Genome-Wide Identification of *Fusobacterium nucleatum* Factors Associated with Oral Squamous Cell Carcinoma

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Abstract

Fusobacterium nucleatum (*Fn*) is an oral commensal bacterium known to promote both oral and extraoral diseases such as periodontitis, colorectal cancer and oral squamous cell carcinoma (OSCC). Despite being the most prevalent and aggressive oral malignancy, the bacterial factors in *Fn* which promote OSCC are still largely known. To identify and characterize OSCC-promoting *Fn* virulence factors, a sequence-defined *Fn*-Tn5 transposon library is applied. A genome-wide high-throughput screening approach was adopted by infecting a GFP-expressing OSCC cell line with this library. By monitoring GFP intensity, we identified mutants with promoted or attenuated spheroid growth. Secondary and tertiary screenings examining spheroid formation, growth, and 2D&3D cell migration excluded false-positive mutants. Two candidates that consistently showed cancer promotion or attenuation were selected for generating marker-less deletion mutants for future confirmation. This large-scale screening identified *Fn* virulence factors contributing to our understanding of *Fn*-promoted OSCC, ultimately aiming to illuminate potential OSCC therapeutic targets.

Background

- *F. nucleatum* (*Fn*) is a key colonizer in oral biofilm development and an opportunistic pathogen when traversing to distal sites like the placenta and colon, where it is associated with significant diseases such as preterm birth and human colorectal cancer. [1]
- Research supports the role of *Fn* as an oncobacterium by promoting tumor proliferation in OSCC. [2]
- Ton-That lab has previously developed a genetic toolbox for *Fn* research. Using Tn5 transposon mutagenesis and DNA-sequencing to map the insertion sites of the transposon, a Tn5 library was generated consisting of roughly 1,400 unique *Fn* mutants. This library was previously used to identify virulence factors associated with *Fn* coaggregation. [3]

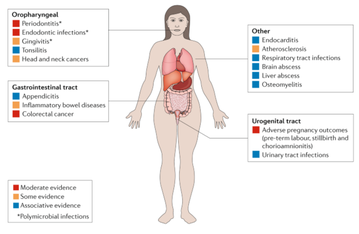

Fig. 1: Oral and extraoral diseases associated with *Fusobacterium nucleatum.* [4]

Methods

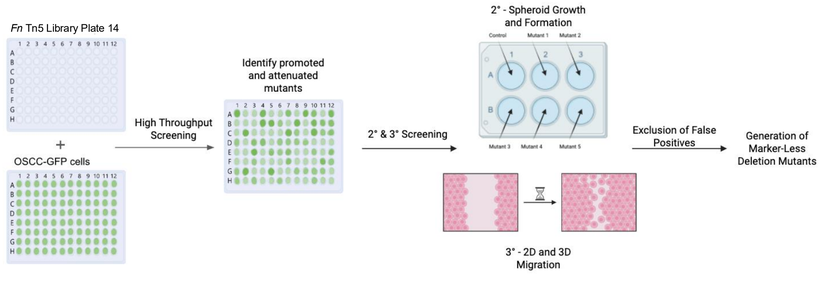

Fig. 1: Schematic of methods. 2,000 SCC9-GFP cells were seeded in each well of a 96-well plate. Plate 14 of the *Fn* Tn5 library was inoculated from frozen stock in a deep well 96-well plate. Infection with the cultured *Fn* library plate occurred 24 hours post seeding. Infection with mutants exhibiting promoted or attenuated growth were selected for secondary and tertiary screening. These methods included spheroid growth and spheroid formation assays, along with wound healing and transwell migration assays. Mutants retaining the promoted or attenuated phenotype produced in the initial screen were selected to be generated as marker-less deletion mutants.

