



# Human papillomavirus oncogenes reprogram the cervical cancer microenvironment independently of and synergistically with estrogen

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**High-risk human papillomaviruses (HPVs) infect epithelial cells and are causally associated with cervical cancer, but HPV infection is not sufficient for carcinogenesis. Previously, we reported that estrogen signaling in the stromal tumor microenvironment is associated with cervical cancer maintenance and progression. We have now determined how HPV oncogenes and estrogen treatment affect genome-wide host gene expression in laser-captured regions of the cervical epithelium and stroma of untreated or estrogen-treated nontransgenic and HPV-transgenic mice. HPV oncogene expression in the cervical epithelium elicited significant gene-expression changes in the proximal stromal compartment, and estrogen treatment uniquely affected gene expression in the cervical microenvironment of HPV-transgenic mice compared with nontransgenic mice. Several potential estrogen-induced paracrine-acting factors were identified in the expression profile of the cervical tumor microenvironment. The microenvironment of estrogen-treated HPV-transgenic mice was significantly enriched for chemokine/cytokine activity and inflammatory and immune functions associated with carcinogenesis. This inflammatory signature included several proangiogenic CXCR2 receptor ligands. A subset of the same CXCR2 ligands was likewise increased in cocultures of early-passage cells from human cervical samples, with levels highest in cocultures of cervical fibroblasts and cancer-derived epithelial cells. Our studies demonstrate that high-risk HPV oncogenes profoundly reprogram the tumor microenvironment independently of and synergistically with estrogen. These observations illuminate important means by which HPVs can cause cancer through alterations in the tumor microenvironment.**

human papillomavirus | cervical cancer | estrogen | stroma | paracrine signaling

Viruses cause ~15% of human cancers (1), and high-risk human papillomaviruses (HPVs) alone are responsible for nearly 5% of human malignancies (2). HPVs infect poorly differentiated, basal keratinocytes within stratified squamous epithelia where they can establish persistent infections. The high-risk mucosotropic HPVs cause malignancies in the stratified epithelia lining the cervix, vagina, and other organs of the lower female reproductive tract, as well as the anus, penis, and epithelia lining the oropharynx. We previously developed transgenic mice expressing the high-risk type HPV16 oncogenes E6 and E7 (3, 4). In these mice, the keratin 14 (K14) promoter directs expression of the HPV viral oncogenes in the basal layer of stratified squamous epithelia, which is the natural site of papillomavirus infection. We have used this *in vivo* model to show how HPV E6 and E7 oncogenes and cellular cofactors contribute to HPV-associated carcinogenesis in various anatomical sites (5–8), including the cervix (9, 10).

Despite the strong etiological link between HPV and cervical cancer, persistent HPV infection is not sufficient for human cervical cancer development (11, 12), and thus other cofactors

likely contribute to carcinogenesis. One such cofactor is estrogen (17 $\beta$ -estradiol) (13, 14). Elevated levels of circulating estrogens have been detected in women with HPV<sup>+</sup> lesions and cancers (15, 16), and multiparity and long-term oral contraceptive use are significant cervical cancer risk factors (17–20). Experiments in HPV transgenic mice have directly linked estrogen and cervical cancer. In *K14E6/E7* mice, expression of HPV16 E6 and E7 in the cervical epithelium is essential but not sufficient to cause cervical cancer, and additional systemic delivery of exogenous 17 $\beta$ -estradiol and expression of its receptor ER $\alpha$  are required for the onset, maintenance, and progression of neoplastic disease (9, 10, 21, 22). Furthermore, treatment with estrogen-signaling inhibitors promotes regression of cervical cancer and precancerous lesions in these mice (23, 24). While caution is reasonable in extrapolating the efficacy of drugs in murine models to therapeutic treatment of human disease (25), human population-based data do correlate long-term antiestrogen use with lower risk of cervical neoplasia (26).

An important observation is that estrogen contributes to cervical carcinogenesis through the underlying stroma. Continued

## Significance

**A subset of human papillomaviruses (HPVs) causes 5% of human cancers, including virtually all cancers of the cervix. In a mouse model of cervical cancer, estrogen is a necessary cofactor that contributes to disease by signaling through the underlying tumor microenvironment. In this study, we discovered that epithelial expression of the HPV oncoproteins reprograms the cervical tumor microenvironment and its response to estrogen. These changes involve the elicitation of paracrine-acting factors implicated in carcinogenesis, and the expression of a subset of these factors was also induced in cocultures of human cervical cancer cells and stromal fibroblasts. We hypothesize that HPV oncogenes cause cancer in part by creating a unique tumor microenvironment that synergizes with estrogen in the cervix.**

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Data deposition: The sequences reported in this paper have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database (accession no. [GSE102232](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE102232)).

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expression of ER $\alpha$  in the cervical stroma of HPV transgenic mice is required for the maintenance of neoplastic disease in the cervical epithelium (27). In human cervical cancers, expression of ER $\alpha$  is, in fact, retained in the stroma but is lost in epithelial cancer cells (28), and human cervical cancer-associated fibroblasts have been shown to mediate estrogen-dependent signaling (29). Moreover, stromal–epithelial interactions mediate the effects of estrogen on female reproductive tract morphogenesis, and stromal ER $\alpha$  facilitates the proliferative effects of estrogen on the adjacent epithelium (30–33). The tumor microenvironment directs the biology of many cancers, as bidirectional communication between epithelial cells and the tumor microenvironment affects tumor initiation, neoplastic progression, metastasis, and therapeutic response (34). These insights suggest that stromal estrogen signaling drives cervical carcinogenesis in concert with epithelial HPV oncogene expression. In the present report, we describe how we used our estrogen-dependent *K14E6/E7* cervical cancer mouse model to investigate the independent and synergistic effects of epithelial HPV oncogene expression and estrogen signaling on genome-wide gene-expression levels in the cervical stroma. We discovered an extensive reprogramming of the cervical stroma in the context of mice expressing the HPV16 E6 and E7 oncogenes in the epithelia. Unique changes were also noted in the microenvironment of these mice when given exogenous estrogen to promote cervical carcinogenesis. Paracrine factors were identified whose expression was increased in the cervical stroma of *K14E6/E7* mice and was enhanced in these mice when treated with estrogen. The expression of these same paracrine factors, which were previously implicated in carcinogenesis, was further enhanced when human cervical cancer-derived cell strains were cocultured with cervical fibroblasts, defining potential modulators of cervical carcinogenesis arising from the tumor microenvironment.

