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Human cytomegalovirus and mucoepidermoid carcinoma of salivary glands: Cell-specific localization of active viral and oncogenic signaling proteins is confirmatory of a causal relationship

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ABSTRACT

Human cytomegalovirus (hCMV) infection is common. Although still controversial, there is growing evidence that active hCMV infection is associated with a variety of malignancies, including brain, breast, lung, colon, and prostate. Given that hCMV is frequently resident in salivary gland (SG) ductal epithelium, we hypothesized that hCMV would be important to the pathogenesis of SG mucoepidermoid carcinoma (MEC). This was initially supported by our finding that purified CMV induces malignant transformation in SG cells in an *in vitro* mouse model, and utilizes a pathogenic pathway previously reported for human MEC. Here we present the histologic and molecular characterizations of 39 human SG MECs selected randomly from a repository of cases spanning 2004–2011. Serial sections were obtained from formalin-fixed, paraffin embedded, tissue blocks from previous incisional or excisional biopsies. Immunohistochemical assays were performed for active hCMV proteins (IE1 and pp65) and the activated COX/AREG/EGFR/ERK signaling pathway. All four prospective causal criteria for viruses and cancer are fully satisfied: (1) protein markers for active hCMV are present in 97% of MECs; (2) markers of active hCMV are absent in non-neoplastic SG tissues; (3) hCMV-specific proteins (IE1, pp65) are in specific cell types and expression is positively correlated with severity; (4) hCMV correlates and colocalizes with an upregulation and activation of an established oncogenic signaling pathway (COX/AREG/EGFR/ERK). Thus, the evidential support reported here and previously in a mouse model is strongly confirmatory of a causal relationship between hCMV and SG mucoepidermoid carcinoma. To our knowledge, this is the first demonstration of hCMV's role in human oncogenesis that fully responds to all of Koch's Postulates as revised for viruses and cancer. In the absence of any contrary evidence, hCMV can reasonably be designated an "oncovirus."

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Introduction

Human cytomegalovirus (hCMV), both active and latent, has a particular tropism for salivary gland (SG) ductal epithelium (Lagenaur et al., 1994; Nichols and Boeckh, 2000; Wagner et al., 1996). hCMV infection is common, 50–95% of adults being seropositive, depending on geographical and socioeconomic status (Boppana and Fowler, 2007; Bruggeman, 1993). Like other herpesviruses, hCMV establishes lifelong persistence and latent infection following primary exposure (Nichols and Boeckh, 2000). Viral major immediate-early (MIE) gene expression (*e.g.* IE1, IE2) is highly reduced or absent during hCMV latency, precluding a productive viral life cycle (Yuan et al., 2009). Specifically, early hCMV gene expression requires *de novo* synthesis of IE and cellular proteins for their transcription (White and Spector, 2007). While it is known that the MIE enhancer/promotor is regulated by coordinated expression of various *cis*-acting elements, the precise triggering mechanisms that promote hCMV reactivation are unknown (Yuan et al., 2009).

hCMV primary, recurrent and secondary infections are associated with various well-documented adverse consequences, including asymptomatic or febrile viruria in immunocompetent hosts, serious congenital disorders (deafness, blindness, mental retardation) in newborns, infants and toddlers, and frequent opportunistic infections in immunocompromised patients that have high morbidity and mortality. There is growing evidence that active hCMV infection is associated with a variety of malignancies, including brain, breast, lung, colon and prostate (Cobbs et al., 2002, 2007, 2008; Giuliani et al., 2007; Harkins et al., 2002, 2010; Lucas et al., 2010; Samanta et al., 2003; Scheurer et al., 2008). Multiple studies indicate that by

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dysregulating host signaling pathways, hCMV likely modulates the cellular environment, as well as initiates and/or promotes tumorigenesis and other pathoses (Slinger et al., 2011; Soroceanu and Cobbs, 2011).

Mucoepidermoid carcinoma (MEC) is the most common malignant tumor originating in major and minor salivary glands (SGs), accounting for about one-third of all SG carcinomas (Lujan et al., 2010; Schwarz et al., 2011). Advanced stage (TNM stage III–IV) and higher grade cancers are often associated with a poor prognosis and necessitate radical surgical resection. MECs are histologically and genetically heterogeneous (Schwarz et al., 2011). For example, approximately 40% contain the t(11;19) chromosomal translocation-generated CRTC1-Maml2 fusion protein which is associated with distinct tumor subtypes and clinicopathologic features (Bell et al., 2010; Kaye, 2009; O'Neill, 2009; Okumura et al., 2011). Further, there is variable overexpression of COX-2, as well as the EGFR/ERK signaling pathway (Akrish et al., 2009; Ito et al., 2009; Lujan et al., 2010). All this notwithstanding, the etiology of MEC is unknown.

