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CRTC1 Expression during Normal and Abnormal Salivary Gland Development Supports a Precursor Cell Origin for Mucoepidermoid Cancer

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Abstract

Dysregulation of the transcription factor CRTC1 by a t(11;19) chromosomal rearrangement mediates the formation of mucoepidermoid salivary gland carcinoma (MEC). Although the *Crtc1* promoter is consistently active in fusion-positive MEC and low levels of *Crtc1* transcripts have been reported in normal adult salivary glands, the distribution of CRTC1 protein in the normal salivary gland is not known. The aim of this study was to determine if CRTC1, like many known oncogenes, is expressed during early submandibular salivary gland (SMG) development and re-expressed in an experimental tumor model. Our results indicate that CRTC1 protein is expressed in SMG epithelia during early stages of morphogenesis, disappears with differentiation, and reappears in initial tumor-like pathology. This stage-dependent expression pattern suggests that CRTC1 may play a role during embryonic SMG branching morphogenesis but not for pro-acinar/ acinar differentiation, supporting a precursor cell origin for MEC tumorigenesis. Moreover, the coincident expression of CRTC1 protein and cell proliferation markers in tumor-like histopathology suggests that CRTC1-mediated cell proliferation may contribute, in part, to initial tumor formation.

Keywords

CREB coactivator; CRTC1; salivary glands; development; tumorigenesis; cell proliferation; mucoepidermoid carcinoma

1. Results and Discussion

Mucoepidermoid carcinoma (MEC) is the most frequent primary malignancy of salivary glands in both children and adults, representing 5% of all salivary gland tumors and 35% of

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all malignant forms (Enlund et al., 2004; Behboudi et al., 2006; Kaye, 2009; O'Neill, 2009). MECs, composed of mucous forming goblet, epidermal and intermediate cells, are thought to originate in the excretory and intercalated ducts of the salivary gland (Behboudi et al., 2006). In approximately 40% of the primary salivary gland MEC carcinomas, the fusion protein CRTC1-Maml2 has been detected (see reviews, Stenman, 2005; Kaye, 2009; O'Neill, 2009). Importantly, the CRTC1-Maml2 fusion protein is associated with distinct tumor subtypes and clinicopathologic features (Behboudi et al., 2006; Okabe et al., 2006; Nakayama et al., 2009; Kaye, 2009).

The *CRTC1* gene (CREB-Regulated Transcription Coactivator 1; also known as Mect1 or Torc1) is a coactivator of cyclic AMP (cAMP)/cAMP response-element binding protein (CREB) transcription (Conkright et al., 2003; Iourgenko et al., 2003). CREB regulates genes involved in cell proliferation and differentiation, and abnormal CREB activity is associated with carcinogenesis (Wu et al., 2005). CRTC1 acts by binding CREB and enhancing its transcription, independent of phosphorylation (O'Neill, 2009). The *Maml2* gene (related to the *Drosophila* gene *Mastermind* and to the mammalian mastermind-like gene *Maml1*) is an essential coactivator for Notch receptor transcriptional activation and signaling for cell proliferation and differentiation (Wu et al., 2005; Tonon et al., 2003). In the t(11;19) chromosomal translocation generated *CRTC1-Maml2* fusion oncogene, the CREB binding domain from *CRTC1* replaces the free intracellular Notch-binding domain from *Maml2* to produce a protein with novel transformational properties (O'Neill, 2009). Since deletions in *CRTC1* abolished transforming activity, and disruption of CREB activity eliminated CRTC1-Maml2-mediated tumorigenicity, indications are that CRTC1 dysregulation is the principal mediator of cell transformation and tumor formation (Coxon et al., 2005; Wu et al., 2005). Although the precise role of the CRTC1-Maml2 fusion oncogene remains unclear, molecular studies suggest that CRTC1-Maml2 plays a role during initial stages of tumor formation (Kaye, 2006, 2009).