## Results

**Gene-Expression Profiling of Cervical Epithelium and Adjacent Stroma.** We used our mouse model of cervical carcinogenesis to identify HPV oncogene- and estrogen-driven gene changes in the stroma associated with cervical cancer development. Four groups of adult female mice representing different grades of disease were included: (i) nontransgenic mice (WT; no disease); (ii) nontransgenic mice treated with estrogen (hereafter abbreviated as “E2”) for 1 mo, a duration sufficient to induce benign hyperplasia (WT+E2; hyperplasia); (iii) *K14E6/E7* bitransgenic mice [E6/E7; largely cervical intraepithelial neoplasia grade 1/2 (CIN1/2) precancerous lesions]; and (iv) *K14E6/E7* bitransgenic mice treated with estrogen for 6 mo, a duration necessary to induce cervical cancers (E6/E7+E2; cancer) (Fig. S1A and Table S1). From the reproductive tracts of the above mice, 20 epithelial regions of histopathological interest (i.e., no disease, hyperplasia, precancerous lesions, or cancer) and matched regions of proximal stroma were laser-capture microdissected. Pre–laser-capture microdissection (LCM) and post-LCM images were used to assess the accuracy of tissue capture before RNA extraction (Fig. S1B). RT-PCR was used to measure levels of the *K14E6/E7* transgene (Fig. S1C), expression of which should be restricted to the epithelial compartment due to the tissue specificity of K14 promoter activity (3, 4). The average number of transgene transcripts was significantly higher in E6/E7 ( $P = 0.0007$ ) and E6/E7+E2 ( $P = 0.03$ ) cervical epithelia than in the adjacent stroma, confirming accurate capture of epithelia and stroma. A significantly higher level of transgene-specific mRNA was measured in E6/E7 epithelia than in E6/E7+E2 epithelia ( $P = 0.006$ ), likely because the squamous cell carcinomas in the E6/E7+E2 group are a more heterogeneous mixture of cells compared with the more homogenous stratified epithelium in the E6/E7 group. Total RNA was then analyzed using Affymetrix GeneChip Mouse Genome 430 2.0 microarrays comprehensively measuring nearly 40,000 murine transcripts. Significant gene-expression changes between

pairwise group comparisons were defined as having a false-discovery rate (FDR)  $\leq 0.05$  and a fold change  $\geq 2$ .

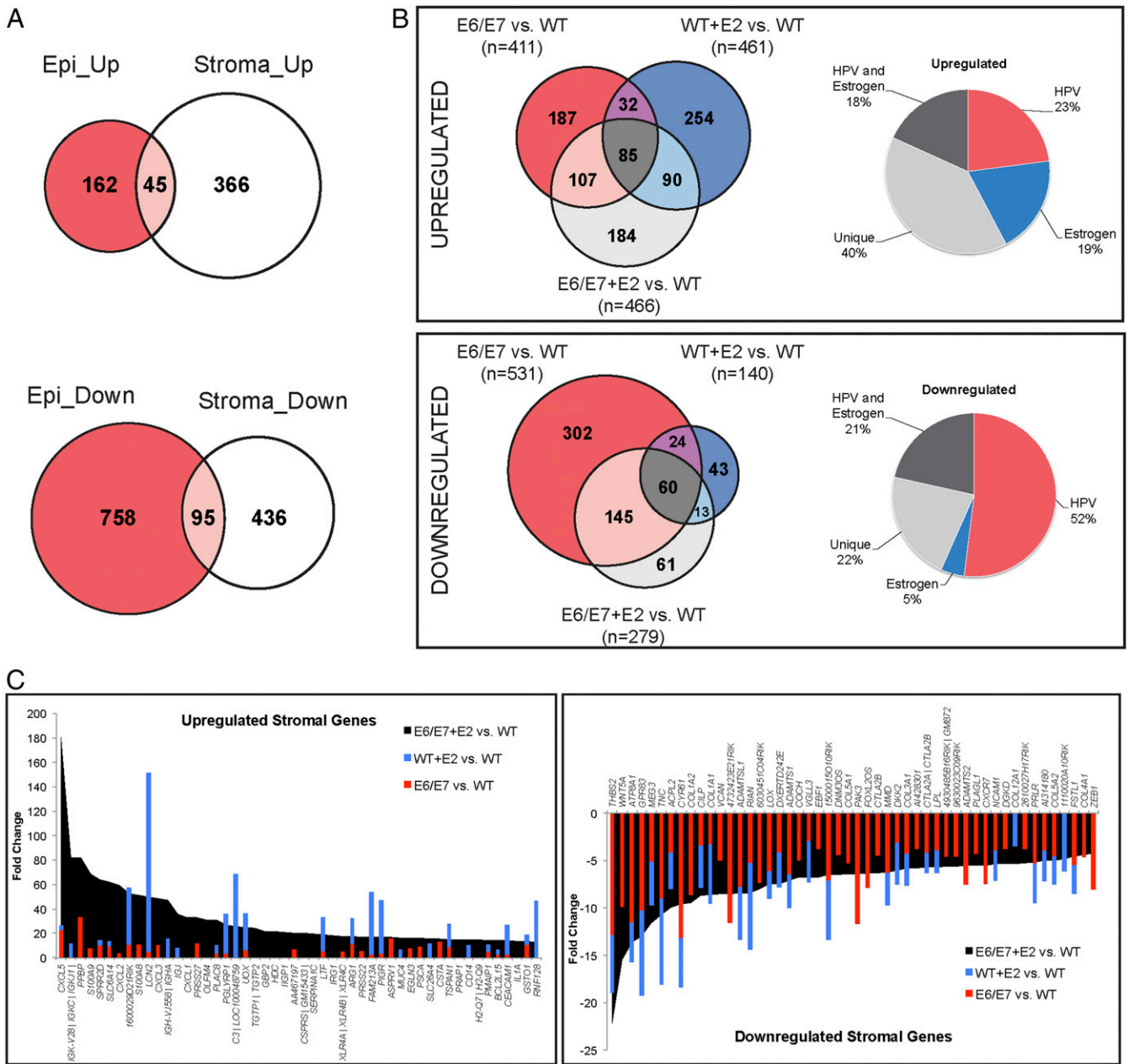
**Epithelial Expression of HPV Oncogenes Dramatically Alters Host Gene Expression in Adjacent Cervical Stroma.** We first compared gene expression in the cervical epithelium and stroma of E6/E7 and WT mice. HPV E6 and E7 significantly altered gene expression in the epithelium: 207 unique annotated genes were up-regulated, and 853 genes were down-regulated, in the *K14E6/E7* cervical epithelium (Fig. 1A and Dataset S1). Strikingly, the HPV oncogenes had substantial effects on gene expression in the surrounding stroma: 411 genes were up-regulated, and 532 genes were down-regulated, in the cervical stroma adjacent to *K14E6/E7* epithelia (Fig. 1A and Dataset S2 A and B). Differentially expressed genes in the *K14E6/E7* epithelium and its adjacent stroma were largely exclusive to each compartment, suggesting that the HPV oncogenes cause gene-expression changes in the stromal microenvironment distinct from those in the epithelium.

Gene ontology (GO) analysis (Fig. 2A and Table S2) showed that many genes up-regulated in the *K14E6/E7* stroma are involved in epithelial processes such as keratinization, keratinocyte differentiation, and epidermis development as well as intermediate filament organization, cell-cycle, metabolic, and apoptotic processes. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with genes up-regulated in the transgenic stroma included the p53 signaling pathway, cell cycle, and DNA replication. Ontology associated with down-regulated genes in the *K14E6/E7* stroma highlighted dysregulation of genes involved in stromal tissue architecture, such as cell adhesion, extracellular matrix (ECM) organization, and collagen fibril organization. KEGG pathway analysis reinforced this observation, indicating significant association with focal adhesion, ECM–receptor interaction, and gap junction. We conclude that HPV oncogene expression in the epithelial compartment changes the expression of genes in the microenvironment that are largely involved in both epithelial and stromal tissue dynamics.

**Estrogen Affects Stromal Gene Expression Differently in *K14E6/E7* Versus Nontransgenic Mice.** To examine how estrogen affects stromal gene expression, differentially expressed genes were determined by comparing WT+E2 and E6/E7+E2 mice with WT mice. All E6/E7+E2 mice developed squamous cell carcinomas, and these stroma samples therefore represented the tumor microenvironment.