Given that hCMV is frequently resident in SG ductal epithelium (Lagenaur et al., 1994), that cancer-related inflammation and its related induction of genetic instability is a hallmark of cancer (Colotta et al., 2009), and that other herpesviruses are documented "oncoviruses" [*e.g.* EBV and KSHV (Ganem, 1997; Young and Rickinson, 2004)], we hypothesized that CMV would be an important component of MEC pathogenesis. To address this hypothesis, we recently developed a novel mouse postnatal SG organ culture model of mouse CMV (mCMV)-induced tumorigenesis that displays histologic and molecular characteristics similar to human MEC (Jaskoll et al., 2011; Melnick et al., 2011). To wit, there is *de novo* re-expression of CRTC1 protein with initial tumor formation, as well as a dramatic upregulation of the activated COX/AREG/EGFR/ERK signaling pathway. Additionally, the CMV-induced neoplastic phenotype was precluded or ameliorated by treatment with either anti-virals or small molecule inhibitors of several key targets in the autocrine loop.

In the present study, we investigate human MEC, and provide a histologic and molecular characterization of 39 human specimens of salivary gland MECs selected randomly from a repository of cases spanning 7 years (2004–2011). Informed by a revision of Koch's Postulates that speaks to the emerging relationship between viruses and cancer (Evans, 1976; Fredricks and Relman, 1996; Soroceanu and Cobbs, 2011), we have now addressed the following five causal criteria: (1) hCMV is present in most cases of human MEC; (2) only neoplastic tissue harbors hCMV, not surrounding normal human SG tissue; (3) hCMV-specific gene expression is demonstrable at the human SG cellular level and is positively correlated with human MEC severity; (4) hCMV infection of human MEC cells is correlated and colocalized with an upregulation of a known oncogenic signaling pathway; (5) purified CMV induces malignant transformation (MEC) in SG cells in an *in vitro* animal model, and utilizes a pathogenetic pathway similar to hCMV-induced MEC. The outcomes of the present study satisfy the first four criteria; extensive prior studies satisfy the fifth (Jaskoll et al., 2011; Melnick et al., 2011). Thus, in the absence of any hitherto unfavorable evidence, these results are confirmatory of a causal relationship between hCMV and salivary gland MEC.

Methods

Institutional review board approval was obtained for this study (USC IRB # UP-11-00091). Human specimens of salivary gland MEC spanning 7 years (2004–2011) from head and neck sites were randomly selected for this study; cases were accessioned from the Oral Pathology Laboratory at Ohio State University as formalin-fixed, paraffin-embedded, tissue blocks from previous incisional or excisional biopsies to allow for routine histologic staining and immunohistochemical studies. All cases were independently diagnosed as MEC by two board certified oral and maxillofacial pathologists. Cases were histologically graded as low, intermediate and high grades using a modified Healey system (Seethala, 2009). For inclusion in this study, cases needed to have adequate tissue bulk available in each block to allow for multiple recuts and staining; patients of any age, sex or ethnicity could be included in the study. Cases were excluded if the diagnosis of MEC was questionable; mucicarmine staining, with or without pancytokeratin staining, was performed on cases when necessary to establish an accurate diagnosis of MEC. A total of 39 tumors were selected for study, including low, intermediate and high grade tumors from minor and major salivary glands, as well as central (primary intraosseous) tumors of the jaw. Minor salivary gland tissue from healthy patients was used as control tissue for histopathologic and immunohistochemical studies and internal controls were also available in most cases as unaffected tumor-adjacent salivary gland tissue.

Histology and immunohistochemistry

All histological and immunohistochemical analyses were performed on 4 μm thick serial sections. For histology, tumor sections were stained with hematoxylin and eosin using routine methodology. Immunohistochemistry for active human CMV (hCMV) proteins IE1-72 (an immediate early gene product) and pp65 (a delayed early gene product) was performed by first heat-induced epitope retrieval in 10 mM citrate buffer (IE1) or 1 mM EDTA pH 8.0 (pp65) for 15 min, followed by blocking for 10 min with 3% peroxidase. Sections were incubated with anti-IE1-72 (MAB810, clone 8B1.2, Millipore, Temecula, CA) or anti-pp65 (NCL-CMVpp65, clone 2 and 6, Leica Microsystems, Newcastle, UK) overnight at room temperature. These antibodies have previously been shown to specifically detect hCMV IE1and pp65 proteins (*e.g.* Harkins et al., 2002; Lucas et al., 2010; Samanta et al., 2003). Detection was performed using a DAKO Envision™ + Dual Link horseradish peroxidase system (DAKO Cytomation, Carpinteria, CA) and the chromagen diaminobenzidine and the sections were counterstained with hematoxylin and eosin as previously described (Melnick et al., 2011). Negative controls consisted of tumor or normal salivary gland sections incubated in the absence of primary antibodies; internal controls consisted of unaffected tumor-adjacent salivary gland tissue. IE1 and pp65 proteins were not detected in normal and tumor sections incubated in the absence of primary antibody. Multiple sections from all 39 tumors and 3 control salivary glands were immunostained for IE1 and 30 tumors and 3 control glands were immunostained for pp65.