In addition to enhancing CREB-dependent transcription, CRTC1 is also a potent modulator of AP-1-mediated cellular proliferation and transformation (Canettieri et al., 2009). In this regard, recent studies demonstrated that disruption of the LKB1/CRTC pathway resulted in tumor formation (Komiya et al., 2010). Though low levels of *Crtc1* transcripts have been reported in normal adult salivary glands (Behboudi et al., 2006), the distribution of CRTC1 protein in the normal salivary gland is not known. The aim of this study was to determine if CRTC1, like many known oncogenes, is expressed during normal submandibular salivary gland (SMG) development and is re-expressed in an experimental tumor model. We determined the cell-specific distribution of CRTC1 proteins during epithelial branching morphogenesis and differentiation of the SMG from embryonic day 13 (E13) to 3-weeks postnatal. Our results demonstrate that CRTC1 protein is present in SMG epithelia during early stages of morphogenesis, disappears with acinar maturation, and reappears in initial tumor-like histopathology.

1.1. Cell-specific distribution of CRTC1 protein during SMG morphogenesis and maturation

Embryonic mouse SMG morphogenesis is initiated with a thickening of the oral epithelium adjacent to the tongue on embryonic days 11.5–12 (E11.5–E12) to form the primitive SMG knot and is best conceptualized in stages (see reviews, Melnick and Jaskoll, 2000; Jaskoll and Melnick, 2005; Fig. 1A). In the *Initial Bud* stage, the oral epithelium proliferates and grows down into the mandibular mesenchyme to form a solid, elongated epithelial stalk, terminating in a bulb. In the *Pseudoglandular* stage, the solid cord of epithelium proliferates, elongates and grows by repeated end-bud branching to produce a bush-like structure, comprised of a network of elongated epithelial branches with buds at their termini. With progressive development, the number of epithelial branches and terminal end-buds

increases and hollow out (canalize/cavitate) to form the ductal system and pro-acini in the *Canalicular* and *Terminal Bud* stages, respectively. By the *Late Terminal Bud* stage, ductal and pro-acinar lumina are surrounded by a single layer of low cuboidal cells and, within the lumina, SMG-specific mucin proteins are expressed. Postnatally, pro-acini continue to differentiate for the next few weeks, with definitive acinar cells being seen around 3 weeks of age (see review, Gresik 1980).

To determine the cell-specific distribution of CRTC1 protein during SMG morphogenesis and differentiation, we used immunolocalization methodologies and anti-CRTC1 antibodies to demarcate the distribution of CRTC1 proteins in embryonic days 13–18 (E13–18), newborn and 3-week-old SMGs; that is, from the *Initial Bud* stage to terminal differentiation. Notable temporal changes in CRTC1 distribution are seen with progressive glandular morphogenesis and maturation (Fig. 1B–H). In the *Initial Bud* stage (Fig. 1B), strong CRTC1 immunostaining is seen in the epithelial stalk and end-bulb, but is relatively absent from the surrounding mesenchyme. In the *Pseudoglandular* (Fig. 1C and D) and *Canalicular* (Fig. 1E) stages, intense CRTC1 immunostaining is primarily found in the cytoplasm of ductal and terminal bud epithelia. In the *Terminal Bud* stage (Fig. 1F), the stage when cytodifferentiation begins (see reviews Melnick and Jaskoll, 2000; Jaskoll and Melnick, 2005), a reduction in immunodetectable CRTC1 is seen in ductal and terminal bud epithelia as compared to earlier stages (compare Fig. 1F to B–E). Postnatally, CRTC1 immunostaining is weakly seen in newborn (*Late Terminal Bud* stage) SMGs (Fig. 1G) and is virtually absent in the differentiated 3-week-old gland (Fig. 1H). CRTC1 immunostaining is also found in developing nerves (data not shown). The relative absence of CRTC1 protein in the mature gland is consistent with the prior report of low levels of *Crtc1* transcripts in adult salivary glands (Behboudi et al., 2006). Taken together, the presence of immunodetectable CRTC1 proteins in embryonic SMG branching epithelia and its disappearance with acinar differentiation suggest that CRTC1 may play a stage-specific, regulatory role during early SMG morphogenesis.

