Deletion of p120-Catenin Results in a Tumor Microenvironment with Inflammation and Cancer that Establishes It as a Tumor Suppressor Gene

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SUMMARY

p120-catenin (p120ctn) interacts with E-cadherin, but to our knowledge, no formal proof that p120ctn functions as a bona fide tumor suppressor gene has emerged to date. We report herein that p120ctn loss leads to tumor development in mice. We have generated a conditional knockout model of p120ctn whereby mice develop preneoplastic and neoplastic lesions in the oral cavity, esophagus, and squamous forestomach. Tumor-derived cells secrete granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), monocyte chemotactic protein-1 (MCP-1), and tumor necrosis factor-α (TNFα). The tumors contain significant desmoplasia and immune cell infiltration. Immature myeloid cells comprise a significant percentage of the immune cells present and likely participate in fostering a favorable tumor microenvironment, including the activation of fibroblasts.

INTRODUCTION

Cell-cell junctions are critical for cell adhesion during normal epithelial homeostasis. The cardinal feature of the adherens junctions is the interaction between the cytoplasmic tail of classical type I (E-cadherin) and type II cadherins, and p120-catenin (p120ctn, also referred to as catenin, delta1, or Ctnnd1), which engenders stability of this specific junctions complex at the cell membrane (Davis et al., 2003; Ireton et al., 2002; Xiao et al., 2003). Recently, it was revealed by X-ray crystallography that p120 isoform 4A is in complex with the juxtamembrane domain core region (JMD(core)) of E-cadherin (Ishiyama et al., 2010). Loss of p120ctn or its phosphorylation on serine-threonine residues is able to destabilize E-cadherin (Fukumoto et al., 2008). Thus, p120ctn regulates cadherin stability and turnover. The balance between adhesion and migration is regulated further by p120ctn’s ability to regulate the activities of RhoA, Rac, and Cdc42 through the amino terminus of p120ctn, thereby orchestrating exquisite actin dynamics (Reynolds and Rocznia-Ferguson, 2004). Furthermore, the bridge between p120ctn and

Significance

Oral squamous cell cancers (OSCCs) and esophageal squamous cell cancers (ESCCs) represent common human cancers worldwide. The molecular pathogenesis underlying these cancers involves p120-catenin (p120ctn), which is critical to epithelial homeostasis through the adherens junctions. p120ctn loss or mislocalization in these specific cancers served as a basis for the generation of a conditional knockout mouse model, revealing a dramatic invasive squamous cell cancer phenotype accompanied by induction of prosurvival signals, desmoplasia, and recruitment of immature myeloid cells. Thus, p120ctn loss in this mouse model proves its tumor suppressor role and establishes a platform for strategies designed for early detection, molecular imaging, and targeted therapeutics in these cancers that have been elusive to date.
phenocopy human disease, but to date, no published mouse in vivo has yet to be demonstrated unequivocally.

various cancer types, but to our knowledge, a causal role data suggest a potential tumor suppressor role for p120ctn in E-cadherin) cell-cell contacts at least in vitro. In aggregate, these retention of membranous p120ctn and cadherin-mediated (P- or 2007), although collective invasion might involve also concurrent tumor but rather in regions of the tumor, similar to that observed for E-cadherin loss (Birchmeier, 1995). This loss or mislocalization of p120ctn frequently leads to E-cadherin destabilization, endowing a cancer cell an advantage in cell migration by virtue of abrogation of cell adhesion. Additionally, knockdown experiments using shRNA to p120ctn have demonstrated that p120ctn loss induces invasion in tumor cell lines with concomitant loss or downregulation of E-cadherin (Macpherson et al., 2007), although collective invasion might involve also concurrent retention of membranous p120ctn and cadherin-mediated (P- or E-cadherin) cell-cell contacts at least in vitro. In aggregate, these data suggest a potential tumor suppressor role for p120ctn in various cancer types, but to our knowledge, a causal role in vivo has yet to be demonstrated unequivocally.

p120ctn loss in the mouse has been modeled in an attempt to phenocopy human disease, but to date, no published mouse model has yielded a cancer phenotype. Deletion of p120ctn by homologous recombination in mice results in embryonic lethality. Tissue-specific targeting of p120ctn has been utilized to study the role of p120ctn in development and tumorigenesis (Davis and Reynolds, 2006). For example, p120ctn loss results in impaired enamel development (Bartlett et al., 2010). Targeted p120ctn deletion in the embryonic salivary gland forces a loss of acinar development and an adoption of a ductal cell fate. Although these mice die immediately in the postnatal period, the salivary glands are distinguished by intraepithelial dysplasia, but not cancer (Davis and Reynolds, 2006), p120ctn loss in the skin results in epidermal hyperplasia and chronic inflammation with loss of hair and body fat (Perez-Moreno et al., 2008). Furthermore, the epidermis has evidence of NFκB activation and mitotic defects such as aneuploidy, but no overt cancer (Perez-Moreno et al., 2008). Recently, conditional p120ctn loss in the small intestine and colon was found to result in death by 21 days, with evidence of mucosal erosion and bleeding, and recruitment of COX-2 expressing neutrophils, suggesting an underlying barrier defect (Smalley-Freed et al., 2010). Thus, to date, and to our knowledge, no tissue-specific p120ctn knockout mouse models have been able to demonstrate that p120ctn loss results in the development of invasive cancer.