Immunolocalization of COX-2, amphiregulin, activated EGFR, and activated ERK1/2

Sections were first subjected to antigen retrieval in citrate buffer, blocked with 3% peroxidase, and incubated overnight at room temperature with anti-phosphorylated ERK1/2 (Thr202/Tyr204) (MAPK phosphor-44/42) (sc-16982R); anti-phosphorylated EGFR (Tyr1173)(sc-101668), anti-amphiregulin (AREG) (H-155) (sc-25436) (Santa Cruz Biotechnology Inc, Santa Cruz, CA) and anti-COX2 (cat #1601063) (Cayman Chemicals, Ann Arbor, MI). Detection was performed as described above. Controls consisted of tumor or normal salivary gland sections incubated in the absence of primary antibodies. COX-2, AREG, pEGFR and pERK1/2 were not detected in normal and tumor sections incubated in the absence of primary antibody. For each antibody, multiple sections from 7 to 10 MEC tumors with active hCMV infection (IE1 immunolocalization) and 3 normal glands were analyzed.

Results

Table 1 summarizes the key demographic, clinical and histopathologic features of the tumors characterized in this study. Histopathologic

Table 1
Demographic and clinicopathologic features of patients with MEC.

Age range/mean	Sex/ratio	Ethnicity	Tumor type	Histologic grade
18 to 89 years	Female = 28	Caucasian = 30	Minor gland MEC = 35	Low = 30
	Male = 11	African-American = 1	(2 clear-cell variants)	Intermediate = 5
Mean = 55 years	F:M = 2.5:1	Unknown = 8	Major gland MEC = 1	High = 4
			Central MEC = 3	

examination of normal salivary gland control tissue as compared to MEC reveals characteristic findings. Normal salivary gland parenchyma is comprised of pale staining lobules containing mucous acini and tubules with associated ducts and fibrous stroma (Figs. 1A–B). The MEC tumors include low, intermediate and high grade lesions from minor and major salivary glands, as well as central intraosseous lesions (Figs. 1C–H). Tumors are characterized by invasive islands of mucous and epidermoid (squamoid) cells, with or without cyst formation, and, in some tumor islands, an intermediate basaloid-like cell type is seen. Two cases do not demonstrate these classic MEC features because they are variants histologically comprised of predominantly clear cells. A host inflammatory response is present in many cases, usually chronic in nature with occasional acute inflammatory cell infiltrates. Mucous cells within tumors vary in size and shape and demonstrate abundant foamy granular cytoplasm and mucicarmine positivity. Epidermoid cells appear ovoid to polygonal and intercellular bridges and individual cell keratinization can be seen. Low grade tumors show prominent cyst formation, predominance of mucous cells, and minimal cellular atypia. High grade tumors show little to no cyst formation, predominance of epidermoid and intermediate cells, and more frequent cellular atypia. Aberrant mitotic figures and prominent nucleoli are seen in higher grade lesions in addition to pleomorphism among tumor cells. Intermediate grade tumors show features that overlap with low and high grade tumors. Within tumor islands of MEC, a cytomegalic "owl-eye" microscopic appearance in addition to viral inclusion bodies is evident in a subset of tumor cells; importantly, these features are seen in minor, major and central tumors which include low (Figs. 1G–H), intermediate (Figs. 1E–F) and high grade (Figs. 1C–D) lesions. These features, when present, are most often seen in epidermoid and intermediate tumor cells and not mucous or stromal cells.

Immunolocalization of hCMV IE1 and pp65 proteins in low, intermediate and high grade salivary gland MECs

We performed immunohistochemical staining of the 39 MECs and 3 normal salivary glands using an antibody specific for the hCMV-encoded IE1 protein, an immediate early gene product of active viral infection. IE1 is detected in 38/39 MECs (Figs. 2B–D), but not in normal salivary glands (data not shown) or unaffected tumor-adjacent regions (Fig. 2A). IE1 immunoreactivity is characterized by a coarse and granular pattern of brown hue, with high chroma and saturation. IE1 immunostaining is seen in all grades of tumors, specifically epidermoid and intermediate tumor cells but not mucous or stromal cells (Figs. 2B–D). The frequent localization of IE1 in nuclei of dysplastic cells and in viral inclusion bodies is indicative of active infection (Yuan et al., 2009). IE1 immunostaining is also seen in the perinuclear cytoplasm (Figs. 2C–D), a reflection of viral protein kinetics. Importantly, there is a marked increase in IE1 immunostaining in higher grade tumors as compared to lower grade lesions (compare Figs. 2D to C, B and C to B).

Antibodies against pp65 determined that delayed hCMV integument protein pp65 is expressed in these tumors. Specifically, pp65 immunostaining is found in neoplastic epidermoid and intermediate tumor cells, as well as in stromal inflammatory cells, in all grades of MECs (Figs. 3B–D). In contrast, pp65 immunostaining is absent in adjacent unaffected tumor-adjacent regions (Fig. 3A), normal glands (data not shown) or tumor sections incubated in the absence primary antibody (data not shown). Compared to IE1, pp65 immunoreactivity is characterized by a finer granular pattern of brown hue, with lower chroma and saturation. The pattern of nuclear and cytoplasmic localization of pp65 immunostaining, as well as its presence in viral inclusion bodies, is similar to that seen for IE1 (compare Figs. 3B–D to 2B–D). Our demonstration of hCMV IE1 and pp65 proteins in human salivary gland MECs is consistent with prior reports of these viral proteins being present in other human tumors, including glioblastoma, and colorectal, mammary gland, and prostate carcinomas (*e.g.*Harkins et al., 2002, 2010; Lucas et al., 2010; Samanta et al., 2003). Taken together, the presence and nuclear localization of hCMV IE1 and pp65 proteins are indicative of active viral infection. Indeed, the IE1 and pp65 immunohistochemical assays performed on these tumors are identical to that used to establish active hCMV infection in clinical patient care.