1.2. Increased CRTC1 protein expression in tumor-like SMG phenotype

Since the CRTC1-Mam12 fusion protein is frequently found in salivary gland MEC tumors (see reviews, Kaye, 2009; O'Neill, 2009) and CRTC1 has been shown to mediate cell transformation and tumor formation (Coxon et al., 2005; Komiya et al., 2005; Canettieri et al., 2009; Komiya et al., 2010), we postulated that CRTC1 protein would be re-expressed with induction of tumor-like histopathology. To address this question, we employed an *in vitro* strategy shown to induce cellular pathology in SMGs which resembles secretory glandular neoplasia (Melnick et al., 2006). In this set of experiments, we infected E15 (mostly *Canalicular* stage) and newborn (*Late Terminal Bud* stage) SMGs with mouse cytomegalovirus (mCMV) for 11–12 days *in vitro*; controls consisted of SMGs cultured for the entire period in control medium. mCMV infection *in vitro* results in a severely dysplastic phenotype as compared to controls (compare Figs. 2B to A; 3B to A). mCMV-infected SMGs exhibit atypical ductal epithelial hyperplasia, severely dilated ductal lumina, apparent mesenchymal-to-epithelium transformation, and oncocytic-like stromal cell metaplasia. Pseudostratified epithelium surround extremely enlarged ductal and pro-acinar lumina which are filled, to varying degrees, with living and dead cells that appear to have budded off the epithelia, alone or in clusters. These abnormal epithelial structures are embedded in a hypercellular stroma composed of two distinct cell types, large basophilic round cells and smaller eosinophilic cells, which resemble oncocytic metaplasia. Note the increase in cytomegalic, basophilic round cells within the stroma of mCMV-infected newborn glands as compared to infected E15 glands (compare Fig. 3B to 2B).

We compared the cell-specific distribution of CRTC1 proteins in control and mCMV-infected SMGs and detected marked differences (compare Figs. 2D and F to C; 3D and E to

C). In cultured E15 and newborn control SMGs, weak CRTC1 immunostaining is found throughout ductal and pro-acinar epithelia (Figs. 2B and 3A). There is a notable upregulation of CRTC1 protein expression in abnormal epithelial and stromal cells in cultured mCMV-infected E15 and newborn SMGs as compared to control glands. In mCMV-infected glands, intense immunostaining is seen in the pseudostratified epithelia surrounding dilated lumina (Fig. 2D and 3E), in the epithelial cells within dilated lumina (Fig. 2D and E; Fig. 3D), and in the cytomegalic, hypercellular stromal cells (Fig. 2D and Fig. 3E). Note the marked increase in CRTC1 immunostaining in the pseudostratified epithelia and cytomegalic, round cells in the mCMV-infected newborn SMGs as compared to mCMV-infected E15 glands (compare Figs. 3D and E to 2D and F). High magnification colocalization of CRTC1 protein and DAPI-stained nuclei in mCMV-infected SMGs clearly demonstrates that CRTC1 protein localization is both cytoplasmic (Fig. 2D insert and Fig. 3E upper left insert) and nuclear (Fig. 2F insert and Fig. 3E upper right insert). Taken together, our results indicate that CRTC1 proteins are routinely re-expressed with epithelial dysplasia and stromal metaplasia, early characteristics of tumor formation.