The overarching goal of this study was to generate and characterize mechanistically a genetic mouse model in which conditional p120ctn loss in the squamous oral cavity, esophagus, and forestomach results in cancer that phenocopies precisely the histologic features of human oral and esophageal squamous cell carcinomas (OSCCs, ESCCs, respectively). In so doing, such a model might provide a platform for the consideration of combinatorial therapeutics in ESCCs, OSCCs, and potentially other squamous cell cancers.

RESULTS

p120ctn Expression Is Lost or Reduced in Esophageal Cancer Tissues and Cell Lines

A human tissue microarray (TMA) with matched normal esophageal and esophageal squamous cell cancer (ESCC) tissues was evaluated for p120ctn expression by immunohistochemistry (IHC) (Figure 1A). p120ctn has the expected membrane-associated localization in the normal esophageal squamous epithelium.
By contrast, 100% of the 69 ESCC tumors have p120ctn loss or cytoplasmic mislocalization (Figure 1B). There is a statistically significant association (p = 0.017) between decreased p120ctn and E-cadherin expression with odds ratio = 3.23 (95% CI: 1.13 < O.R. < 9.24). These data reveal that p120ctn and E-cadherin are coordinately decreased or lost in the majority of ESCCs, and substantiate previous findings (Chung et al., 2007).

A panel of 14 human esophageal squamous tumor cell lines was analyzed for p120ctn and E-cadherin expression (Figure 1C). p120ctn expression was detected in all cell lines with different isoforms observed in a subset of them. E-cadherin expression was moderate to high in 11 of 14 cell lines, low in one (TE8), and absent in two cell lines (HCE4, HCE7). p120ctn and E-cadherin expression correlated well in the cell lines. Interestingly, the mesenchymal p120ctn isoform appears to be expressed in HCE-4 and HCE-7 cells along with N-cadherin, consistent with N-cadherin’s pattern of known mesenchymal expression.

Conditional Deletion of p120ctn in the Oral Cavity, Esophagus, and Forestomach Results in Invasive Squamous Cell Cancer, Desmoplasia, and Inflammation

To elucidate the mechanistic roles of p120ctn in vivo, we generated spatially targeted p120ctn deletion in the squamous oral cavity, esophagus, and forestomach. We bred L2Cre and p120loxP/loxP mice together to generate L2Cre;p120loxP/loxP mice, where the L2 promoter is active specifically in the squamous oral cavity, esophagus, and forestomach (Nakagawa et al., 1997; Opitz et al., 2002). LacZ (Gt(Rosa) 26Tm1Sor) reporter mice were crossed with L2Cre mice to determine the efficiency of Cre recombination in the oral cavity, esophagus, and forestomach of mice. LacZ staining demonstrates effective recombination and, therefore, Cre expression in these specific tissues (see Figure S1 available online). To verify p120ctn loss in L2-Cre;p120loxP/loxP mice, p120ctn protein expression was assessed by immunofluorescence (IF). p120ctn was effectively lost in the epithelium in 2-month-old L2-Cre;p120loxP/loxP mice compared to L2-Cre;p120+/+ control mice (Figure 2A).

Gross pathology examination of the oral cavities, esophagi, and forestomachs of L2Cre;p120loxP/loxP mice revealed significant masses in each of these tissues, along with splenomegaly and local lymphadenopathy (Figures 2B and 2C). Histologic analysis at 4–6 months of age revealed epithelial dysplasia (data not shown). Histologic analysis at 9–12 months of age demonstrated severe dysplasia with invasive squamous cancer into the submucosa and muscle in L2Cre;p120loxP/loxP mice, whereas all control mice (p120loxP/loxP, L2-Cre;p120+/+, and L2-Cre;p120+/loxP) had no abnormalities (Figure 3A). Approximately 70% of L2Cre;p120loxP/loxP mice develop tumors between 9 and 12 months of age, whereas no tumors were detected in age-matched littermate control mice (Table S1; p < 0.0001). Invasive esophageal cancer was detected as early as 4 months of age in one L2Cre;p120loxP/loxP mouse. Some esophageal tumors were poorly differentiated but displayed no evidence of any obvious distant metastasis, although we cannot

from a L2Cre;p120loxP/loxP mouse. See also Table S1 and Figure S1. A total of 23 L2Cre;p120loxP/loxP mice and 23 control (five p120ctn+/loxP, ten L2Cre;p120ctn+/+, and eight L2Cre;p120ctn+/loxP) mice were evaluated.
Cancer Cell

p120ctn Loss Results in Inflammation and Cancer

exclude the possibility of micrometastasis in other organs (Figure 3A). The submucosa was altered with marked desmoplasia and the striking presence of immune cell infiltration (Figure 3A).