Estrogen treatment up-regulated 461 genes in the WT+E2 stroma and 466 genes in the E6/E7+E2 cervical stroma (Dataset S2 C and E). E6/E7+E2 stroma and WT+E2 stroma shared some Gene Ontology (GO) associations, but a large proportion in the E6/E7+E2 stroma was distinct (Fig. 2B and Tables S3 and S4). For instance, inflammatory response, keratinization, antigen processing and presentation, neutrophil chemotaxis, cellular response to IFN- $\gamma$ , and NOD-like receptor signaling were significantly associated with up-regulated genes in E6/E7+E2 stroma but not in WT+E2 stroma. Estrogen treatment down-regulated 140 genes in WT+E2 stroma and 279 genes in E6/E7+E2 stroma (Dataset S2 D and F). A large overlap in processes negatively affected by estrogen in the WT+E2 and E6/E7+E2 cervical stroma included GO groups such as focal adhesion, ECM–receptor interaction, collagen fibril organization, and cell adhesion. However, these processes were more significantly associated with down-regulated genes in E6/E7+E2 mice than in the WT+E2 mice (compare Tables S3 and S4). Other GO associations were unique to genes down-regulated in E6/E7+E2 stroma, including cell migration, negative regulation of epithelial cell proliferation, and the TGF- $\beta$ –signaling pathway. Collectively, the gene-expression patterns indicated that estrogen treatment affected distinct biological processes in the cervical stroma of *K14E6/E7* versus nontransgenic mice.

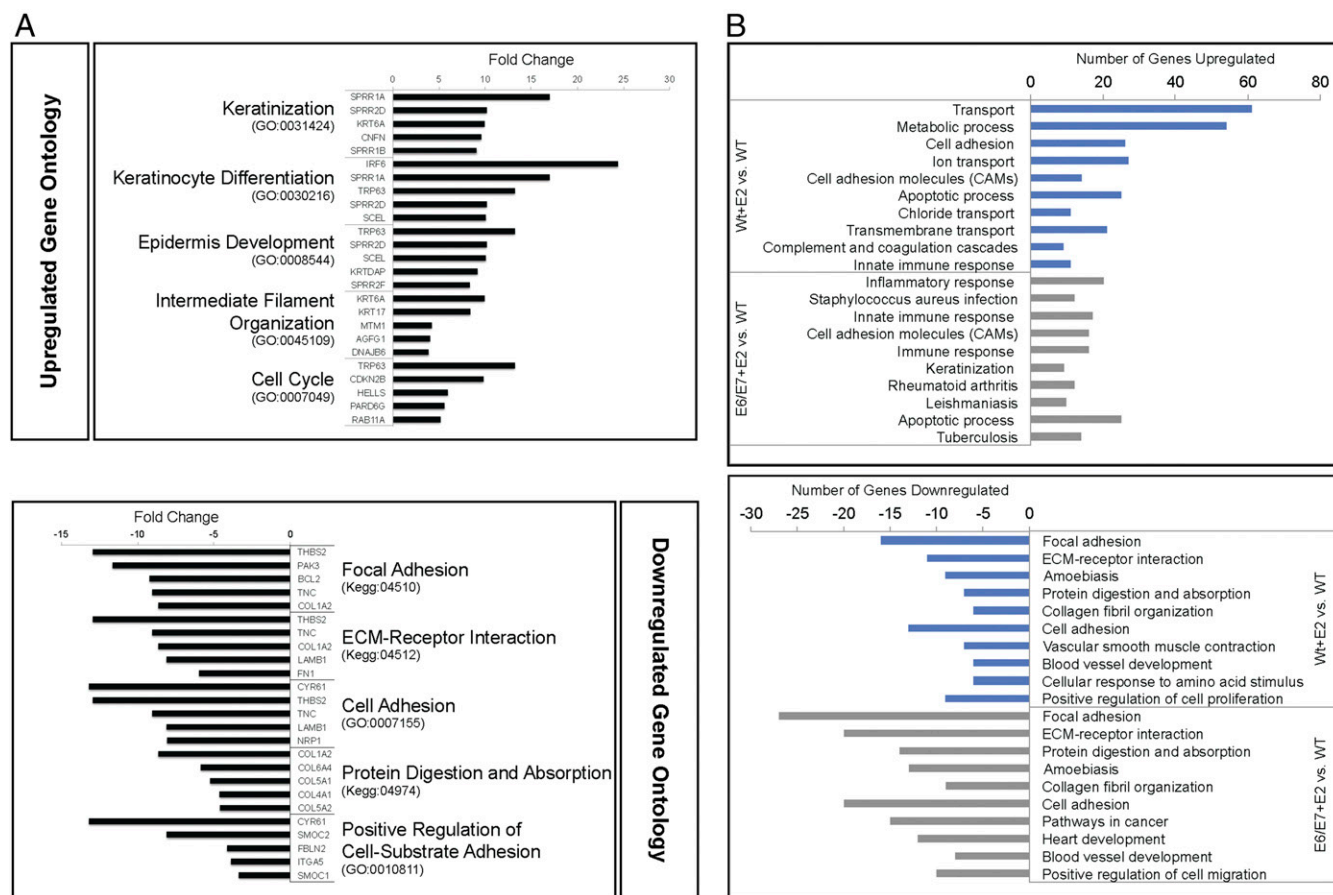
Interestingly, 40% (184 of 466) of the up-regulated genes and 22% (61 of 279) of the down-regulated genes in the E6/E7+E2 cervical stroma were unique, meaning their differential expression required both E6/E7 and estrogen (Fig. 1B and Dataset S2 G and H).



**Fig. 1.** Epithelial expression of HPV oncogenes alters host gene expression in the stroma and affects the transcriptional response to estrogen. (A) Venn diagrams showing comparative analyses of differentially expressed genes up-regulated (Upper) and down-regulated (Lower) in the *K14E6/E7* cervical epithelium (red) and stroma (white) compared with nontransgenic (E6/E7 vs. WT). Genes shared between the epithelium and stroma are shown in pink. (B, Left) Three-way Venn diagram analysis of differentially expressed genes in the stroma of E6/E7 (red), WT+E2 (blue), and E6/E7+E2 (light gray) compared with WT mice. The total number of differentially expressed genes for each given comparison is indicated in parentheses. See [Dataset S2 G and H](#) for detailed lists of genes in this comparative analysis. (Right) Pie charts showing the percentage of total genes up-regulated ( $n = 466$ ) (Upper) and down-regulated ( $n = 279$ ) (Lower) in E6/E7+E2 versus WT stroma that are also differentially expressed in E6/E7 (HPV, red segment), WT+E2 (Estrogen, blue segment), all three groups (HPV and Estrogen, black segment), or are unique to the E6/E7+E2 stroma (Unique, gray segment). (C) The 50 genes with the highest increased (Left) and decreased (Right) fold changes between E6/E7+E2 and WT cervical stroma are shown in black. Gene names are given on the x axis, and fold change is indicated on the y axis. For each gene, the fold changes measured for the WT+E2 versus WT comparison (blue bars) and E6/E7 versus WT comparison (red bars) are superimposed. Genes unique to the E6/E7+E2 cervical stroma lack superimposed blue or red bars.