1.3. Cell proliferation in CRTC1 positive tumor cells

Given that CRTC1 induces AP1-dependent and, to a lesser degree, cAMP-dependent cell proliferation (Canetti et al., 2009), and the CRTC1-Maml2 fusion oncoprotein exerts its transforming activity through CRTC1-mediated cell proliferation (Coxon et al., 2005; Komiya et al., 2005; Canetti et al., 2009; Komiya et al., 2010), it was reasonable to postulate that CRTC1-positive abnormal cells are proliferating. To begin to address this question, we determined the cell-specific distribution of proliferating cell nuclear antigen (PCNA), a marker of cells in early G1 and S phases of the cell cycle, in control and mCMV-infected SMGs. As shown in Figure 4, marked differences in the pattern of cell proliferation are found between cultured control and mCMV-infected E15 SMGs (compare Fig. 4B and C to A). In control glands, PCNA-positive nuclei are infrequently seen in ductal and pro-acinar epithelia (Fig. 4A). In mCMV-infected glands, there is a marked increase in PCNA-positive nuclei found in abnormal epithelial cells within dilated lumina and the surrounding pseudostratified epithelia, as well as in cytomegalic, hypercellular stromal cells (Fig. 4B and C). This increase in cell proliferation correlates with the cell-specific increase in CRTC1 protein expression (compare Fig. 4B and C to 2E and F). A similar correlation between the cell-specific distribution of proliferating cells and intense CRTC1 immunostaining is seen in mCMV-infected newborn SMGs (data not shown). Taken together, the coincident expression of CRTC1 proteins and cell proliferation suggests that CRTC1 signaling is a mediator of cell proliferation. Our observation of reduced cell proliferation (Jaskoll and Melnick, 1999) and CRTC1 protein expression (Fig. 1B-H) in embryonic SMG epithelia with progressive development and differentiation, provides additional evidence supporting this conclusion.

Concluding remarks—In conclusion, the results reported here demonstrate that CRTC1 proteins are expressed in epithelia during early SMG morphogenesis, disappear with differentiation and are re-expressed with initial tumor formation. This stage-dependent CRTC1 expression pattern indicates that CRTC1 may play a role during embryonic SMG branching morphogenesis but not for subsequent pro-acinar/acinar differentiation. Mucoepidermoid carcinoma (MEC) of the salivary gland has long been thought to originate from precursor (stem) cells in excretory and intercalated ducts (Auclair et al., 1991). The dedifferentiation and transformation observed in our experimental SMG tumor model (Figs. 2 and 3; Melnick et al., 2006), and re-expression of CRTC1 proteins in these “embryonic” cells (Figs. 2 and 3), would support a precursor cell origin of MEC. Moreover, the coincident expression of CRTC1 protein and cell proliferation markers in tumor-like histopathology suggests that CRTC1-mediated cell proliferation may contribute, in part, to

initial tumor formation. Given that the current model proposes that CRTC1 activity requires nuclear localization to mediate gene expression (Bittinger et al., 2004), our results suggest that tight regulation of CRTC1 activity during normal early salivary gland development results in very low, but still relevant, levels of nuclear-localized CRTC1, while mCMV-mediated cell proliferation is associated with higher levels of nuclear-localized CRTC1. Functional studies are needed to determine if CRTC1 signaling is necessary and/or sufficient as a mediator of cell proliferation and cell transformation during tumorigenesis.

2. Experimental Procedures

2.1. Animals and Tissues

Inbred C57/BL6 female mice (Charles River, Wilmington, MA) were mated overnight [plug day = day 0 of gestation] and embryonic days 13–14 (E13-E14) heads and E15–18, newborn and 3-week-old SMGs were dissected and collected for immunolocalization or organ culture experiments as previously described (Melnick et al., 2006, 2009). All protocols involving mice were approved by the Institutional Animal Care and Use Committee (USC, Los Angeles, CA).