Analysis of L2-Cre; p120loxP/loxP forestomachs revealed severe hyperplasia, severe dysplasia, and invasive cancers into the underlying desmoplastic and inflammatory stroma (Figure 3A). The forestomachs of all control mice (p120lox/lox, L2-Cre; p120lox/lox, and L2-Cre; p120loxP/loxP) revealed no morphologic changes (Figure 3A). Nearly identical results are evident in the oral cavities of L2-Cre; p120loxP/loxP mice, namely invasive squamous cell cancer with desmoplasia and immune cell infiltration (Figure 3A).

We next evaluated E-cadherin expression in L2-Cre; p120loxP/loxP mice. Significant loss of E-cadherin expression is noted in L2-Cre; p120loxP/loxP mice compared to L2-Cre; p120lox/lox control mice, with residual E-cadherin expression in some L2-Cre; p120loxP/loxP mice where it is cytoplasmic (Figure 3B). In some mice, whereas p120ctn is lost and E-cadherin is either lost or cytoplasmic, there are occasional focal areas within tumors that retain both p120ctn and E-cadherin at the cell membrane (Figure 3C), the latter consistent with the notion of collective invasion observed in A431 cells in vitro (Macpherson et al., 2007).

**Alterations in Proliferation and Differentiation in L2-Cre; p120loxP/loxP Mice**

Ki-67 and keratin 14 (K14), markers of proliferation, were evaluated in esophageal tumors from L2-Cre; p120loxP/loxP mice. Normal proliferating basal cells express Ki-67 and K14, whereas normal differentiated suprabasal cells express keratins 4 and 13 (K4, K13, respectively) and harbor little proliferation. Ki-67 expression was significantly increased in the esophageal epithelium and in the invasive cancers in L2-Cre; p120loxP/loxP mice compared to control mice (Figure S2; p = 3 x 10^-5). K14 expression was increased in the esophageal epithelium at 5 months of age, which ultimately expanded to the luminal surface by 12 months of age (data not shown). K4 expression, as evaluated by qPCR, is decreased 2.5-fold (p = 0.014) in L2-Cre; p120loxP/loxP mice compared to L2-Cre; p120lox/lox control mice (data not shown). Invading tumor cells are K14 positive (Figure 3C). Interestingly, β-catenin expression was membranous in the esophagus of L2-Cre; p120loxP/loxP mice (Figure 3C) but lost in the oral cavity of the L2-Cre; p120loxP/loxP mice. Furthermore, no alterations were apparent for epidermal growth factor receptor (EGFR), c-myc, or K-ras by IHC in the tumors (data not shown). However, p-Akt (but not p-Erk1/2 [p-Mapk]) was increased in the tumors, as was p-Stat3, which might contribute to tumorigensis (Figure S2).

**NFκB and Inflammatory Cells in the Tumor Microenvironment**

Given the significant recruitment of immune cells in the tumor stroma, we hypothesized that NFκB activation may be present in these lesions, as has been reported previously (Perez-Moreno et al., 2006; Perez-Moreno et al., 2008). Indeed, this was confirmed with robust NFκB induction in dysplasia and tumors, thereby suggesting that this is an early event (Figure 4A). The NFκB induction was evident also in the immune cells found in the tumor stroma (Figure 4A).

We then established cells from an esophageal tumor (designated as 714ET) as well as esophageal cell lines from p120ctnloxP/loxP mice that in turn were infected with retroviral Cre recombinase or a control vector (designated as F2-Cre and F2-Tomato, respectively). Western blot analysis revealed complete p120ctn loss in both F2-Cre and 714ET cells and retained p120ctn expression in the control F2-Tomato cells (Figure 4B and quantified in Figure 4C, left panel). In F2-Cre and 714ET cells, there is activated p-NFκB (8-fold), p-Akt (4-fold), and p-Stat3 (2-fold) (Figures 4B and 4C). Additionally, 714ET cells were found to secrete significant amounts of granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), monocyte chemotactic protein-1 (MCP-1), and tumor necrosis factor-α (TNFα) (Figure 4D), but not other cytokines tested (IL-1β, IL-2, IL-4, IL-5, IL-6, or IFNγ; data not shown). GM-CSF has been suggested to contribute to immature myeloid cell recruitment (Marigo et al., 2010).