Of the down-regulated genes, 52% (145 of 279) and 5% (13 of 279) were shared exclusively with E6/E7 and WT+E2 conditions, respectively, suggesting that HPV oncogenes drive much of the gene down-regulation in the E6/E7+E2 stroma. We conclude that the HPV oncogenes influence how estrogen affects gene expression in the microenvironment, resulting in a subset of genes responding differently to estrogen in *K14E6/E7* versus nontransgenic mice.

To determine the relative magnitude of the effect of the HPV oncogenes or estrogen on gene dysregulation in the E6/E7+E2 cervical stroma, the 50 highest-fold gene-expression changes between the E6/E7+E2 and WT cervical stroma (Fig. 1C, black) were compared with their respective fold changes in E6/E7 (red) and WT+E2 (blue) stroma. These fold changes in the E6/E7+E2 cervical stroma reflected either their unique nature, indicated by



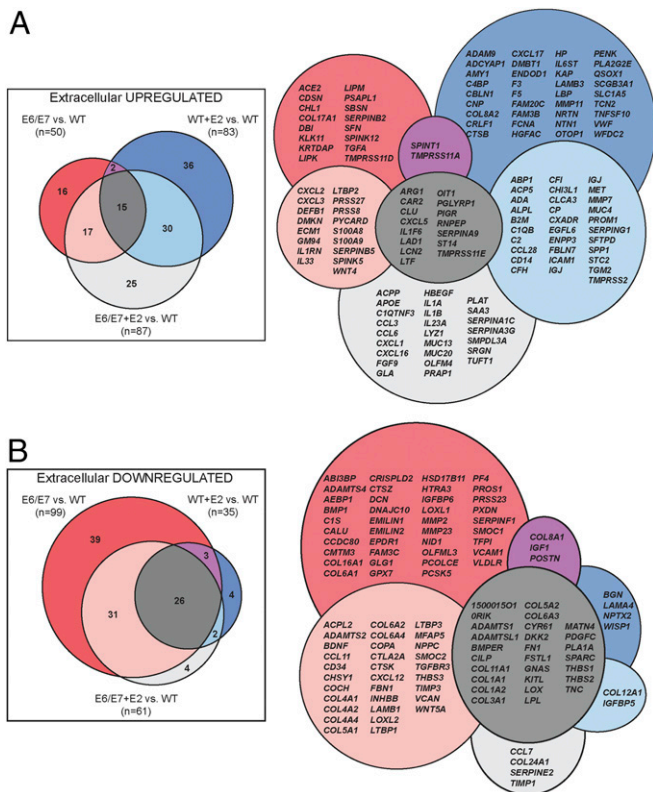
**Fig. 2.** GO analysis of differentially expressed genes. (A) GO of up-regulated (Upper) and down-regulated (Lower) genes differentially expressed in *K14E6/E7* versus nontransgenic stroma. The top five most significant GO groups are shown (for *P* values, see Table S2) along with GO identifiers in parentheses. The five genes classified in each GO group with the highest fold-change values measured between E6/E7 and WT groups are shown in the bar graphs. (B) GO comparisons of genes differentially expressed in WT+E2 versus WT stroma (blue bars) and E6/E7+E2 versus WT stroma (gray bars). The top 10 most significant GO groups are shown (for more details, see Tables S3 and S4) for the up-regulated genes (Upper) and down-regulated genes (Lower). The number of genes in each GO group that are differentially expressed in each comparison is indicated in the bar graphs.

genes shown in black that have no overlap with genes in red or blue (e.g., *CXCL1*, *GBP2*, *HDC*) or strong synergy compared with either E6/E7 or estrogen alone (e.g., *CXCL5*, *SPRR2D*, *IGJ*). Conversely, nearly all the top 50 genes most down-regulated in the E6/E7+E2 stroma showed overlap with both factors, particularly with E6/E7. We conclude that the HPV oncogenes and estrogen not only function individually but also cooperate to alter stromal gene expression.

**Candidate Paracrine Factors in the Cervical Microenvironment.** We hypothesized that estrogen induces paracrine factors in the *K14E6/E7* stroma to promote cervical carcinogenesis in the adjacent HPV<sup>+</sup> epithelia. Therefore, we compared differentially expressed stromal genes ontologically annotated as components of the extracellular space, extracellular region, ECM, and/or proteinaceous ECM (Fig. 3). In E6/E7+E2 cervical stroma, 87 extracellular genes were up-regulated, and 61 genes were down-regulated. Consistent with the unique effects of estrogen on gene expression in the *K14E6/E7* stroma, more than 25% (25 of 87) of the up-regulated extracellular genes were unique to the E6/E7+E2 condition. With this list of “extracellular” genes, we thus identified a collection of candidate paracrine factors in the cervical microenvironment regulated by estrogen and/or epithelial HPV oncogene expression.

**Genes Involved in Inflammatory Response Are Increased by Epithelial E6/E7 Expression and Further Enhanced by Estrogen.** We narrowed our focus to the extracellular genes up-regulated in the E6/E7+E2 stroma, as they could function as positive-acting factors on the nearby epithelia. GO of these genes identified a strong inflammatory and immune response signature (Fig. 4A). The top five most significant GO classes included cytokine activity, chemokine activity, inflammatory response, immune response, and cytokine–cytokine receptor interaction. A core group of genes driving these signatures were proinflammatory cytokines, including *CCL3*, *CCL6*, *CCL28*, *CXCL1*, *CXCL2*, *CXCL3*, *CXCL5*, *IL1A*, and *IL1B*, as well as the *S100A8* and *S100A9* genes that have well-characterized roles in inflammation and carcinogenesis (Fig. 4A) (35). *CXCL1*, *CXCL2*, *CXCL3*, and *CXCL5* were among the genes most up-regulated in the E6/E7+E2 stroma (Dataset S2E). These members of the ELR<sup>+</sup> CXC family of chemokines are CXCR2 receptor ligands and comprise a signaling axis involved in several facets of tumorigenesis (36). Their identification as some of the most highly up-regulated genes in the E6/E7+E2 cervical stroma makes them prime candidates for paracrine factors in epithelial–stroma communication.

Interestingly, many of the inflammation-associated genes were highly up-regulated in the E6/E7 stroma even in the absence of estrogen, and their expression was further increased by estrogen treatment. Estrogen treatment alone in nontransgenic mice had little, if any, effect on inflammation-associated gene expression in the



**Fig. 3.** Identification of candidate paracrine factors in the cervical stroma. Overview of analysis of differentially expressed extracellular genes. Significantly differentially expressed genes determined previously for E6/E7, WT+E2, and E6/E7+E2 compared with WT were used as input for cellular component GO analysis. Results that were classified as extracellular (GO:0005578, GO:0005576, GO:0031012, and GO:0005615) were pooled to generate a consolidated list of extracellular genes. The lists of up-regulated (A) and down-regulated (B) extracellular genes were compared using Venn diagrams. The total numbers of significantly differentially expressed extracellular genes are shown in parentheses under each comparison label. The diagrams on the right contain gene symbols corresponding to the Venn diagrams.

stroma, although *CXCL5* and *S100A9* were increased slightly over WT stroma (Fig. 4B). Real-time qPCR specific to several of the inflammation-associated genes performed on independently laser-captured cervical stroma validated these observations (Fig. 4C).