2.2. Immunofluorescent localization of CRTC1

Embryonic heads and SMGs were fixed for 4 hrs in Carnoy's fixative at 4°C, embedded in low melting point paraplast, serially-sectioned at 8 µm and immunostaining conducted essentially as previously described (Melnick et al., 2006, 2009) using 2 anti CRTC1 polyclonal rabbit antibodies raised against amino acids 19–34 of human CRTC1 (formerly Mect1) purchased from ProSci Incorporated (Poway, CA) and Rockland Immunochemicals (Gilbertsville, PA), biotin-labeled anti-rabbit IgG (Fab)₂ fragment (Invitrogen, Camarillo, CA) and Alexa Fluor 488 streptavidin (Invitrogen). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen). A minimum of 3 SMGs per day and treatment were analyzed. Negative controls were performed in parallel under identical conditions and consist of sections incubated without anti CRTC1 antibodies. Since CRTC1 is highly expressed in the brain and in nerves whereas CRTC2 is highly expressed in skeletal muscles, we analyzed the distribution of immunostain in E13 and E14 heads to confirm specificity of these antibodies for CRTC1. Our observations that brain and nerves stained positive and that the skeletal muscles of the head stained negative with these antibodies demonstrates CRTC1 specificity (data not shown).

2.3. Organ Culture Experiment

E15 (mostly *Canalicular* stage) or newborn (*Late Terminal Bud* stage) SMGs were cultured for 11–12 days using a modified Trowell method essentially as previously described (Melnick et al., 2006). For mCMV infection, SMGs were incubated with 1×10^{-5} plaque-forming units (PFU)/ml of *lacZ*-tagged mCMV RM427+ on day 0 for 24 hrs and then cultured in virus-free BGJb media (Invitrogen) for an additional 10–11 days; controls consisted of SMGs cultured in control medium for the entire period. SMGs were collected and processed for hematoxylin and eosin histology, immunolocalization or cell proliferation analysis.

2.4. Cell proliferation assay

The cell-specific localization of PCNA (proliferating cell nuclear antigen) was determined in control and mCMV-infected E15 and newborn SMGs cultured for 11–12 days using the Zymed mouse PCNA kit (Invitrogen, Carlsbad, CA.) and counterstained with hematoxylin as previously described (Melnick et al., 2006). In this set of experiments, the cytoplasm

appears blue and PCNA-positive nuclei appear dark brown. For cell proliferation analysis, 3–4 SMGs per treatment per age were analyzed.

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Abbreviations

AP-1	activator protein –1
E13	embryonic day 13
cAMP	cyclic AMP
CREB	cAMP response-element binding protein
CRTC1	CREB-regulated transcription coactivator 1
CRTC2	CREB-regulated transcription coactivator 2
DAPI	4,6-diamidino-2-phenylindole
LKB1	serine/threonine liver kinase B1
Maml	mammalian mastermind-like gene
MEC	mucoepidermoid carcinoma
mCMV	mouse cytomegalovirus
PCNA	proliferating cell nuclear antigen
SMG	submandibular salivary gland

References

- Auclair, PL.; Ellis, GL. Mucoepidermoid carcinoma. In: Ellis, GL.; Auclair, PL.; Gnepp, DR., editors. *Surgical Pathology of the Salivary Gland*. W.B. Saunders Company; Philadelphia: 1991. p. 269–298.
- Behboudi A, Enlund F, Winnes M, Andren Y, Nordkvist A, Leivo I, Flaberg E, Szekely L, Makitie A, Grenman R, Mark J, Stenman G. Molecular classification of mucoepidermoid carcinomas--prognostic significance of the MECT1-MAML2 fusion oncogene. *Genes Chromosomes Cancer* 2006;45:470–481. [PubMed: 16444749]
- Bittinger MA, McWhinnie E, Meltzer J, Iourgenko V, Latario B, Liu X, Chen CH, Song C, Garza D, Labow M. Activation of cAMP response element-mediated gene expression by regulated nuclear transport of TORC proteins. *Curr Biol* 2004;14:2156–2161. [PubMed: 15589160]
- Canettieri G, Coni S, Guardia MD, Nocerino V, Antonucci L, Di Magno L, Screaton R, Screpanti I, Giannini G, Gulino A. The coactivator CRTC1 promotes cell proliferation and transformation via AP-1. *Proc Natl Acad Sci USA* 2009;106:1445–1450. [PubMed: 19164581]
- Conkright MD, Canettieri G, Screaton R, Guzman E, Miraglia L, Hogenesch JB, Montminy M. TORCs: transducers of regulated CREB activity. *Mol Cell* 2003;12:413–423. [PubMed: 14536081]
- Coxon A, Rozenblum W, Park YS, Joshi N, Tsurutani J, Dennis PA, Kirsch IR, Kaye FJ. Mect1-Maml2 fusion oncogene linked to the aberrant activation of cyclic AMP/CREB regulated genes. *Cancer Res* 2005;65:7137–7144. [PubMed: 16103063]
- Enlund F, Behboudi A, Andren Y, Oberg C, Lendahl U, Mark J, Stenman G. Altered Notch signaling resulting from expression of a WAMTP1-MAML2 gene fusion in mucoepidermoid carcinomas and benign Warthin's tumors. *Exp Cell Res* 2004;292:21–28. [PubMed: 14720503]
- Gresik EW. Postnatal developmental changes in submandibular glands of rats and mice. *J Histochem Cytochem* 1980;28:860–870. [PubMed: 6160181]