In order to characterize the immune cells in the tumor microenvironment, we undertook a number of experiments. Splenic weights in L2-Cre; p120loxP/loxP mice were 5-fold greater than in control mice (p = 0.0004), and there was also leukocytosis with increased segmented neutrophils, band neutrophils, eosinophils, and monocytes (Table S2). These findings suggested that there may be an elevated production of circulating myeloid lineage cells and that the invasive squamous cell cancers in the L2-Cre; p120loxP/loxP mice might contribute to the recruitment of these immune cells. Therefore, we analyzed the leukocyte fraction (CD45+ cells) for immature myeloid cells (defined phenotypically as Gr-1+ CD11b+ cells), macrophages (F4/80+), T cells (CD3+), B cells (CD19+), dendritic cells (CD11c+), and natural killer (NK) cells (CD49b+). The percentage of CD45+ cells was approximately the same for normal versus dysplastic versus tumor tissues and spleens (Figure 4E). However, there was a statistically significant increase (Figure S3) in immature myeloid cell infiltration in dysplastic and tumor tissues (Figure 4E). In dysplastic esophagi or forestomachs, the percentage of immature myeloid cells increased 4- to 8-fold compared to control tissues (<5% of CD45+ cells) (Figure 4F). The percentage of immature myeloid cells was higher also in esophageal and forestomach tumors (Figure 4F). Other types of immune cells (macrophages, T cells, B cells, dendritic cells, NK cells) did not increase with dysplasia and cancer (Figures 4E; Figure S3).

The fact that only one subpopulation of immune cells (immature myeloid cells) is specifically increased suggests that p120ctn loss may lead to a highly selective process of immune cell recruitment. We next purified splenic Gr-1+ CD11b+ cells and prepared cytospins for cytology (Figure S3). Staining and quantification revealed that the Gr-1+ CD11b+ cells represent a range of cells, including myelocytes/metamyelocytes (11.7%), segmented neutrophils (48.1%), band neutrophils (12.5%), and monocyteid cells (7.7%). These cells have activated nuclear NFκB (Figure S3), consistent with what was found in the mouse tumors (Figure 4A).

In order to evaluate the functional properties of the immature myeloid cells, we again purified Gr-1+ CD11b+ cells from tumor-bearing L2-Cre; p120loxP/loxP mice and found that these cells inhibited peptide-specific T cell responses in vitro (using OVA-specific TCR-transgenic T cells) (Figure 4G).
Additionally, these immature myeloid cells produce nitric oxide and have been suggested to suppress T cells (Figure 4H) (Bronte et al., 2003; Bronte and Zanovello, 2005). We next treated L2-Cre; p120^loxP/loxP mice with dexamethasone and found a dramatic attenuation of esophageal tumor invasion to that of hyperplasia (Figures 5A; Figure S4). CD45+ cells and Gr-1+CD11b+ cells were quantified by flow cytometry and were reduced (Figure 5B; data not shown). We analyzed further these Gr-1+CD11b+ cells and found decreased IL-4R expression in the dexamethasone-treated mice (Figure 5C). Notably, IL-4R expression on Gr-1+CD11b+ cells has been reported to correlate with suppression of CD8+ T cells (Gallina et al., 2006; Mandruzatto et al., 2009). These data suggest that inflammatory cells, including the Gr-1+CD11b+ cells, are fostering tumorigenicity.

**Desmoplasia Is Present in the Tumor Microenvironment**

To evaluate the specific features of the microenvironment in the invasive cancers that develop in L2-Cre; p120^loxP/loxP mice, a pronounced increase in fibrillar collagen was present in the tumor stroma consistent with the desmoplasia in human ESCC and OSCC (Figure 5D). By contrast, fibrillar collagen is localized to a narrow region below the epithelium (Figure 5D). Additionally, there was increased fibroblast specific protein-1 (Fsp1) expression in mouse OSCC and ESCC (Figure 5E). Similarly, α-smooth muscle actin (αSMA) was increased in the mouse tumors (data not shown). Interestingly, dexamethasone-treated mice had resolution of the desmoplasia with little residual Fsp1+ fibroblasts (Figures 5E and 5F; Figure S4), suggesting a possible role for the immune system in regulating desmoplasia in our model system.

To evaluate a potential link between immature myeloid cell inflammation and desmoplasia, we next conducted coculture of purified Gr-1+CD11+ cells from tumor-bearing L2-Cre; p120^loxP/loxP mice with normal fibroblasts (from C57BL/6 mice) and cancer (OSCC, ESCC) associated fibroblasts (CAF’s) (from L2-Cre; p120^loxP/loxP mice) that revealed activation of the normal fibroblasts and further augmentation of CAF’s based upon αSMA and Fsp1 expression (Figures 6A–6C). In fact, some of the activated CAF’s have an interesting “dendritic” appearance in coculture and display a similar morphology in mouse tumors (Figure 6C). Furthermore, the Gr-1+CD11+ cells survived better and expressed higher IL-4Rα levels in coculture with CAF’s (Figure 6D).

