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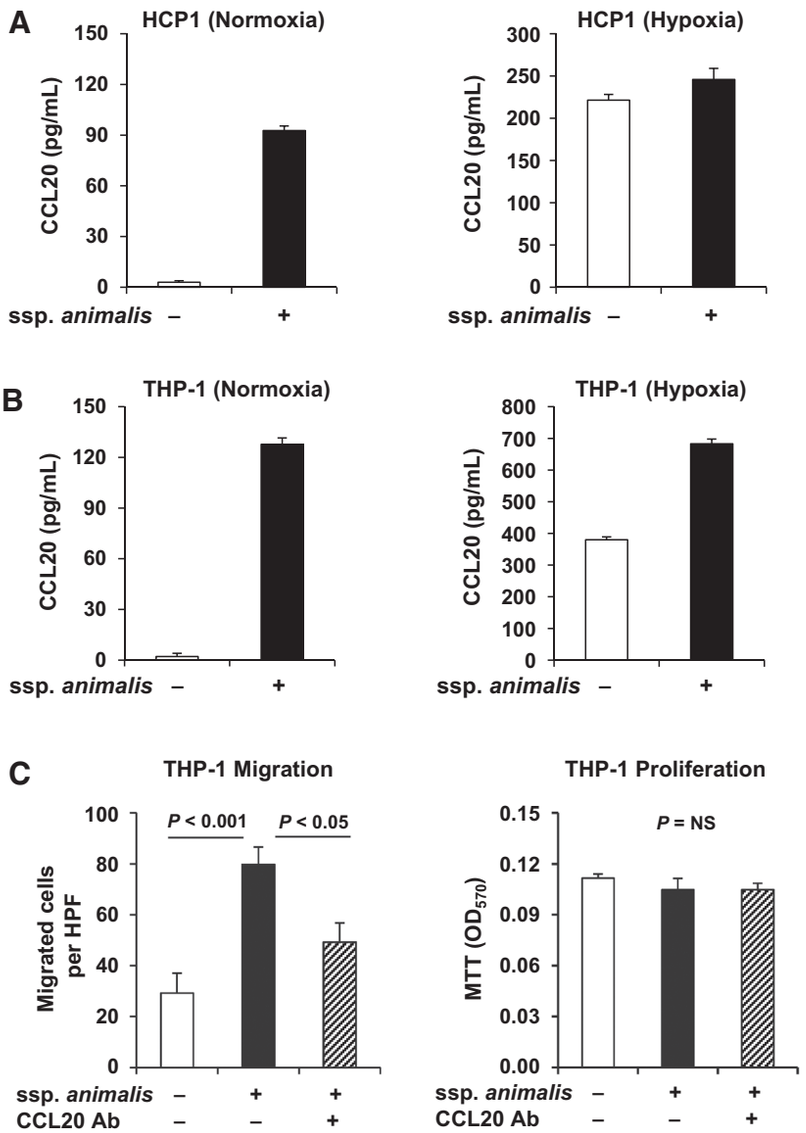

Figure 5.

F. nucleatum ssp. *animalis* induces CCL20 expression and monocyte migration in coculture. **A,** ELISA-based measurement of the CCL20 concentrations in the conditioned media from coculture of HCP1 colorectal cancer cells with or without *F. nucleatum* ssp. *animalis* under normoxic or hypoxic conditions. **B,** The CCL20 concentrations in the conditioned media from coculture of the THP1 monocytes with or without *F. nucleatum* ssp. *animalis* under normoxic or hypoxic conditions. **C,** *F. nucleatum* ssp. *animalis* stimulates monocyte migration that can be partially blocked by a CCL20-neutralizing antibody. The migration of THP-1 monocytes was assessed in Boyden chamber systems with or without *F. nucleatum* ssp. *animalis* or a CCL20-neutralizing antibody under normoxic conditions. The cell proliferation of THP-1 monocytes under the same treatments was measured by MTT assay. The growth rate was not significantly different between the control and treated cells.

dynamics in the specimens. In addition to the sequencing analysis, we established a qPCR assay and demonstrated differential distribution of *Fusobacterium* in the colorectal specimens. Further improvement of the assay specificity for detecting the disease-relevant species will help resolve some inconsistencies regarding the role of *Fusobacterium* in colorectal cancer (41–43).

Fusobacterium spp. and ssp. are very heterogeneous (10, 11). In our in-depth taxonomic analysis, we found that *F. nucleatum* was the prevalent species present in the tumor specimens we profiled. Our analysis further revealed that *F. nucleatum* ssp. *animalis* was the most dominant subspecies associated with colorectal cancer in the same specimens. Interestingly, a recent study of the fecal samples from colorectal cancer patients showed that *F. nucleatum* ssp. *vincentii* and *F. nucleatum* ssp. *animalis* were the top-ranked gut microbial species associated with colorectal cancer (44). Although we agree that the predominance of *F. nucleatum* sp. *animalis* was associated with colorectal cancer, our data did not support *F. nucleatum* ssp. *vincentii* as an abundant species in the tissue specimens of colorectal tumors (Fig. 3A; Supplementary Fig. S2). Further validation is critical for the identification of the specific *Fusobacterium* pathogen and its oncogenic mechanism(s) that, in turn, may theoretically be targets for prevention and possibly even treatment of colorectal cancer.