We next compared the effects of HPV oncogenes and estrogen on inflammation-associated gene expression in the cervical epithelium and stroma (Fig. 4D). In WT mice, estrogen treatment had minimal effect on inflammation-associated gene expression in the stroma but significantly up-regulated expression in the epithelia. In E6/E7 mice, inflammation-associated gene expression increased in both the cervical epithelia and surrounding stroma. Expression of inflammation-associated genes in the epithelia of E6/E7+E2 mice increased modestly compared with E6/E7 cervical epithelia but increased dramatically in the stromal compartment. The level of inflammation-associated gene expression was much higher in the E6/E7+E2 stroma than in the adjacent E6/E7+E2 epithelia or in either WT+E2 or E6/E7 stroma. Thus, HPV oncogenes induced the expression of proinflammatory genes in both the epithelial and stromal compartments of the cervix. However, estrogen appeared to function synergistically with epithelial HPV oncogene expression to specifically enhance stromal proinflammatory gene expression.

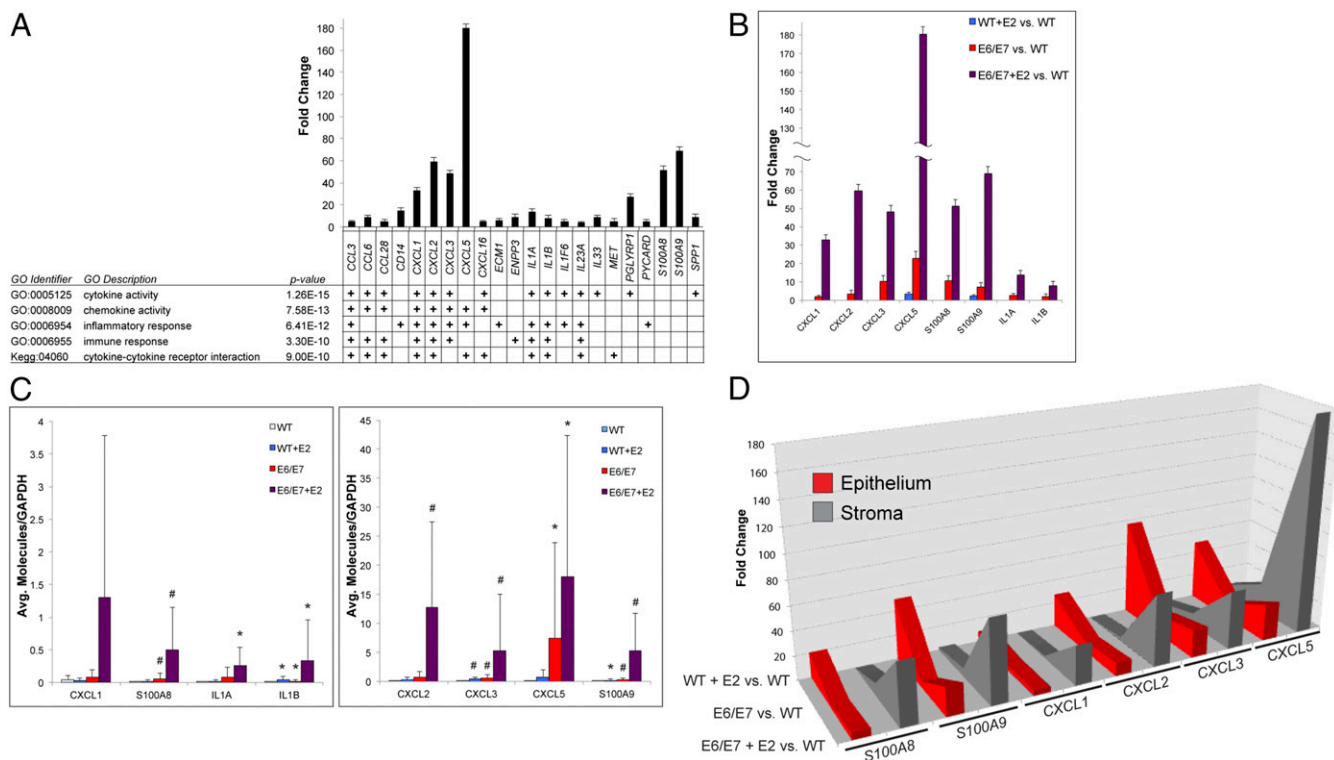
**Chemokine Expression Increases upon Coculture of Primary Human Cervical Cancer Cells with Cervical Fibroblasts.** Since stromal chemokine expression in E6/E7+E2 mice increased significantly

(Fig. 4), we evaluated whether it was likewise affected in human cervical cancer cells. Fresh normal human cervix and cervical cancers were evaluated for tissue histopathology and immunohistochemistry for p16, a well-validated marker of HPV infection (Fig. 5A). The cancers generally showed histopathological markers of HPV<sup>+</sup> cervical carcinomas, such as keratin pearls and p16<sup>+</sup> cells, whereas normal cervical samples did not. From these same tissue samples, primary epithelial and fibroblast cell cultures were established (Fig. 5B). HPV-specific PCR and immunoblots verified that normal samples were HPV<sup>-</sup> and that cancer samples were positive for high-risk HPV16 (cancer tissue no. 1) or HPV18 (cancer tissue no. 2) (Fig. 5B).

We hypothesized that interaction between HPV<sup>+</sup> cervical epithelia and the surrounding microenvironment contributes to the inflammatory signature we observed in mice. To test this, 3D cultures were generated from primary epithelial cells only, primary cervical fibroblasts only, or cocultures of both cell types. After 72 h of culture, levels of two representative chemokines, CXCL1 and CXCL5, were measured in conditioned medium (CM). Results from one representative normal cervical sample and one cervical tumor (normal tissue no. 2 and HPV18<sup>+</sup> cancer tissue no. 2 in Fig. 5B) are shown in Fig. 5C. The CXCL5 level in CM from cocultures was significantly higher than in CM from monocultured normal epithelial cells ( $P = 0.041$ ) and cancer epithelial cells ( $P = 0.018$ ). Likewise, CXCL5 levels were higher in cocultures containing both normal ( $P = 0.001$ ) and tumor ( $P = 9.1 \times 10^{-5}$ ) epithelial cells and fibroblasts than in cultures of fibroblasts alone. The CXCL5 concentration was also significantly higher in CM from cocultured cancer epithelial cells and fibroblasts than in CM from cocultured normal epithelial cells and fibroblasts ( $P = 0.039$ ). An additional comparison of an early-passage normal and a cervical cancer sample (normal tissue no. 1 and cancer tissue no. 1 in Fig. 5B and Fig. S24) showed similar results. CXCL5 secretion was also increased when cervical cancer epithelial cells were cocultured with human dermal lymphatic endothelial cells (HDLECs) (Fig. S2B), suggesting that, in addition to fibroblasts, other cell types may contribute to the inflammatory signature. The amount of CXCL1 produced from cocultured normal epithelial cells and fibroblasts ( $P = 0.005$ ) and cancer epithelial cells and fibroblasts ( $P = 0.0007$ ) was significantly higher than that produced from fibroblast monocultures. The level of CXCL1 produced from cocultured cells was also increased over that from epithelial cell monocultures, but this difference did not reach statistical significance (normal epithelial cell coculture,  $P = 0.095$ ; cancer cell epithelial cell coculture,  $P = 0.188$ ) (Fig. 5C). These results indicated that the expression of two representative inflammation-associated chemokines, CXCL5 and CXCL1, increases when early-passage human cervical epithelial cells and fibroblasts are cocultured. Interestingly, levels of CXCL1 and CXCL5 secreted from cocultured cells were much higher when early-passage cancer epithelial cells were cultured with their autologous fibroblasts (Fig. S2C), suggesting an inherent relationship between an individual tumor and its microenvironment that promotes high chemokine secretion. The analysis of primary human cervical cells showed that HPV<sup>+</sup> human cervical tumors interact with their surrounding microenvironment to increase inflammation-associated gene expression, as is consistent with the observations in the cervix of *K14E6/E7* mice.

