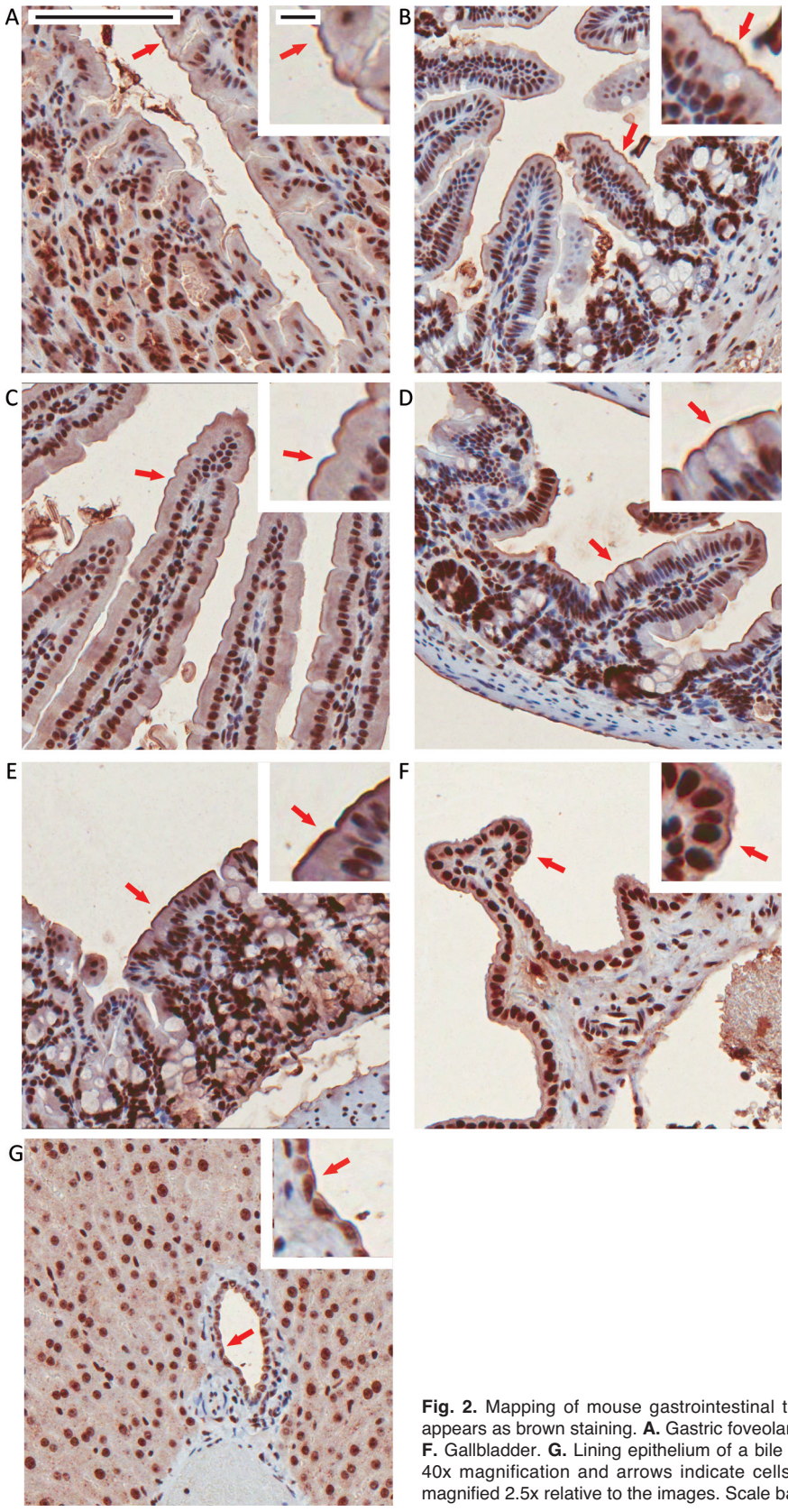

Fig. 2. Mapping of mouse gastrointestinal tract for cell-surface nucleolin expression. Nucleolin appears as brown staining. **A.** Gastric foveolar cells. **B.** Duodenum. **C.** Jejunum. **D.** Ileum. **E.** Colon. **F.** Gallbladder. **G.** Lining epithelium of a bile ductule in a portal triad. All images were captured at 40x magnification and arrows indicate cells with surface nucleolin expression. The insets are magnified 2.5x relative to the images. Scale bars: 100 µm; insets, 10 µm.