COX-2, amphiregulin, activated EGFR and activated ERK1/2 localization

Prior work in our laboratory has demonstrated the importance of the COX/AREG/EGFR/ERK pathway for mCMV-induced mouse salivary gland tumorigenesis (Melnick et al., 2011). Other studies have demonstrated: (1) COX-2 overexpression in malignant salivary gland tumors, including MECs, as well as in other types of tumors; (2) the correlation between active hCMV infection and COX-2 expression in colorectal carcinoma; (3) that the COX-2/PGE2/EP4 pathway can induce amphiregulin (AREG), a EGFR ligand, and thereby activate EGFR signaling and cell proliferation and (4) that the EGFR/ERK pathway is activated in high grade MEC of the salivary glands and correlated with overall survival (Akrish et al., 2009; Harkins et al., 2002; Liu and Yang, 2007; Lujan et al., 2010; Melnick et al., 2011; Saba et al., 2009; Yonesaka et al., 2008). Given the above, we postulated that we would see an overexpression of COX-2, AREG, activated EGFR [phosphorylated (pEGFR)], and activated ERK1/2 [phosphorylated (pERK1/2)] proteins in human salivary gland MECs which exhibit hCMV IE1 and pp65 expression. Thus, we determined the cell-specific distribution of COX-2, AREG, pEGFR, and pERK proteins in hCMV-infected low, intermediate and high grade MECs as compared to normal salivary gland tissues. As shown in Figs. 4A–D, COX-2, AREG, pEGFR and pERK colocalize in epidermoid and intermediate tumor cells, areas of IE1 localization (Fig. 4, inset). In contrast, these proteins are mostly absent in normal salivary glands (data not shown) and unaffected tumor-adjacent regions (data not shown). Our observation of COX-2, EGFR, and ERK1/2 overexpression in salivary gland MECs is consistent with previous reports on human salivary gland MECs (Akrish et al., 2009; Ito et al., 2009; Lujan et al., 2010), and in an *in vitro* mouse model of MEC (Melnick et al., 2011). Importantly, our data suggests that hCMV induction of the COX-2/AREG/EGFR/ERK pathway may play an important role in human salivary gland tumorigenesis.

Discussion

The evidence presented above (Figs. 1–4), strongly implicates hCMV in the etiology and pathogenesis of human salivary gland MEC. All four prospective causal criteria for viruses and cancer (see Introduction) have been fully satisfied: (1) histologic and immunohistochemical (protein) markers of active hCMV infection are *present*