**Discussion**

The activities of the multifunctional HPV oncogenes E6 and E7 (37) are not sufficient for cervical carcinogenesis (11). Previous work identified estrogen as a cocarcinogen that appears to function through signaling in the stroma (9, 10, 21, 27–29). In this study, we show that epithelial expression of the HPV oncogenes dramatically affects gene expression in the cervical stroma of *K14E6/E7* transgenic mice and influences the effects of estrogen on gene expression in the microenvironment (Figs. 1



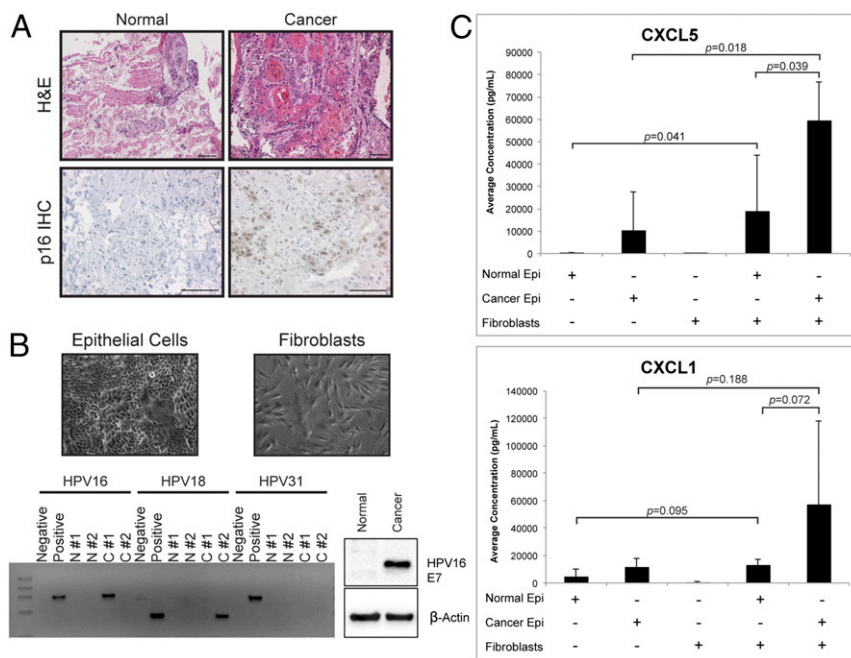
**Fig. 4.** Inflammation-associated gene expression is increased by epithelial E6/E7 expression and is exacerbated by chronic estrogen treatment. (A) GO was performed on the 87 extracellular genes up-regulated in the E6/E7+E2 stroma identified in Fig. 3A. The most significant GO groups associated with these genes are shown. Individual genes driving the significant GO associations are shown; a plus sign denotes that the gene is classified in the corresponding GO group. The fold change of each gene in the E6/E7+E2 stroma compared with WT stroma is shown in the bar graph. Error bars indicate SD. (B) Fold-change values are shown for a representative group of core genes (x axis) in the inflammatory signature identified in A. The fold change of each gene in the stroma of WT+E2 (blue bars), E6/E7 (red bars), and E6/E7+E2 (purple bars) compared with WT stroma is shown. Error bars indicate SD. (C) RT-PCR validation of selected inflammation-associated genes. RNA from laser-captured stroma was used to measure transcript levels of the indicated genes using RT-PCR. Statistical analysis was performed using a Wilcoxon rank-sum test. # $P \leq 0.01$ , \* $P \leq 0.05$  compared with WT. Error bars indicate SD. (D) Comparison of fold-change values for selected inflammation-associated genes in the epithelium (red) and stroma (gray) in WT+E2 (rear-most values), E6/E7 (middle values), and E6/E7+E2 (foremost values) compared with WT stroma.

and 2). Thus, HPV oncogenes and estrogen, individually and synergistically, affect gene expression in the murine cervical stroma. We reasoned that HPV-dependent, estrogen-induced paracrine factors in the stroma contribute to cervical carcinogenesis, and we identified differential expression of several candidates in the cervical stroma (Fig. 3). The stroma of estrogen-treated *K14E6/E7* mice had a strong inflammatory and immune-related gene-expression signature, specifically for chemokine and cytokine activity (Fig. 4). Primary human cervical cancer epithelial cells and fibroblasts similarly showed increased chemokine expression, but only when cocultured (Fig. 5). Therefore, inflammation-associated chemokines in *K14E6/E7* cervical stroma are candidate genes that may function in estrogen-driven epithelial–stromal crosstalk during carcinogenesis. While several studies have analyzed gene expression in cervical cancer epithelial cells (28, 38–40), few have performed such analyses on the cervical stroma (41–43). Our results define potentially important mechanisms through which HPV-infected epithelia and the microenvironment cooperate during cervical cancer development.

The primary objective of our study was to determine mechanisms of estrogen-dependent stroma-to-epithelium signaling. Surprisingly, we discovered additional epithelium-to-stroma signaling. Because we detected (i) low *K14E6/E7* transgene transcript levels in the stroma (Fig. S1C), (ii) little overlap in differentially expressed genes between stroma and epithelium (Fig. 1A), and (iii) strong opposing trends in gene-expression patterns, such as for cytokines, between the epithelium and stroma

(Fig. 4D), the profound changes in the stroma of *K14E6/E7* mice cannot be due to epithelial contamination of laser-captured stroma but rather is due to epithelium-to-stroma signaling between the HPV<sup>+</sup> epithelia and the surrounding microenvironment.

At least two nonexclusive hypotheses could explain this paracrine effect. First, the HPV oncogenes might alter the epithelial secretion repertoire in vivo as they do in keratinocytes cultured in organotypic rafts (44), leading to stromal changes. Second, changes in the number and/or content of extracellular vesicles secreted by HPV<sup>+</sup> epithelial cells and delivered to stromal cells might alter stromal gene expression. Exosomes, for instance, deliver proteins, RNA, miRNA, and/or DNA to recipient cells to modulate gene expression (45, 46). Virus infections can alter vesicle profiles (47). For example, Epstein–Barr virus–positive cancer cells release exosomes containing viral proteins, miRNAs, and signal transduction molecules that can activate protumorigenic signaling pathways in recipient cells (48, 49). Exosomes from HPV<sup>+</sup> keratinocytes contain several anti-apoptotic proteins (50), unique miRNAs (51, 52), and even E6 and E7 transcripts (52), suggesting transferable oncogenic potential. Thus, HPV-infected epithelial cells could use exosomes to alter gene expression in the microenvironment to support tumorigenesis. It will be important to determine not only the extent to which HPV<sup>+</sup> epithelial cells alter gene expression in a paracrine fashion but also the functions of individual HPV oncogenes in this process and whether these effects are reversible, as has been done previously in epithelial cells (53).