Given the different lines of evidence for the tumor-promoting role of Gr-1+CD11+ cells in L2-Cre; p120^loxP/loxP mice, we next sought to determine a relationship between myeloid cells and p120ctn loss in human ESCC. Increased myeloperoxidase (MPO) staining in the tumor stroma for myeloid lineage cells correlates with p120ctn loss (Figure 7A; p = 0.05). This raises the possibility that p120ctn loss might induce the recruitment of MPO+ cells in the pathogenesis of human ESCC.

**Secondary Alterations in Invasive Squamous Cell Cancers**

Apart from enhanced activation of NF-κB, Akt, and Stat-3 in the tumor cells, and the probability that GM-CSF, MCP-1, and TNFα are induced by NfκB in the tumor cells (Schreck and Baeuerle, 1990), we have begun to identity potential secondary alterations in p120ctn-deficient squamous epithelia that may contribute to cancer development. RNA microarray analysis was performed using isolated epithelia from the esophagi and forestomachs from 12-month-old L2-Cre; p120^loxP/loxP mice and L2-Cre/ p120^+/+ control mice. Unsupervised hierarchical clustering was performed and identified significantly altered genes (Figure S5). Several genes involved in cytokine signaling, IL-13Ra1, IL-1R2, and Pias1, are altered in p120-deficient squamous epithelia based upon microarray analysis and were confirmed by qPCR (Figure S5).

**DISCUSSION**

**p120-ctn Loss Results in Invasive Squamous Cell Cancer**

The complex between E-cadherin and p120-ctn is critical for the formation and maintenance of the adherens junctions. Disruption of this complex is a hallmark feature of epithelially derived cancers. p120ctn loss or mislocalization can destabilize E-cadherin. Definitive proof for p120ctn’s functional role as a tumor suppressor gene in vivo has been elusive to date. Targeted disruption of murine p120ctn in the salivary gland (MMTV-cre;p120ctn^flox/flox), skin (K14-cre;p120ctn^flox/flox), and intestine (Villin-Cre; p120ctn^flox/flox) does not result in cancer (Davis and Reynolds, 2006; Perez-Moreno et al., 2006; Smalley-Freed et al., 2010). p120ctn may not be critical for tumor suppression in these specific tissues. Alternatively, a lack of tumor formation in these mice may be due to early death. Generation and characterization of L2-Cre;p120^loxP/loxP mice result in a dramatic phenotype of invasive cancer in the oral cavity, esophagus, and forestomach. These cancers may be either unifocal or multifocal, and there is preceding severe dysplasia, underscoring a temporal progression from preneoplasia to neoplasia, mirroring human OSCC and ESCC progression.

How does p120ctn loss mechanistically cause invasive squamous cell cancers? Concordant p120ctn and E-cadherin loss is likely favoring cancer cell migration and invasion. Because
Figure 4. Immature Myeloid Cells Are Recruited to the Esophagi and Squamous Forestomachs of L2Cre;p120ctn$^{loxP/loxP}$ Mice

(A) NFκB upregulation in L2Cre;p120ctn$^{loxP/loxP}$ mice. IHC staining for the NFκB p65 subunit revealed significant upregulation in hyperplastic, dysplastic, and invasive cancer lesions in the esophagi of L2Cre;p120ctn$^{loxP/loxP}$ mice (n = 6). Arrows denote NFκB nuclear staining in immune cells within the stroma in L2Cre;p120ctn$^{loxP/loxP}$ mice. Interestingly, these cells were found in early hyperplastic lesions. Scale bars, 50 μM.
p120ctn loss precedes E-cadherin loss in our model system, it is unclear to what extent there is a dependence upon E-cadherin loss.

E-cadherin destabilization or loss contributes to tumorigenesis. These squamous cancers induce prosurvival signals with NF-κB, Akt, and p-Stat3 activation. In contrast to p120ctn loss in skin with activated MAPK (Perego et al., 2006), p120ctn loss in oral and esophageal keratinocytes does not have enhanced MAPK signaling. These squamous cell cancers have striking features of desmoplasia and immune cell infiltration, with significant increases in immature myeloid cells, but not in T cells, B cells, NK cells, dendritic cells, and macrophages. The precise recapitulation of human ESCC (along with OSCC) by our model system related to angiogenesis, secretion of chemokines/cytokines, and production of ECM-degrading enzymes (DeNardo et al., 2009, 2010; Ferrara, 2010; Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg and Sinha, 2009; Yang et al., 2004, 2008). Because dexamethasone has protean effects, it will be important to ablate immature myeloid cells in a specific fashion in our model system and in those models where immature myeloid cells are thought to be critical.