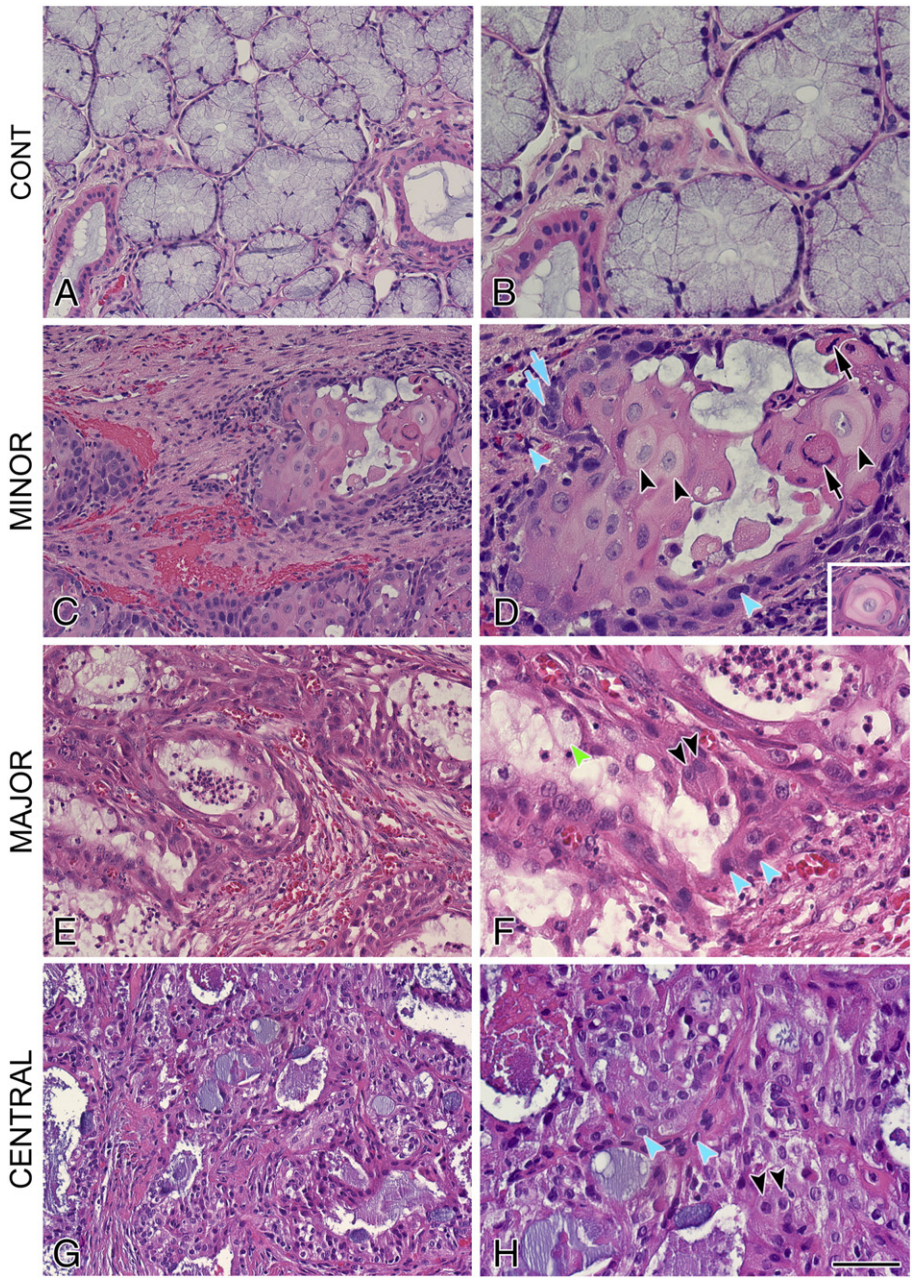

Fig. 1. Histological characteristics of normal salivary glands and salivary gland mucoepidermoid carcinomas. A. Representative normal salivary gland parenchyma which is comprised of characteristic pale staining lobules of mucous acini and tubules with associated ducts and fibrous stroma. B. Higher magnification of normal salivary gland tissue shown in A shows the tubular arrangement of mucous cells which have dense appearing nuclei compressed at the basal end of the cell with small central patent tubules evident at the apical portion where cells converge. A normal salivary duct is seen in the lower left and appears as a single layer to bilayer of cuboidal cells with granular eosinophilic cytoplasm; pale staining mucous is seen within the central lumen. C. Mucoepidermoid carcinoma of a minor salivary gland is characterized by invasive islands of mucous and epidermoid (squamoid) cells within a fibrocollagenous connective tissue that demonstrates erythrocyte extravasation and a chronic inflammatory cell infiltrate. The tumor islands to the left and bottom right show more squamoid features and the island to the right shows a mixture of squamoid and mucoid features. D. Higher magnification of the cancer in C shows a characteristic tumor island with mucous and epidermoid cells. A third or intermediate basaloid cell type (blue arrows) can also be seen at the periphery of the tumor island and is thought to be the progenitor cell of mucous and epidermoid cells. The mucous cells vary in size and shape and contain abundant foamy cytoplasm that appears slightly basophilic and stains positively with mucin stains (data not shown). The epidermoid cells appear ovoid to polygonal and intercellular bridges and individual cell keratinization can be seen. Mitotic figures (black arrows) can be seen within some of the pleomorphic tumor cells. Note the characteristic "owl-eye" cell appearance (black arrowheads) and the presence of viral inclusion bodies (blue arrowheads) in a subset of tumor cells. Inset: High magnification of a cytomegalic tumor cell exhibiting "owl-eye" appearance. E. Mucoepidermoid carcinoma of a major salivary gland (parotid) characterized by invasive islands and cords of mucous and epidermoid cells within a fibrovascular connective tissue that has erythrocyte extravasation and inflammation. Again, mucous-producing tumor cells can be seen that contain abundant foamy cytoplasm that appear more granular and eosinophilic. F. Higher magnification of the cancer shown in E showing "owl-eye" cells (black arrowheads) and viral inclusion bodies (blue arrowheads) in addition to a neutrophilic microabscess (top) which indicates an acute inflammatory response in this tumor. Cytoplasmic granularity is seen in mucous-producing tumor cells (green arrowhead) which can be characteristic of mucoepidermoid carcinoma. G. A central (primary intraosseous) mucoepidermoid carcinoma showing histopathologic features similar to those seen with the minor and major glands above. Islands and cords of invasive mucoepidermoid cancer within a fibrocollagenous stroma are again evident in addition to several microcystic regions containing mucinous debris. H. Higher magnification of the cancer shown in G reveals aberrant tumor cells with increased nuclear-to-cytoplasmic ratios, pleomorphism, hyperchromatism and prominent nucleoli in some cases. Several tumor cells show evidence of viral inclusion bodies (blue arrowheads), as well as the characteristic "owl-eye" appearance (black arrowheads). Bar: A, C, E, G = 60 μm; B, D, F, H = 30 μm; inset D = 25 μm.