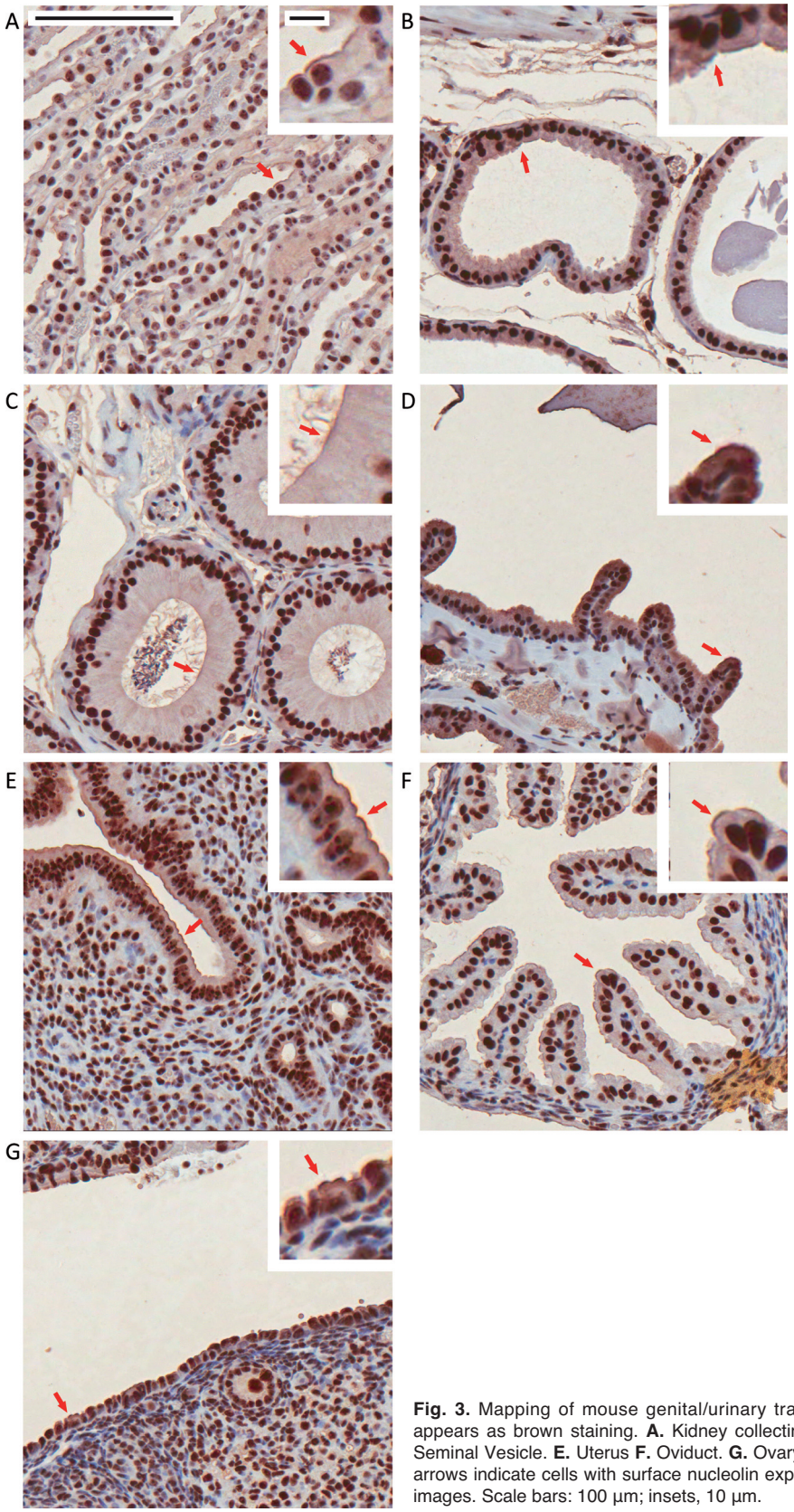

Fig. 3. Mapping of mouse genital/urinary tract for cell-surface nucleolin expression. Nucleolin appears as brown staining. **A.** Kidney collecting duct. **B.** Lateral prostate. **C.** Epididymal duct. **D.** Seminal Vesicle. **E.** Uterus **F.** Oviduct. **G.** Ovary. All images were captured at 40x magnification and arrows indicate cells with surface nucleolin expression. The insets are magnified 2.5x relative to the images. Scale bars: 100 μm; insets, 10 μm.