In summary, conditional p120ctn loss in the esophagus, oral cavity, and forestomach leads to invasive squamous cell cancers, involving NFκB, Akt, Stat-3 activation, increased proliferation, and production of GM-CSF, M-CSF, MCP-1, and TNFα from tumor-derived cells. The accompanying tumor microenvironment is replete with desmoplasia and immature myeloid cells. These events likely contribute to cancer arising from p120ctn-deficient cells (Figure 7), which provide compelling evidence that p120ctn is indeed a bona fide tumor suppressor gene. Because the L2-Cre;p120loxP/loxP mice phenocopy human OSCC and ESCC, they serve as a model for the translational development of diagnostic and therapeutic agents, goals that have been hindered by the absence of robust, genetically based models. Furthermore, our model might be applicable to other squamous cancers given common genomic alterations between ESCC and lung SCC where SOX-2 is amplified (Bass et al., 2009). Several approaches merit consideration in this context. First, strategies designed to restore wild-type p120ctn may provide a venue to ameliorate tumor progression. Given the complex nature of the tumor microenvironment, targeting of components, either individually or in combination, might advance the field. For example, prodrugs have been demonstrated to be efficacious against fibroblast activation protein (FAP) in prostate cancer (Lebeau et al., 2009). Strategies to target immature myeloid cells might be attractive based upon our studies as well in pancreatic cancer (Clark et al., 2007).

The Tumor Microenvironment Is Critical in Mediating the Effects of p120-ctn Loss

The tumor microenvironment is an array of diverse cell types that cooperate to foster tumor cell migration and invasion. The extracellular matrix includes invading tumor cells, fibroblasts (CAFs), endothelial cells, pericytes, and immune cells (Andreu et al., 2010; DeNardo et al., 2010; Kalluri and Zeisberg, 2006; Lewis and Pollard, 2006).

Immature myeloid cell recruitment has been noted by IL-1β overexpression in the stomach with resulting inflammation and cancer (Tu et al., 2008), disruption of TGFβ signaling in mammary carcinomas (Yang et al., 2008), knock in of mutant K-ras to the pancreas (Clark et al., 2007), and preparing the local pulmonary environment for metastasis (Yang et al., 2010). We observed a significant increase in immature myeloid cells in the tumor microenvironment and the spleen. It is conceivable that GM-CSF secreted by tumors contributes to the recruitment of immature myeloid cells (Marigo et al., 2010). We do not find evidence of increased IL-1β, IL-6, VEGF, and prostaglandins from the tumor cells, which have been linked also to the recruitment of immature myeloid cells (Ostrand-Rosenberg and Sinha, 2009). Other cell types in the tumor microenvironment might serve as the sources for these other cytokines.

How do immature myeloid cells contribute to tumorigenesis? Immature myeloid cells have the ability to suppress antigen-specific T cell responses in vitro (Gabrilovich et al., 2001; Gallina et al., 2006; Sinha et al., 2005). In our model system, immature myeloid cells isolated from tumor-bearing mice also have the ability to inhibit T cell activation. Additionally, the immature myeloid cells activate normal fibroblasts and augment activation of CAFs based upon Fsp1 and sSMA expression, which are novel findings. In turn, CAFs in our system contribute to prolonged survival of immature myeloid cells. We cannot exclude other protumorigenic functions of immature myeloid cells in our model system related to angiogenesis, secretion of chemokines/cytokines, and production of ECM-degrading enzymes (DeNardo et al., 2009, 2010; Ferrara, 2010; Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg and Sinha, 2009; Yang et al., 2004, 2008).
**Figure 5. The Immune Response Is Involved in the Tumor Microenvironment in L2Cre;p120ctnloxP/loxP Mice**

(A) Dexamethasone (Dex) or PBS (control) was administered to 8-month-old L2Cre;p120ctnloxP/loxP mice (five in each group) for 7 days. Histologic examination of the esophagi revealed hyperplasia without desmoplasia or inflammation in the Dex-treated mice, whereas invasive esophageal squamous cancer with desmoplasia and inflammation was present in PBS-treated mice. Arrows point to regions of invasion. Scale bars, 100 μM.

(B) FACS analysis for CD45+ cells contained within the esophagi from Dex-treated mice (red bars) and PBS control mice (green bars) (five in each group; p = 0.01). Dex treatment reduced CD45+ and Gr-1+CD11b+ cells by 6-fold.

(C) FACS analysis for IL-4Rα+ cells contained within the Gr-1+CD11b+ population of cells from Dex-treated mice (red bars) and PBS control mice (green bars) (five in each group; p = 0.0016). Dex reduced the percentage of Gr-1+CD11b+ cells that express IL-4Rα by about 50%.