- Iourgenko V, Zhang W, Mickanin C, Daly I, Jiang C, Hexham JM, Orth AP, Miraglia L, Meltzer J, Garza D, Chirn GW, McWhinnie E, Cohen D, Skelton J, Terry R, Yu Y, Bodian D, Buxton FP, Zhu J, Song C, Labow MA. Identification of a family of cAMP response element-binding protein coactivators by genome-scale functional analysis in mammalian cells. *Proc Natl Acad Sci* 2003;100:12147–12152. [PubMed: 14506290]
- Jaskoll T, Melnick M. Submandibular gland morphogenesis: stage-specific expression of TGF- α /EGF, IGF, TGF- β , TNF, and IL-6 signal transduction in normal embryonic mice and the phenotypic effects of TGF- β 2, TGF- β 3, and EGF-r null mutations. *Anat Rec* 1999;256:252–268. [PubMed: 10521784]
- Jaskoll T.; Melnick, M. Embryonic salivary gland branching morphogenesis. In: Davies, J., editor. *Branching Morphogenesis*. Landes Bioscience/Eureka Press; Georgetown: 2005. p. 160-175.
- Kaye FJ. Emerging biology of malignant salivary gland tumors offers new insights into the classification and treatment of mucoepidermoid cancer. *Clin Cancer Res* 2006;12:3878–3881. [PubMed: 16818681]
- Kaye FJ. Mutation-associated fusion cancer genes in solid tumors. *Mol Cancer Ther* 2009;8:6.
- Komiya T, Coxon AB, Park Y, Chen WD, Zajac-Kaye M, Meltzer P, Karpova T, Kaye FJ. Enhanced activity of the CREB co-activator Crtc1 in LKB1 null lung cancer. *Oncogene* 2010;29:1672–1680. [PubMed: 20010869]
- Komiya T, Park Y, Modi S, Coxon AB, Oh H, Kaye FJ. Sustained expression of Mect1-Maml2 is essential for tumor cell growth in salivary gland cancers carrying the t(11;19) translocation. *Oncogene* 2005;25:6128–6132. [PubMed: 16652146]
- Melnick M, Jaskoll T. Mouse submandibular gland morphogenesis: a paradigm for embryonic signal processing. *Crit Rev Oral Biol* 2000;11:189–215.
- Melnick M, Mocarski ES, Abichaker G, Huang J, Jaskoll T. Cytomegalovirus-induced embryopathy: mouse submandibular salivary gland epithelial-mesenchymal ontogeny as a model. *BMC Dev Biol* 2006;6:42. [PubMed: 16959038]
- Melnick M, Phair RD, Lapidot SA, Jaskoll T. Salivary gland branching morphogenesis: a quantitative systems analysis of the Eda/Edar/NF κ B paradigm. *BMC Dev Biol* 2009;9:32. [PubMed: 19500387]
- Nakayama T, Miyabe S, Okabe M, Sakuma H, Ijichi K, Hasegawa Y, Nagatsuka H, Shimozato K, Inagaki H. Clinicopathological significance of the CRTC3-MAML2 fusion transcript in mucoepidermoid carcinoma. *Mod Pathol* 2009;22:1575–1581. [PubMed: 19749740]
- Okabe M, Miyabe S, Nagtsuka H, Terada A, Hanai N, Yokoi M, Shimozato K, Eimoto T, Nakamura S, Nagai N, Hasegawa Y, Inagaki H. Mect1-Maml2 fusion transcript defines a favorable subset of mucoepidermoid carcinoma. *Clin Cancer Res* 2006;12:3902–3907. [PubMed: 16818685]
- O'Neill ID. t(11;19) translocation and CRTC1-MAML2 fusion oncogene in mucoepidermoid carcinoma. *Oral Oncol* 2009;45:2–9. [PubMed: 18486532]
- Stenman G. Fusion oncogenes and tumor type specificity--insights from salivary gland tumors. *Semin Cancer Biol* 2005;15:224–235. [PubMed: 15826837]
- Tonon G, Modi S, Wu L, Kubo A, Coxon AB, Komiya T, O'Neil K, Stover K, El-Naggar A, Griffin JD, Kirsch IR, Kaye FJ. t(11;19)(q21;p13) translocation in mucoepidermoid carcinoma creates a novel fusion product that disrupts a Notch signaling pathway. *Nat Genet* 2003;33:208–430. [PubMed: 12539049]
- Wu L, Liu J, Gao P, Nakamura M, Cao Y, Shen H, Griffin JD. Transforming activity of MECT1-MAML2 fusion oncoprotein is mediated by constitutive CREB activation. *EMBO J* 2005;24:2391–2402. [PubMed: 15961999]