vesicle (Fig. 3D) and epididymal duct (Fig. 3C) in males; and uterus (Fig. 3E), oviduct (Fig. 3F) and ovary (Fig. 3G) in females. We also found surface nucleolin expression in bone marrow reticular cells (Fig. 4A) and the fibroblastic reticular cells within the medullary sinus of lymph nodes (Fig. 4B). In the eye, cell-surface nucleolin staining was observed in the conjunctiva (Fig. 5A); the lens and the retina were negative. Positive immunostaining for cell-surface nucleolin was also observed in olfactory bulb (Fig. 5B), secretory cells of the choroid plexus (Fig. 5C), lining cells of the spinal cord central canal (Fig. 5D), and within peripheral nerve fascicles (Fig. 5E). No cell-surface immunostaining for nucleolin was observed in the endocrine organs examined (pituitary, thyroid, pancreas, adrenal), mammary gland, salivary gland, skin, skeletal muscle, adipose tissue or cartilage (not shown). Table 1 summarizes the results of positive cell-surface nucleolin immunostaining in the mouse tissues examined. All mouse tissues examined showed a similar distribution of

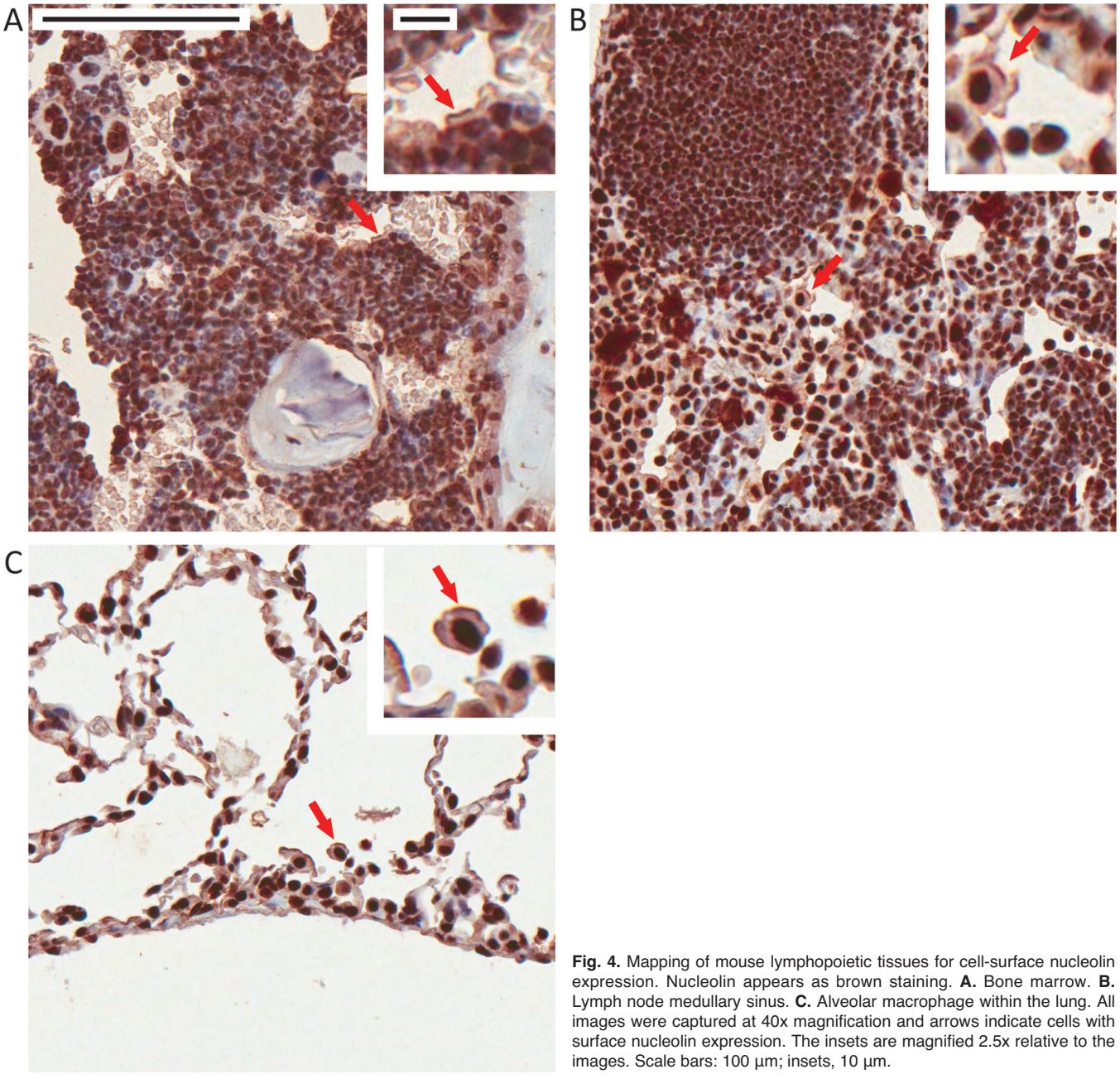

Fig. 4. Mapping of mouse lymphopoietic tissues for cell-surface nucleolin expression. Nucleolin appears as brown staining. **A.** Bone marrow. **B.** Lymph node medullary sinus. **C.** Alveolar macrophage within the lung. All images were captured at 40x magnification and arrows indicate cells with surface nucleolin expression. The insets are magnified 2.5x relative to the images. Scale bars: 100 µm; insets, 10 µm.