(D) Masson’s trichrome staining on esophagi of L2Cre;p120ctnloxP/loxP mice (n = 4) and control (L2Cre;p120ctn+/+) mice. Asterisks indicate the fibrillar collagen layer in the control L2Cre;p120ctn+/+ esophagi. Arrows indicate the abundant fibrillar collagen surrounding invasive esophageal tumors (upper right and lower right panels). Invasive oral and forestomach tumors in L2Cre;p120ctnloxP/loxP mice are depicted in the bottom row. Arrows indicate the staining surrounding invasive cancers. Scale bars, 50 μM.
Cell Lines

TE and HCE cells, all established human esophageal squamous cancer cell lines, were cultivated in DMEM media supplemented by 10% fetal calf serum (Sigma-Aldrich) and 1% penicillin/streptomycin (Invitrogen) and grown in 5% CO₂ conditions as described previously (Okano et al., 2000). Cell lines were established from mouse esophageal tumors (designated as 714ET), mouse fibroblasts (designated as OF3 from normal oral cavity), and mouse CAFs (designated as 1136CF from OSCC and designated as 955EF from ESCC) and verified to be of epithelial or fibroblast origin. The mouse cell lines were cultivated in DMEM as above.

To generate pBABE-tdTomato-SV40-nlsCre (6982 bp), puromycin acetyl-transferase of pBABE-puro was replaced with nlsCre cDNA derived from pHRnlsCre (Addgene), and then tdTomato cDNA (gift of Roger Y. Tisen, University of California, San Diego, San Diego, CA, USA) was subcloned. The parental F2 cells were established through the isolation of esophageal epithelia (Opitz et al., 2002) from p120loxP/loxP mice, infected with pBABE-tdTomato-SV40-nlsCre (designated as F2-Cre) or pBABE-tdTomato (designated as F2-Tomato), and stable clones were FACS selected for tdTomato. The F2 Cre lines were analyzed in coculture with Gr-1+CD11b+ cells for Fsp1 expression by IF. Cocultured fibroblasts had increased Fsp1 expression (n = 6). Scale bars, 50 μM. (C) An esophageal CAF cell line (955EF) had high basal Fsp1 expression when cultured alone. Coculturing of 955EF cells (n = 6) resulted in even higher Fsp1 expression. Note that the highest Fsp1 + 955EF cells have "dendrite-like" projections, which is evident also in esophageal tumors from L2Cre;p120ctnloxP/loxP mice (n = 4). Scale bars, 50 μM. (D) FACS revealed increased survival of Gr-1+CD11b+ cells in coculture with CAFs (n = 4; p = 0.021). (E) FACS demonstrated increased IL-4Rα expression in Gr-1+CD11b+ cells in coculture with CAFs (n = 4; p = 0.016). Error bars indicate SEM.

**Figure 6. Crosstalk between Immature Myeloid Cells and Fibroblasts in the Tumor Microenvironment of L2Cre;p120ctnloxP/loxP Mice**

(A) Fibroblasts from the oral cavity of wild-type mice (designated as OF3) or oral CAFs (designated as 1136CF) were cocultured with Gr-1+CD11b+ cells for Fsp1 expression by IF. Cocultured fibroblasts had increased Fsp1 expression (n = 6). Scale bars, 50 μM. (B) Cocultured fibroblasts had increased αSMA expression (n = 6). Scale bars, 50 μM.

EXPERIMENTAL PROCEDURES

**Cell Lines**

TE and HCE cells, all established human esophageal squamous cancer cell lines, were cultivated in DMEM media supplemented by 10% fetal calf serum (Sigma-Aldrich) and 1% penicillin/streptomycin (Invitrogen) and grown in 5% CO₂ conditions as described previously (Okano et al., 2000). Cell lines were established from mouse esophageal tumors (designated as 714ET), mouse fibroblasts (designated as OF3 from normal oral cavity), and mouse CAFs (designated as 1136CF from OSCC and designated as 955EF from ESCC) and verified to be of epithelial or fibroblast origin. The mouse cell lines were cultivated in DMEM as above.

To generate pBABE-tdTomato-SV40-nlsCre (6982 bp), puromycin acetyl-transferase of pBABE-puro was replaced with nlsCre cDNA derived from pHRLnlsCre (Addgene), and then tdTomato cDNA (gift of Roger Y. Tisen, University of California, San Diego, San Diego, CA, USA) was subcloned. The parental F2 cells were established through the isolation of esophageal epithelia (Opitz et al., 2002) from p120loxP/loxP mice, infected with pBABE-tdTomato-SV40-nlsCre (designated as F2-Cre) or pBABE-tdTomato (designated as F2-Tomato), and stable clones were FACS selected for tdTomato. The parental F2 cells were cocultured with Gr-1+CD11b+ cells for Fsp1 expression by IF. Cocultured fibroblasts (n = 6) resulted in even higher Fsp1 expression. Note that the highest Fsp1 + 955EF cells have "dendrite-like" projections, which is evident also in esophageal tumors from L2Cre;p120ctnloxP/loxP mice (n = 4). Scale bars, 50 μM. (C) An esophageal CAF cell line (955EF) had high basal Fsp1 expression when cultured alone. Coculturing of 955EF cells (n = 6) resulted in even higher Fsp1 expression. Note that the highest Fsp1 + 955EF cells have "dendrite-like" projections, which is evident also in esophageal tumors from L2Cre;p120ctnloxP/loxP mice (n = 4). Scale bars, 50 μM. (D) FACS revealed increased survival of Gr-1+CD11b+ cells in coculture with CAFs (n = 4; p = 0.021). (E) FACS demonstrated increased IL-4Rα expression in Gr-1+CD11b+ cells in coculture with CAFs (n = 4; p = 0.016). Error bars indicate SEM.