Figure 1. Cell-specific localization of CRTC1 protein with progressive SMG morphogenesis and differentiation

A. Stages of embryonic SMG development. B–H. Stage-dependent immunolocalization of CRTC1 proteins. B. *Initial Bud* stage. C. *Early Pseudoglandular* stage. D. *Pseudoglandular* stage. E. *Canalicular* stage. F. *Terminal Bud* stage. G. Newborn (*Late Terminal Bud* stage) SMG. H. 3-week-old SMG. In the *Initial Bud* stage, intense CRTC1 immunostaining is seen in the epithelial stalk and end bud (e) and is relatively absent from mesenchyme (m). In the *Pseudoglandular* and *Canalicular* stages, strong CRTC1 immunostaining is localized to epithelial ducts (d) and buds (b). CRTC1 immunostaining is primarily seen in the cytoplasm and, to a lesser extent, in nuclei, of epithelia. By the *Terminal Bud* stage, reduced CRTC1 immunostaining is found in epithelial cell surrounding distinct ductal and terminal bud (pro-acinar) lumina (arrows) as compared to that seen in earlier stages. Postnatally, CRTC1 immunostaining is weakly detected in the newborn SMG and is relatively absent in the differentiated 3-week-old SMG. Bar scale: 30 μ m.

Figure 2. mCMV infection of E15 SMGs induces tumor-like histopathology

A, B. Histological analysis of E15 control (A) and mCMV-infected (B) SMGs cultured for 12 days. Control SMGs (A) display clusters of epithelial ducts (open red arrows) and terminal buds (pro-acini) (red arrowheads) with distinct lumina surrounded by a single layer of cuboidal cells. mCMV-infected SMGs (B) exhibit a notable histopathology. Abnormal ducts are characterized by a pseudostratified epithelium (blue arrows) surrounding severely dilated lumina, with clusters of abnormal, hyperplastic epithelial cells (red *) found within the enlarged lumina. These abnormal epithelial structures are embedded in a hypercellular stroma which no longer resembles mesenchyme, but is composed of giant basophilic round cells (black double arrowheads) and smaller eosinophilic cells (black *). B–D, F. Immunolocalization of CRTC1 protein. In control SMGs (C), immunodetectable CRTC1 is diffusely and weakly distributed throughout ductal and pro-acinar epithelia. With mCMV infection (D, F), there is a substantial increase in immunodetectable CRTC1 in the pseudostratified epithelia (D, black arrows) surrounding enlarged ductal lumina and in epithelial cells found within dilated lumina (triple black arrows), as well as in cytomegalic, hypercellular stromal cells (D, black arrowheads). D, F. Inserts. Colocalization of CRTC1 protein (green) and DAPI-stained nuclei (dark blue) was assessed as merged images. In areas of colocalization, the DAPI dark blue stain appears light blue. High magnification images demonstrate that CRTC1 proteins are localized in the cytoplasm (green) (D insert) and colocalized CRTC1 protein and DAPI-stained nuclei appear light blue (D, F inserts, white arrow). E. Negative control section of control SMG showing absence of immunostaining. Bar scale: A–F. 30 μ m. Inserts, D, F. 4 μ m.