F. nucleatum ssp. *animalis* infections associated with human diseases have been documented in the literature. Studies have demonstrated an association of *F. nucleatum* ssp. *animalis* virulence with inflammatory periodontal diseases and adverse pregnancy outcomes (45, 46). Interestingly, *F. nucleatum* ssp. *animalis* was found more

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frequently in the isolates from gastrointestinal tract than in the isolates from oral sites (47). A draft genome sequence of *F. nucleatum* ssp. *animalis* was reported (48), but information of its unique genetic features is lacking. In addition, *F. nucleatum* ssp. *animalis* has two bacteriophage variants (49). Because bacteriophages are known to contribute to the host strain's virulent phenotype, the *F. nucleatum* ssp. *animalis* bacteriophage may be another layer of the complexity of this subspecies' virulence.

Investigators have explored the function of *Fusobacterium* spp. in carcinogenesis. Early studies demonstrated *Fusobacterium* strains that are invasive and proinflammatory in the oral mucosa (50). The strains can also invade colorectal cancer cells and induce cytokine secretion (51–53). In colorectal adenomas and carcinomas, invasive *Fusobacterium* spp. can be visualized using FISH (8, 54). In an $Apc^{Min/+}$ mouse model of colorectal cancer, *F. nucleatum* promoted intestinal carcinogenesis via the bacterial virulence factor FadA, which binds to E-cadherin on the host cell surface and activates β-catenin signaling and inflammatory response (52). On the other hand, *F. nucleatum* increased tumor multiplicity and selectively recruited tumor-infiltrating myeloid cells, predominantly myeloid-derived suppressor cells, with potent immunosuppressive activity (40). *F. nucleatum* infection has also been associated with NF-κB activation and proinflammatory gene expression (40, 53). Similarly, in the present study, we found that expression/secretion of proinflammatory cytokines IL17A and TNF was markedly higher in colorectal tumors than in adjacent mucosal tissue. In addition, we found that CCL20 protein was increased in the colorectal adenomas and carcinomas compared with mucosa.

CCL20 and its receptor CCR6 are known for their important roles in the recruitment of immune cells and their paradoxical functions in regulation of both immunological tolerance and inflammation (55). Clinical studies have established that overexpression of CCL20 and CCR6 is associated with colorectal cancer progression (19, 26, 27, 56). In preclinical studies, the CCL20/CCR6 axis has proven to be critical for intestinal tumorigenesis in mice. For example, using the mutagen MNU plus *Helicobacter pylori* to induce colorectal tumorigenesis in wild-type C57BL mice, investigators showed that tumor-associated macrophages recruited $CCR6^+$ regulatory T cells to the tumor microenvironment via CCL20 signaling and promoted tumor growth (24). However, in $Apc^{Min/+}$ mice, knockout of CCR6 decreased spontaneous intestinal tumorigenesis via reduction of macrophage recruitment to the inflamed intestinal mucosa (57). Taking our own data into consideration for the *F. nucleatum* ssp. *animalis* interaction with colorectal cancer cells and monocytes, the response was stronger in the monocytes and showed a macrophage activation phenotype with increased migration and CCL20 protein expression, particularly under a hypoxic condition mimicking the tumor microenvironment. In a recent study of *Fusobacterium* and T-cell density in colorectal cancer, investigators showed that *Fusobacterium*-high cases were inversely associated with the density of $CD3^+$ pan-T cells (58). Future studies need to address whether the *Fusobacterium*-induced cytokine/chemokine signaling, including CCL20/CCR6 axis, selectively regulates the inflammation and immune suppression in tumor microenvironment, thus promoting the colorectal tumor growth and progression.

In summary, we identified five species of *Fusobacterium* in clinical colorectal cancer specimens, of which *F. nucleatum* was the most predominant. We also identified and confirmed that *F. nucleatum* ssp. *animalis* was the most prevalent subspecies of *F. nucleatum* in colorectal tumors. Moreover, we found that colorectal tumors differentially expressed the proinflammatory cytokines IL17A, IL21, TNF, and CCL20, with CCL20 being highly expressed at every stage of CRC. In *in vitro* assays, coculture with *F. nucleatum* ssp. *animalis* markedly induced CCL20 protein expression in certain colorectal cancer cells. Similarly, the coculture strongly stimulated CCL20 protein expression in monocytes and induced monocyte/macrophage activation and migration, suggesting that *F. nucleatum* ssp. *animalis* interacts directly with monocytes, which may recruit other immunoregulatory cells via CCL20 signaling and subsequently promote colorectal cancer progression. These findings provide unique insight into the tumorigenic mechanism mediated by *Fusobacterium* spp. and mucosal immunity. More importantly, we identified a specific subspecies of tumor-associated *F. nucleatum* ssp. *animalis* that may be useful for early-stage detection of colorectal cancer and for antibiotic therapy or vaccination against for colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: X. Ye, R. Bhattacharya, L.M. Ellis
Development of methodology: X. Ye, L. Xia, J.F. Petrosino
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Ye, R. Wang, F. Fan, L. Xia, N.J. Ajami, J.F. Petrosino, S. Venable, D. Maru, L.M. Ellis
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Ye, R. Bhattacharya, N.J. Ajami, M.C. Wong, D.P. Smith, J.F. Petrosino, W. Qiao, V. Baladandayuthapani
Writing, review, and/or revision of the manuscript: X. Ye, D.R. Boulbes, N.J. Ajami, S. Venable, L.M. Ellis
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.R. Boulbes, D.P. Smith, D. Maru, L.M. Ellis
Study supervision: L.M. Ellis

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Cancer Prevention Research

Fusobacterium Nucleatum Subspecies *Animalis* Influences Proinflammatory Cytokine Expression and Monocyte Activation in Human Colorectal Tumors

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