(E) Fsp1 IF with increased expression in esophageal and oral cavity tumors from L2Cre;p120ctnloxP/loxP mice (n = 4) compared to control L2Cre;p120ctnloxP mice (n = 4) tissues. Scale bar, 50 μM. (F) Fsp1 IF of esophagi from L2Cre;p120ctnloxP/loxP mice treated with either PBS or Dex from Figure 5A. Fsp1 expression is decreased in Dex-treated mice (n = 5) when compared to PBS treated mice (n = 5). Scale bars, 50 μM. See also Figure S4. Error bars indicate SEM.
F2-Cre and F2-Tomato cell lines were cultivated in KSFM as described previously (Andl et al., 2003; Harada et al., 2003).

**Histology and IHC/IF**

Paraffin tissue sectioning and staining for hematoxylin and eosin (H&E) and IHC were performed as described previously (Stairs et al., 2008). Antibodies used for IHC were the following: p120ctn (610134; BD Transduction); E-cadherin (610182; BD Transduction); K14 (PRB-155P; Covance); pAKT (9271; Cell Signaling); pMAPK(9101; Cell Signaling); NFκB-p65 subunit(4764; Cell Signaling); and Ki67 (NCL-KI67p; Novacastra). For IF the following antibodies were used: K14 (PRB-155P; Covance); Fsp1 (DakoCytomation); β-catenin (9652; Cell Signaling); p120ctn (61634; BD Transduction); E-cadherin (61634; BD Transduction); E-cadherin (3195; Cell Signaling); c-myc (1472-1; Epitomics); MPO (45977; Abcam); p-Stat3 (9145; Cell Signaling); and sSMA (A2547; Sigma). Secondary antibodies used were: Alexa Fluor 488-anti-rabbit, Alexa Fluor 568 anti-rat, or Alexa Fluor 568 anti-mouse. Slides were cover-slipped using VectaShield mounting medium with DAPI.

**Figure 7. MPO Expression in Human ESCC**

(A) MPO expression was analyzed by IF in 14 ESCC samples with matched adjacent normal esophageal mucosa. Representative image is depicted with MPO-positive cells present in the tumor stroma (right panel). MPO staining was increased in the tumor stroma and correlates with p120ctn loss (p = 0.05, Fisher’s exact analysis). Scale bars, 50 μM.

(B) Model of OSCC and ESCC in conditional p120ctn-deleted mice. Solid lines represent cell-cell interactions emerging from experiments in this study. Dashed lines are potential areas of interactions. See also Figure S5.
for the test samples to a standard curve generated by serial dilution of 0.1 mM sodium nitrite. Student’s t test was used.

Cytoculture of Immature Myeloid Cells and Fibroblasts
Immature myeloid cells were cocultured with fibroblasts for 96 hr, and fibroblasts were evaluated for αSMA and Fsp1. Immature myeloid cells were evaluated for survival and IL-Rx expression. Student’s t test was used.

Cytology of Gr-1+ CD11b+ Cells
Gr-1+ CD11b+ cells were purified (>98%) from spleens of tumor-bearing mice using a MoFlo fluorescence activated cell sorter (DakoCytomation). Cytospin preparations were stained with Diff-Quick reagent and by IF for NF-κB.

Cytokine Analysis
The following cytokines were measured either by BD Cytokine Bead Array (BD Biosciences) or by ELISA (R&D Systems): IL-1α, IL-2, IL-4, IL-5, IL-6, IFN-γ, TNF-α, GM-CSF, G-CSF, MCP-1, KC, M-CSF, SCF, and VEGF.

RNA Microarrays
The epithelia from mouse esophagi and squamous forestomachs were isolated by digesting the tissues with a 1:2 dilution dispase (BD) in deionized water at 37°C for 5–10 min. The epithelia were peeled and snap-frozen and stored at −80°C for RNA isolation. RNA was purified, and cRNA was prepped (Affymetrix) and run on Affymetrix Mouse 1.0ST Affymetrix Arrays.

Statistical Analysis of RNA Microarray Data
Gene expression differences were considered statistically significant if the p value was less than 0.01. A global test was done as to whether the expression profiles differed between the classes by permuting the labels of which arrays corresponded to which classes. For each permutation the p values were recomputed, and the number of genes significant at the 0.01 level was noted. The false discovery rate was estimated to be less than 10%. Cluster analysis was performed with Cluster and TreeView software (Eisen et al., 1998).

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