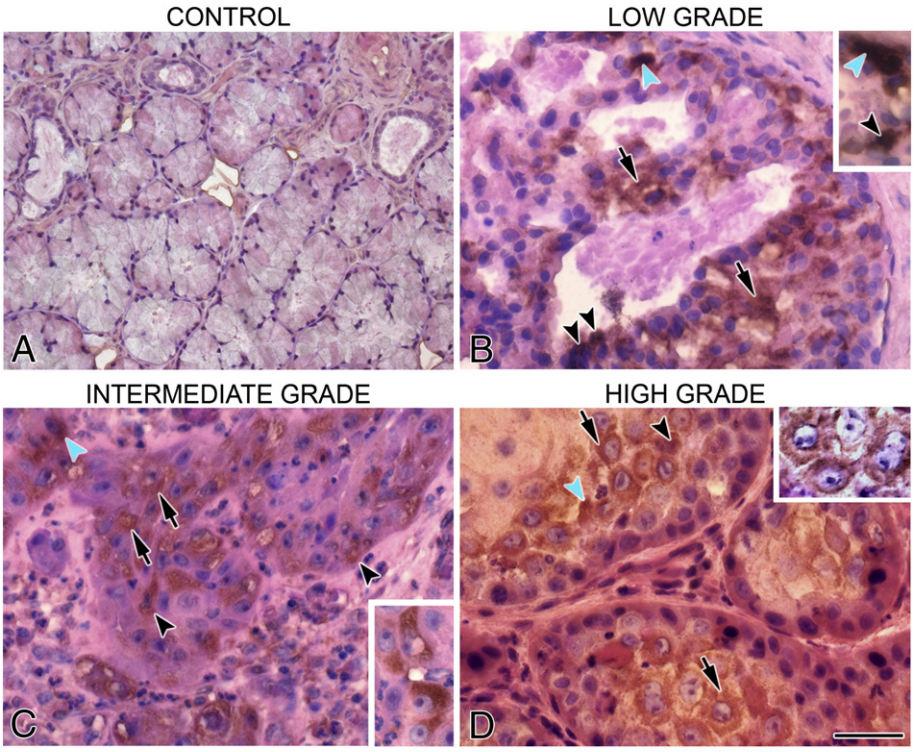

Fig. 2. Immunohistochemical localization of HCMV IE1 in all grades of MEC tumors. A. Low magnification of representative normal salivary gland tissue showing the absence of IE1 immunostaining in unaffected tumor-adjacent salivary gland tissue in a low grade tumor. B–D. Immunolocalization of IE1 in low (B), intermediate (C) and high (D) grade MECs. B. Low grade MEC exhibiting nuclear (black arrowheads) and cytoplasmic (black arrows) localization of IE1 immunostaining; IE1 is also seen in viral inclusion bodies (blue arrowheads). Inset: Higher magnification showing IE1 staining in nuclei and viral inclusion bodies. C. Intermediate grade MEC exhibiting IE1 immunostaining in nuclei (black arrowheads), cytoplasm (black arrows) and viral inclusion bodies (blue arrowheads). Inset: Higher magnification showing IE1 immunostaining in "owl-eye" tumor cells. D. High grade mucoepidermoid carcinoma exhibiting IE1 immunostaining. Nuclear (black arrowheads) and cytoplasmic (black arrows) IE1 immunostaining are seen; IE1 staining is also found in viral inclusion bodies (blue arrowheads). There is a notable increase in IE1 immunolocalization in higher grades of tumors (compare D to B, C and C to B). There is also a noted increase in cytoplasmic localization of IE1 in higher grades of tumors (compare D to B, C and C to B). Inset: Higher magnification showing IE1 immunostaining. Bar: A = 60 μm; B–D = 30 μm; insets = 20 μm.

in 97% (38/39)of MECs; (2) histologic and immunohistochemical (protein) markers of active hCMV are *not present* in non-neoplastic tumor-adjacent regions or in normal salivary glands; (3) hCMV-specific protein expression (IE1, pp65) is clearly demonstrable in specific MEC cell types and the extent of this expression is positively correlated with the severity of malignancy (grade); (4) hCMV infection of human MEC cells is correlated and colocalized with a clear upregulation and activation of an established oncogenic signaling pathway [COX-2/AREG/EGFR/ERK (see Melnick et al., 2011 for details)]. To our knowledge, this is the first demonstration of hCMV's likely role in human SG oncogenesis, and is all the more remarkable because in most cancers caused by viral infections the DNA is present in very small copy number, most often less than one DNA copy per 10 tumor cells (Sampson and Mitchell, 2011).

Importantly, these findings are corroborated by the prior satisfaction of the fifth causal criterion (Evans, 1976), namely the ability of purified virus to induce malignant transformation of SG cells in an *in vitro* animal model, using a similar oncogenic signaling pathway (Jaskoll et al., 2011; Melnick et al., 2011). Using small molecule inhibitors to target several key steps in the signaling pathway, and in this way ameliorate pathology, we previously demonstrated that ERK phosphorylation is necessary for initial mouse CMV-induced tumorigenesis, and that ErbB receptor family phosphorylation and downstream signaling will be likely targets for drug discovery.

Although the precise mechanism of human CMV-related SG tumorigenesis is unknown, there is sufficient human and mouse data to provide an informed, albeit putative, framework within which future studies might proceed (Fig. 5). MEC of the salivary gland has long been thought to originate from excretory duct pluripotent progenitor cells (Auclair and Ellis, 1991; Eversole, 1971; Regezi and Batsakis, 1997; Feng et al., 2009). It has also been demonstrated in a variety of craniofacial tissues (*e.g.* brain and salivary gland) that CMV has tropism for pluripotent progenitor cells and not uncommitted stem cells (Kawasaki et al., 2011; Melnick et al., 2006; Michaelis et al., 2009; Jaskoll et al., 2008a, 2008b, 2010, 2011; Tsutsui, 2009). Taken together, these observations suggest two, not mutually exclusive, possibilities (Fig. 5A): CMV directly initiates malignant transformation of SG progenitor cells and subsequent oncomodulation of these cells *and/or* CMV promotes oncomodulation of already precancerous SG stem cells (see reviews Barami, 2010; Michaelis et al., 2009, 2011; Soroceanu and Cobbs, 2011). Evidence for one seemingly key oncomodulatory pathway (Fig. 5B) is presented here (Fig. 4) and in multiple other human and mouse studies (Akrish et al., 2009; Ito et al., 2009; Lujan et al., 2010; Melnick et al., 2011; Michaelis et al., 2009).