Figure 3. mCMV-infected newborn SMGs exhibit a tumor-like histopathology

A, B. Histological analysis of control (A) and mCMV-infected (B) newborn SMGs cultured for 12 days (NB + 12). Control SMGs are characterized by epithelial ducts and pro-acini, with distinct lumina being surrounded by a single layer of cuboidal cells. mCMV-infected SMGs are severely dysmorphic, characterized by abnormal ducts composed of pseudostratified epithelia surrounding severely dilated lumina and clusters of abnormal, hyperplastic epithelial cells within these enlarged lumina. These abnormal epithelial structures are embedded in a hypercellular stroma composed of giant basophilic round cells and smaller eosinophilic cells. Note the consolidation of the cytomegalic, basophilic cells around pseudostratified epithelial ducts and pro-acini (double arrowheads). C–E. Immunolocalization of CRTC1 protein. In control SMGs (C), immunodetectable CRTC1 is diffusely distributed throughout ductal and pro-acinar epithelia. In mCMV-infected SMGs (D, E), there is a marked increase in immunolocalized CRTC1 in the pseudostratified epithelia surrounding enlarged ductal lumina (D–E, double arrows), in hyperplastic epithelial cells within dilated lumina (D, arrows), and in cytomegalic, hypercellular stromal cells (E, black arrowheads). E inserts. Colocalization of CRTC1 protein (green) and DAPI-stained nuclei (dark blue) was assessed as merged images. In areas of colocalization, the DAPI dark blue stain appears light blue. High magnification images demonstrate that CRTC1 proteins are localized in the cytoplasm (green)(left insert) and colocalized CRTC1 protein and DAPI-stained nuclei appear light blue (right insert, white arrow). Bar scale: A–E. 30 μ m; Inserts, E. 5 μ m.

Figure 4. PCNA-positive nuclei are seen in mCMV infected and affected cells

The cell-specific localization of PCNA-positive nuclei in E15 control (A) and mCMV-infected (B, C) SMGs cultured for 12 days. In controls (A), PCNA-positive nuclei are infrequently seen in ductal and proacinar epithelia. B. In mCMV-infected SMGs (B, C), PCNA-positive nuclei are found in the epithelial cells within dilated lumina (*) and pseudostratified ductal epithelia as well as in cytomegalic, hypercellular stromal cells (black arrowheads). C. Higher magnification of section shown in B. The dotted line outlines the ductal epithelia surrounding the dilated lumen. Note the presence of PCNA-positive nuclei in the hyperplastic epithelial cells within dilated lumina (*) and in the cytomegalic, round stromal cells (black arrowheads). Bar scale: A, B. 40 μ m; C. 20 μ m.