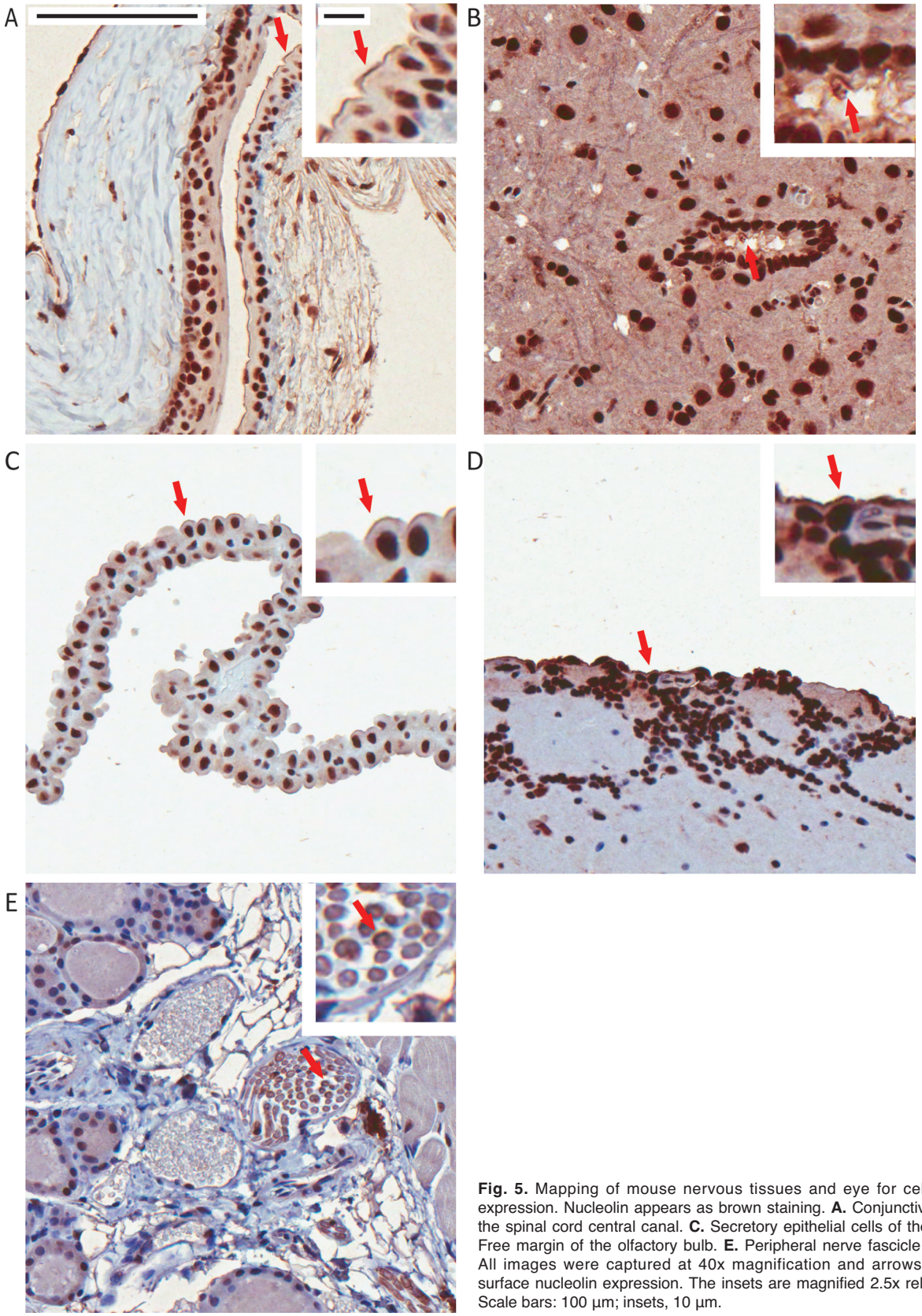

Fig. 5. Mapping of mouse nervous tissues and eye for cell-surface nucleolin expression. Nucleolin appears as brown staining. **A.** Conjunctiva. **B.** Lining cells of the spinal cord central canal. **C.** Secretory epithelial cells of the choroid plexus. **D.** Free margin of the olfactory bulb. **E.** Peripheral nerve fascicle adjacent to thyroid. All images were captured at 40x magnification and arrows indicate cells with surface nucleolin expression. The insets are magnified 2.5x relative to the images. Scale bars: 100 µm; insets, 10 µm.

positive immunostaining for cell-surface nucleolin.

Discussion

Expression of cell-surface nucleolin in the respiratory tract and myocardium

Our observations of cell-surface nucleolin expression by epithelial cells lining bronchioles and larynx, alveolar lining cells and alveolar macrophages are in agreement with the classical understanding of RSV as a respiratory tract pathogen and serve as a positive control for the evaluation of cell-surface nucleolin expression in other tissues. Interestingly, human larynx epidermoid carcinoma (HEp-2) cells are widely used for propagating human RSV *in vitro* (Wang et al., 2009) and our results confirm this cell type also expresses cell-surface nucleolin *in vivo*.

One of the main aims of this study was to examine whether the pattern of cell-surface nucleolin expression in mouse is congruent with the published descriptions of extrapulmonary manifestations of RSV infections. The presence of cell-surface nucleolin expression in cardiomyocytes correlates with numerous reports of cardiovascular pathologies observed in RSV infections. Myocarditis is the inflammation of heart muscle causing damage leading to cardiac dysfunction and possibly heart failure (Feldman and McNamara, 2000). While the most common viral agents of myocarditis are enteroviruses and adenoviruses, myocarditis has been reported in RSV-infected patients (Eisenhut, 2006; Menchise, 2011) and one study used PCR to document RSV nucleic acid in the heart tissue of a patient with myocarditis (Bowles et al., 2003). It is noteworthy that we found no evidence of surface nucleolin expression in skeletal muscle cells examined from various mouse body locations. By contrast, the expression of nucleolin on the surface of developing myotubes has been demonstrated (Alete et al., 2006) and underscores the complicated temporal aspects of nucleolin expression which should be noted in studies of its normal function.