Conclusion

If we are to propose that hCMV is etiologically related to MEC, then we must show evidence that the revised and relevant Koch's Postulates are operative. Failure to do so would negate the etiologic claim. Success in doing so, however, does not provide etiologic certainty, for such a claim would mire us in the fallacy of affirming the consequent (aka "proving the null hypothesis"). Nevertheless, in the absence of any contrary evidence, the evidential support reported here (Figs. 1–4) and elsewhere (Jaskoll et al., 2011; Melnick et al., 2011) are strongly confirmatory of a causal relationship between hCMV and salivary gland mucoepidermoid carcinoma. Thus, hCMV can reasonably be designated an "oncovirus."

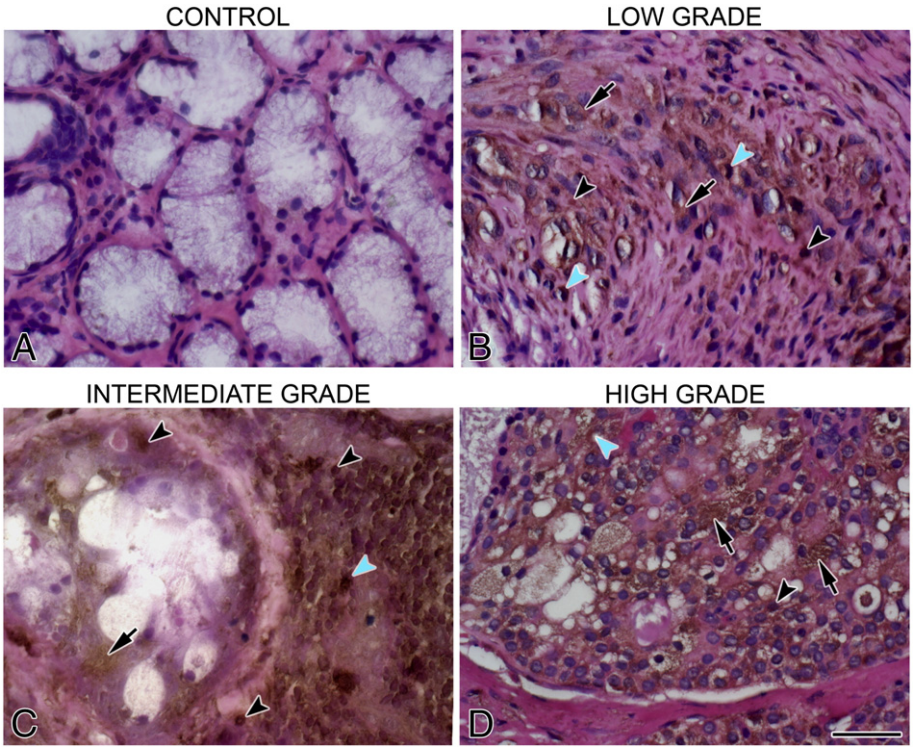

Fig. 3. Immunohistochemical localization of CMV pp65. A. Representative normal salivary gland in unaffected tumor-adjacent salivary gland tissue in a low grade tumor. Note the absence of pp65 immunostaining in serous and mucinous acini. B–D. pp65 immunolocalization in low (B), intermediate (C) and high (D) grade MECs. In all grades of tumors, pp65 immunostaining is seen in nuclei (black arrowheads) and cytoplasm (black arrows), as well as in viral inclusion bodies (blue arrowheads). There is a notable increase in immunodetectable pp65 in high grade tumors as compared to low and intermediate grade tumors (compare D to B, C). Bar: A–D = 30 μm.