**Fig. 5.** Chemokine expression increases upon coculture of primary human cervical cancer cells with cervical fibroblasts. (A) Tissue sections of representative primary human normal cervical and cervical cancer samples (normal tissue no. 1 and cancer tissue no. 1) were stained with H&E (Upper) or were used for immunohistochemistry for the human p16 protein (Lower). (Scale bars, 100  $\mu$ M.) (B) Characterization of primary human cervical cell strains. (Upper) Representative brightfield images of epithelial (Left) and fibroblast (Right) cell strains isolated from primary human cervical samples. (Lower Left) HPV16-, HPV18-, and HPV31-specific PCR analysis of total DNA isolated from primary epithelial cell strains. Negative controls were DNA from untransfected normal cervical epithelial cells. Positive controls were DNA from normal cervical epithelial cells transfected with HPV16, HPV18, or HPV31 genomes. N#1, normal cervical epithelial cells, sample WICVX-1; N#2, normal cervical epithelial cells, sample WICVX-3; C#1, cervical cancer epithelial cells, sample WICVX-2; C#2, cervical cancer epithelial cells, sample no. 6204. (Lower Right) Immunoblot analysis of protein lysates harvested from normal (N#2) and cervical cancer epithelial (C#1) cells probed with an antibody specific to HPV16 E7 protein.  $\beta$ -Actin is shown as a loading control. (C) In vitro coculture experiments and analysis of secreted chemokines. The average concentration (pg/mL) of CXCL5 (Upper) and CXCL1 (Lower) in the CM of monocultured and cocultured cervical normal (normal tissue no. 2) and cancer (cancer tissue no. 2) epithelial cells and fibroblasts measured using a multiplexed bead-based screening assay. Shown are composite data from normal fibroblasts and cancer-associated fibroblasts, as no significant difference was observed between the different sources in either monoculture or coculture. Results represent three independent experiments. Epithelial-only and fibroblast-only concentration values were compared with coculture values using a multiple-experiment statistical test to preserve each experiment structure. Normal coculture and cancer coculture values were compared using a two-sided Wilcoxon rank-sum test. Error bars indicate SD.

Likewise, establishing whether exosomes are involved and which stromal cell types contribute to the differentially expressed gene patterns in the *K14E6/E7* cervical stroma will provide further mechanistic insight.

The striking increase in markers for epithelium-associated processes in the *K14E6/E7* stroma indicated that HPV16 E6 and E7 induce an epithelium-like stromal signature (Fig. 2A and Table S2). While surprising, epithelium-specific gene expression has been reported in cervical cancer stroma. Gius et al. (42) observed an increase in the transcription of *DSG3*-encoding desmoglein 3, involved in epithelial cell-cell contacts, in the stromal compartment of human cervical cancers. We did not detect an increase in *DSG3*, but *DSG1* and *DSG2* were increased significantly in the *K14E6/E7* stroma compared with nontransgenic stroma. Explanations for this epithelial signature in the stroma include the possibility that the HPV oncogenes elicit mesenchymal-to-epithelial transition or epithelial-mesenchymal plasticity in the stroma (54, 55). Another possibility is that HPV oncogenes increase the abundance of normally infrequent cervical mesenchymal stem cells expressing cytokeratins (56). The HPV oncogenes may induce such changes in the stroma to promote tissue regeneration through reepithelialization (57), a process that can occur by reprogramming stromal cells to become epithelial cells (58). This could also reflect a stromal response to the metabolic demands of HPV<sup>+</sup> epithelia, as suggested by signatures observed in a previous report (42). Future studies will investigate this epithelial signature and determine

whether the HPV oncogenes induce cellular plasticity in the stroma to facilitate neoplastic progression.

The ontology of down-regulated genes in *K14E6/E7* stroma was associated with ECM organization and stromal architecture (Fig. 2A and Table S2), driven by fibronectin (*FN1*), more than 15 collagen genes, tenascin C (*TNC*), thrombospondin 2 (*THBS2*), and cysteine-rich protein 61 (*CYR61*). These data are consistent with the reported association of precancerous lesions in *K14HPV16* mice with stromal collagen fibril degradation and extensive ECM remodeling (59). HPV16 E7 inhibits the fibronectin promoter (60), and the HPV oncogenes reduce *THBS1* gene expression in human keratinocytes (61). Interestingly, the potent angiogenesis inhibitors *THBS1*, *THBS2*, and *THBS3* thrombospondins (62) were all down-regulated in *K14E6/E7* stroma, suggesting a proangiogenic effect of HPV oncogenes on the microenvironment. However, the proangiogenic *CYR61* gene was among the most down-regulated genes in the *K14E6/E7* cervical stroma, in contrast to its previously reported increased expression in cultured fibroblasts isolated from the stroma of *K14HPV16* transgenic mice and human cervical cancers (29, 41). We postulate that HPV oncogenes reorganize the underlying stroma to accommodate processes such as vasculature reorganization, immune cell recruitment, and epithelial cell invasion. Our results indicate that, in addition to altering the local microenvironment through enzymatic means (63), the HPV oncogenes may also regulate ECM dynamics through transcriptional regulation.

In addition, our results show that the HPV oncogenes condition the stroma to respond differently to estrogen (Figs. 1B and

C and 2). Many differentially expressed genes, particularly those up-regulated, were uniquely expressed in the estrogen-treated *K14E6/E7* stroma (Fig. 1B). Moreover, the HPV oncogenes and estrogen synergistically increased the expression of several genes, including proinflammatory chemokines (Figs. 1C and 4), and may cooperate to dysregulate genes involved in collagen/ECM dynamics and focal adhesion in the cervical microenvironment of E6/E7+E2 mice (compare Tables S3 and S4). Therefore, while broadly affecting some pathways, estrogen distinctly affects biological processes in the cervical stroma of *K14E6/E7* mice compared with nontransgenic mice (Fig. 2B). We expect that among these HPV-dependent, estrogen-induced stromal genes will be microenvironmental factors critical for cervical carcinogenesis.

Many of the up-regulated extracellular genes in the estrogen-treated *K14E6/E7* cervical stroma encode proinflammatory cytokine/chemokines (Figs. 3 and 4), including *CCL3*, *CCL6*, *CCL28*, *CXCL1*, *CXCL2*, *CXCL3*, *CXCL5*, *IL1A*, *IL1B*, *S100A8*, and *S100A9*. We confirmed that *CXCL1* and *CXCL5* are similarly increased in CM of cocultured early-passage human cervical cancer cells and fibroblasts (Fig. 5), suggesting that cell-to-cell signaling elevates these chemokines in human cervical cancer. Cytokines have well-characterized roles in immune cell recruitment, tumor cell proliferation, angiogenesis, and modulation of the tumor microenvironment (36, 64), in large part through their effects on immune and endothelial cells. The cluster of *CXCL1*, *CXCL2*, *CXCL3*, and *CXCL5* genes in the inflammation signature are members of the ELR<sup>+</sup> C-X-C family of chemokines that bind the CXCR2 receptor. Despite the increase in expression of several CXC ligands in the E6/E7+E2 stroma, we did not observe significant variation in CXCR2 receptor gene expression among various conditions. The CXCL/CXCR2 signaling axis is considered proangiogenic and is implicated in a variety of human cancers (65–70). Strikingly similar to our report here, proinflammatory signatures have been observed in stromal fibroblasts adjacent to prostate cancer (71), melanoma (72), breast cancer (73), and HPV-associated skin and cervical cancers (29, 41).