Expression in the gastrointestinal tract

To our knowledge, cell-surface nucleolin expression in the healthy gastrointestinal tract has not been reported to date. Others have demonstrated surface nucleolin expression in human colonic carcinoma cells (Reyes-Reyes and Akiyama, 2008) and our results suggest that this may also be true for healthy colonic epithelial cells *in vivo*. The gastrointestinal tract is one of the few body sites which has surface nucleolin expression and which classically has not been associated with extrapulmonary manifestations of severe RSV infections. We postulate that for the gastrointestinal tract, the issue may be one of access of viable RSV to the cells: for example, the virus might not survive conditions of gastric acidity to successfully infect the stomach or move distally into the intestines.

Although RSV has a reported association with hepatitis (Eisenhut and Thorburn, 2002), our results did not demonstrate positive nucleolin immunostaining on the cell surface of hepatocytes. Rather, surface immunostaining was detected in the epithelial cells lining intrahepatic bile ductules, extrahepatic bile ducts and gallbladder. It may be that these epithelial cells were infected in the case of a child with RSV-positive liver tissue (Nadal et al., 1990).

Expression in the genitourinary and reproductive tract

In children, RSV has been associated with an exacerbation of nephrotic syndrome (MacDonald et al., 1986); in rats, nephropathy of RSV-inoculated animals was associated with the detection of RSV RNA in glomerular and tubular epithelial cells (Liu et al., 2007). While RSV-associated nephropathy may be related to a host immunological response to viral infection, our findings of cell-surface nucleolin immunostaining on collecting duct epithelium supports the possibility of direct RSV infection, as occurs with RSV infection of polarized renal epithelial cells *in vitro* (Roberts et al., 2005).

With respect to the reproductive tissues, investigators have demonstrated the susceptibility of wild-type Chinese hamster ovary cell line to RSV infection *in vitro* (Martinez and Melero, 2000). Although our findings of cell-surface nucleolin expression by the ovarian germinal epithelium are in agreement with this observation, we were not able to find literature reporting RSV infection of the male or female reproductive systems *in vivo*. Similar to the gastrointestinal tract, the lack of overt RSV infection of the reproductive system *in vivo* may reflect the inaccessibility of these body sites in a manner conducive to establishing a productive infection.

Expression in the hematopoietic and lymphoid systems

RSV is believed to spread to extrapulmonary sites via the hematogenous route (Thorburn and Hart, 2006) and this may be mediated by virus-infected leukocytes rather than the free virus carried within the bloodstream (Domurat et al., 1985; Halfhide et al., 2011). There is literature reporting that RSV can infect human bone marrow stromal cells *in vitro* (Rezaee et al., 2011), in agreement with our current observations. Moreover, alveolar macrophages obtained from immuno-compromised organ transplant recipients are permissive to RSV *in vivo* (Panuska et al., 1992). Although there is less known about the *in vivo* infection of alveolar macrophages of immunocompetent patients, several studies report cell-surface nucleolin expression on cultured alveolar macrophages (Wang et al., 2011) and permissiveness of alveolar macrophages to RSV infection *in vitro* (Panuska et al., 1990). In guinea pigs, RSV antigens have been found in lung Bronchus Associated Lymphoid Tissue (BALT) in the context of

chronic RSV persistence (Bramley et al., 1999). Our findings confirm the existence of cells potentially susceptible to RSV infection within hematopoietic and lymphoid tissues *in vivo*, attributable to cell-surface nucleolin expression.

Expression in eye and nervous system

Our observation of surface nucleolin in conjunctival epithelium was unexpected but lends support to the concept of RSV involvement in the pathogenesis of some cases of conjunctivitis (Fujishima, 2002). Interestingly, RSV-induced expression of interleukin-4 by conjunctival cells has been implicated in the development of allergy (Fujishima et al., 1998) and our observations are consistent with this finding. A recent paper (Espinoza et al., 2013) also provides evidence for the susceptibility of the nervous system to RSV infection in the context of post-infection cognitive impairment in rats. These investigators detected RSV proteins in the choroid plexus and the olfactory bulb of infected animals and demonstrated that RSV can gain access to the central nervous system after intranasal inoculation. Here, we observed cells with surface nucleolin expression in a number of neural tissues (Fig. 5) that correspond well to these findings, including cells expressing surface nucleolin in the margins of olfactory bulb and on the turbinate epithelium, which lends plausibility to the mechanism of intranasal inoculation of RSV described by Espinoza et al. We also noted that a subgroup of secretory epithelial cells of the choroid plexus branching fronds express nucleolin on their surface, as do cells lining the central canal of the spinal cord. Interestingly, there is a case report of detection of RSV nucleic acid in cerebrospinal fluid of an infant with RSV pneumonia (Zlateva and Van Ranst, 2004). We also showed surface nucleolin expression within peripheral nerve fascicles. The link between RSV and central apnea in infancy is now uncertain (Ralston and Hill, 2009) but the ability of RSV to infect nervous tissue may offer a potential mechanism for this finding. Taken together, our observations of surface nucleolin-expressing cells within nervous tissues is compatible with previous results that RSV can infect nerve cells *in vitro* and *in vivo* (Li et al., 2006) and are consistent with cell-surface nucleolin being a determinant for the susceptibility of the nervous system to RSV infection *in vivo*.