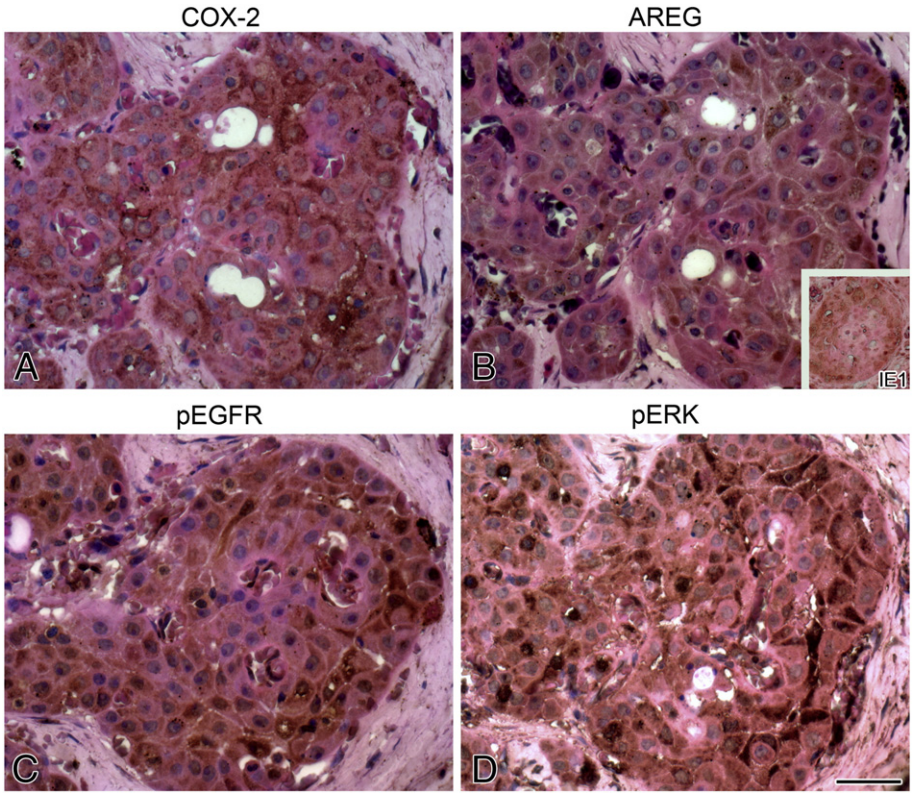

Fig. 4. Colocalization of COX-2, AREG, activated EGFR and activated ERK1/2 in serial sections of an intermediate grade, CMV-infected MEC. The presence of HCMV-IE1 immunostaining (inset B) indicates that this salivary gland tumor has active viral infection. A. COX-2 immunostaining. B. AREG immunostaining. C. Activated EGFR (pEGFR) immunostaining. D. Activated ERK1/2 (pERK) immunostaining. COX2, AREG, pEGFR, and pERK colocalize in epidermoid tumor islands but are mostly absent in adjacent normal tissue (data not shown). A–D = 30 μm; inset B = 40 μm.

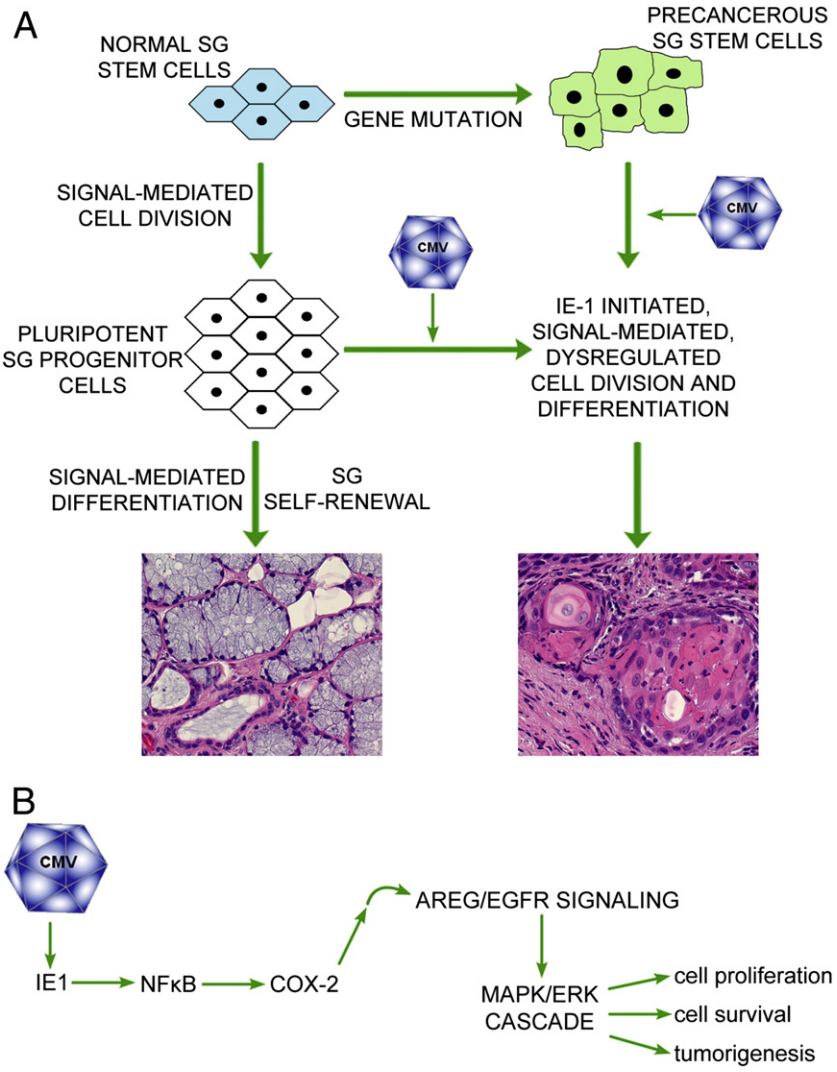

Fig. 5. Model of putative pathogenesis for CMV-induced mucoepidermoid carcinoma of salivary glands. See text for details.

Conflicts of interest

We declare that we have no conflicts of interest.

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