Inflammation is implicated in several facets of HPV-associated carcinogenesis (74). In *K14HPV16* skin, an influx of inflammatory cells was proposed to cause stromal ECM remodeling (75), and inflammation regulates the angiogenic switch (76). Our results contrast with an earlier report by Erez et al. (41), who identified a highly similar proinflammatory signature including CXCR2 ligands in fibroblasts adjacent to dysplastic skin, but not the cervix, of HPV transgenic mice. Conversely, moderate expression of these proinflammatory genes was reported in fibroblasts isolated from human cervical cancers (29). Our results identified inflammation-associated genes as some of the most highly differentially expressed genes in the E6/E7 and E6/E7+E2 cervical stroma and revealed the origins of this response. Our tissue culture-based experiments further support the hypothesis that communication between epithelial cells and multiple stromal cell types, including fibroblasts (Fig. 5) and endothelial cells (Fig. S2B), contributes to proinflammatory gene expression. Therefore, the discordant observations between reports may be due to evaluating a single cell type rather than the microenvironment in its entirety. Nonetheless, our results show that proinflammatory cytokines and chemokines are clear candidate extracellular genes that are induced synergistically by the HPV oncogenes and estrogen in the *K14E6/E7* stroma. There is evidence that prostate epithelial cell-derived IL-1 can induce the expression of several of the same CXCR2 ligands in prostate stromal cells (71). Interestingly, the *IL-1* gene was significantly increased in the *K14E6/E7* cervical epithelia in our study, and this factor could therefore be part of a mechanism that induces proinflammatory gene expression in the cervical stroma. Antiestrogen drugs reduced the expression of several genes in the proinflammatory signature in

human cervical cancer-associated fibroblasts (29), suggesting that estrogen signaling may also contribute to chemokine expression. It will be interesting to determine the contribution of proinflammatory chemokines to cervical cancer development in estrogen-treated *K14E6/E7* mice and how their stromal expression correlates with cervical cancer regression and recurrence following antiestrogen therapy (23, 77).

Additional candidate paracrine factors unique to the estrogen-treated *K14E6/E7* cervical stroma were identified (Fig. 3), including *IL1A* and *IL1B*, fibroblast growth factor 9 (*FGF9*), and heparin-binding epidermal growth factor-like growth factor (*HBEGF*). IL-1 $\alpha$ , a proinflammatory cytokine, has been suggested to provide a selective growth advantage to HPV<sup>+</sup> epithelia (78). Secreted FGF9 is highly induced in gastric cancer-associated fibroblasts and promotes invasion and antiapoptotic mechanisms in gastric cancer epithelial cells (79). Moreover, estrogen and FGF9 synergize to increase the number of breast cancer stem cells (80). *HBEGF*, an EGF receptor (EGFR) ligand, is detected in and produced by stromal fibroblasts adjacent to cervical cancers (29, 81). Such fibroblasts increase cervical cancer epithelial cell proliferation in an *HBEGF*-dependent manner (81). Antiestrogen drugs reduce *HBEGF* gene expression in cervical cancer-associated fibroblasts, suggesting that estrogen promotes its induction (29).

In conclusion, using our *in vivo* mouse model of HPV-associated cervical cancer, we have shown that the HPV oncogenes and estrogen dramatically alter host gene expression in the cervical stroma. With the extensive prior results supporting stromal-to-epithelium signaling (27, 28), this reveals that the epithelial–stromal crosstalk in cervical carcinogenesis is bidirectional. These results provide a foundation for future investigations in several important facets of the progression and maintenance of cervical cancers and potentially other HPV<sup>+</sup> cancers. This includes further focus on key paracrine factors and molecular pathways implicated here; their effects on angiogenesis, immune cell recruitment, and so forth; and the sequence of these events. Since our principal aim was to analyze genome-wide gene expression in the tumor microenvironment of HPV<sup>+</sup>, estrogen-driven cervical cancer, the current study analyzed time points soon after cancers appeared, i.e., after 6-mo estrogen treatment of *K14E6/E7* mice. Our recent preliminary evidence from 1-mo estrogen treatment of *K14E6/E7* mice indicates that at least some of the changes in stromal gene expression observed here are already apparent well before the appearance of cancers. Thus, in the future it will be useful to analyze intermediate estrogen treatment times to more fully define the order of stromal and epithelial changes that accompany neoplastic progression. Such studies should further illuminate how HPV oncogenes and estrogen interact across tissue compartments to drive progression and maintenance of cervical cancer and further assist the identification of therapeutic interventions.

## Materials and Methods

**Animals, Treatment, and Tissue Processing.** *K14E6/K14E7* (referred to as *K14E6/E7* herein) bitransgenic mouse lines maintained on the *FVB/N* genetic background have been described previously (3, 82). Mice were housed and treated in the American Association of Laboratory Animal Care-approved Wisconsin Institute for Medical Research Vivarium of the University of Wisconsin School of Medicine and Public Health (Madison, WI) according to a protocol approved by the University of Wisconsin Institutional Animal Care and Use Committee. Nontransgenic *FVB/N* and *K14E6/E7* transgenic female mice were either untreated or treated with exogenous estrogen (17 $\beta$ -estradiol). Frozen reproductive tracts were cryosectioned and used for histopathological analysis to assign lesion location and grade. Regions of interest (ROIs) in the epithelial or stromal compartments were sampled using LCM.

**RNA Processing and Microarray-Based Gene-Expression Analysis.** Comprehensive mRNA levels were measured essentially as described previously (40). Briefly, RNA was extracted from laser-captured material and used to generate T7 RNA



polymerase promoter-linked, oligo(dT)-primed double-stranded cDNA, which was then used as a template to produce T7 transcripts complementary to all mRNAs (cRNA). These T7 transcripts were then subjected to a second round of cDNA synthesis and T7 RNA polymerase-based amplification. The resulting cRNA was hybridized on Affymetrix Mouse Genome430 2.0 microarrays.

**Biostatistics and GO.** Robust microarray analysis (RMA)-normalized data were used as input, and analysis was performed using the MeV microarray analysis suite (83). Rank product analysis was performed to compare gene expression between pairs of sample groups. The resulting *P* values were corrected for multiple analyses using the Benjamini–Hochberg method, which sets the FDR. Statistical cutoffs were set at twofold changes in gene expression and FDR  $\leq 0.05$  unless otherwise stated. The GeneCodis program was used for ontology analysis (84–86), using significantly differentially expressed genes as input. All Venn diagrams were created using BioVenn (87).

**Coculture Experiments in Early-Passage Cell Strains.** The use of human tissue samples deidentified before receipt was determined to be exempt from the need for Institutional Review Board (IRB) approval and informed consent. Primary human cervical epithelial and fibroblast cell strains were isolated from cervical normal and tumor tissues within 24–48 h of surgical resection. Epithelial cells were cultured in F medium plus 10  $\mu$ M ROCK inhibitor Y-27632, a Rho kinase inhibitor that extends the life span of keratinocytes (88), with a

feeder layer of mitomycin C-treated murine fibroblast J2 3T3 cells. Fibroblasts were cultured in F12 Ham's medium supplemented with 10% FBS and 1% penicillin/streptomycin (P/S). For all in vitro experiments, derived epithelial cell strains were used before six passages, and fibroblast cell strains were used before 12 passages. hTert-immortalized HDLECs (hTert-HDLECs) have been described previously (89). 3D monocultures or cocultures were generated with fibroblasts embedded in collagen and with epithelial cells layered on top of the collagen. All cultures were maintained in F medium without ROCK inhibitor. At 72 h, CM was assayed for several analytes using a magnetic bead-based multiplex assay.

For more detailed materials and methods, please refer to *SI Materials and Methods*.

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