Conclusions

Relationship between cell-surface nucleolin expression and sites of RSV infection

Nucleolin is an essential protein that is expressed within the nucleus all eukaryotic cells and is expressed on the cell surface by a more limited spectrum of cells, particularly epithelial cells lining various visceral and tubular structures of the body, cells in the hematopoietic and lymphoid systems, central and peripheral nervous system, reproductive system and other sites such as ocular conjunctiva. Our observations suggest that cell-surface nucleolin expression provides a plausible mechanism for many reports describing extrapulmonary manifestations of severe RSV infections. Moreover, the apparent absence of cell-surface nucleolin in endocrine glands, mammary gland, salivary gland, skeletal muscle, cartilage and skin is consistent with RSV not being a known cause of infection of these particular body sites. Importantly, there were several body sites where cell-surface nucleolin was documented but for which there are no known clinical correlates of RSV infection at these sites (e.g., stomach, bowel and reproductive systems). In these instances, cell-surface nucleolin expression does not by itself explain RSV tropism, and alternative mechanisms that might affect the ability of infectious virus to access or to establish productive infection at these body sites, require further investigation.

Caveats

There are several important caveats to consider relating to the expression of cell-surface nucleolin for a given cell type that pertain to different physiological states of a cell, or its pathological alteration. One example is skeletal muscle, in which surface nucleolin expression has been documented for developing myotubes (Alete et al., 2006); by contrast, we did not observe surface nucleolin staining on any skeletal muscle tissue examined from mature mice. Another example is the vascular endothelium, in which "quiescent" endothelial cells do not express cell-surface nucleolin but can do so in certain circumstances, such as endothelial cells associated with angiogenic vessels (Christian et al., 2003) or with malignant tumors (Folkman, 2007). Cell-surface nucleolin expression on endothelium is of practical importance in the context of clinical trials currently underway in human cancer patients, with the idea that targeting nucleolin expressed on the surface of cancer-related blood vessels will preferentially kill the tumor cells while not adversely affecting normal cells and tissues. Another relevant example pertains to the liver. It has been demonstrated that hepatocellular carcinoma (Hep G2) cells express nucleolin on the cell surface (Deng et al., 1996) but our samples did not show such expression in hepatocytes *in vivo*. This latter example is consistent with the concept that cell-surface nucleolin expression is a feature of cells in pathological conditions, and that caution is required in drawing parallels to their healthy *in vivo* counterparts.

What does all this say about RSV tropism?

When we first identified nucleolin as a cellular RSV receptor we felt it was insufficient to explain RSV tropism since it is found in all eukaryotic cells. The results of this study provide an improved understanding of the distribution of cell-surface nucleolin expression in

a variety of tissues, and when considered together with current knowledge about RSV infections described in some of these same tissues, the model of viral tropism that pivots on a tissue/cell-type specific receptor accounts very well for many of the types of RSV infections that occur *in vivo*. The opportunity for viable virus to access susceptible body sites is likely a major reason to explain why RSV infections occur primarily in the respiratory tract; importantly, the findings of this study also suggest that RSV should not necessarily be excluded when determining the source of a viral infection of various non-respiratory tissues. In addition, understanding the tissue distribution of cell-surface nucleolin expression may have practical implications that affect the design and implementation of novel interventions for the treatment and prophylaxis of RSV infections *in vivo*.

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