Results

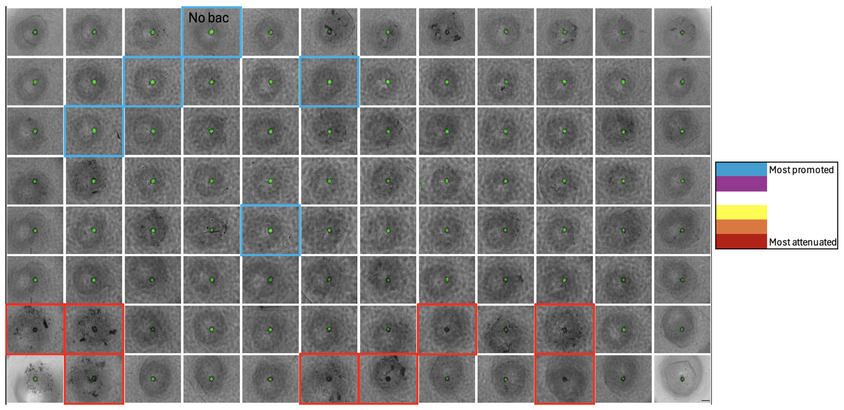

Fig. 2: High-throughput screening with plate 14 of the *Fn* Tn5 Library. Infection of spheroids with individual *Fn* mutants induces differing spheroid growth phenotypes 72 hours post-infection. Spheroid phenotype can be categorized into six groups based on GFP intensity and spheroid size. Scale: 500 µm.

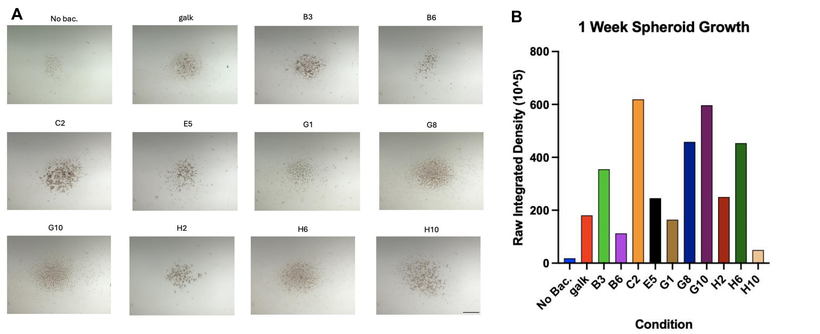

Fig. 3: Infection of OSCC spheroids with individual *Fn* mutants 24 hours post seeding. $1x10^5$ OSCC cells infected at MOI 50 one day post-seeding. **(A)** Infection with individual mutants resulted in promoted, normal, or attenuated growth compared to parental control one week post infection. **(B)** Quantification of spheroid growth 1 week post infection. Scale: 1 cm.

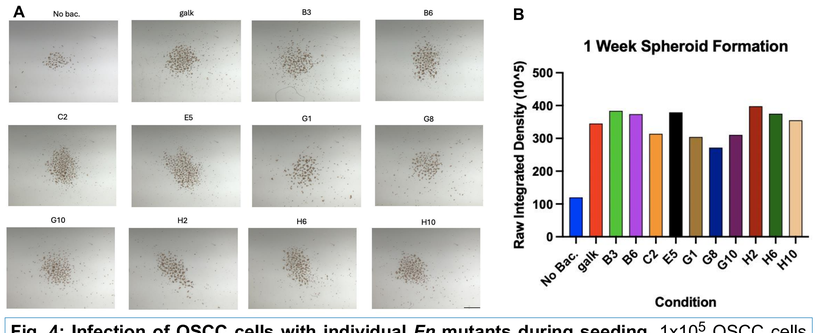

Fig. 4: Infection of OSCC cells with individual *Fn* mutants during seeding. $1x10^5$ OSCC cells infected at MOI 50 immediately after seeding. **(A)** Infection with individual mutants resulted in promoted, normal, or attenuated growth compared to parental control one week post infection. **(B)** Quantification of spheroid growth 1 week post infection. Scale: 1 cm.

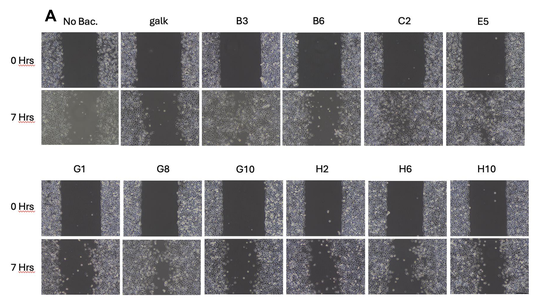

Fig. 5: Infection of wound healing assay with individual *Fn* mutants. Cells infected at MOI 50 one day post-seeding in ibidi silicone inserts. **(A)** Infection with individual mutants resulted in promoted, normal, or attenuated migration compared to parental control one week post infection. Scale: 100 µm. **(B)** Quantification of wound healing.

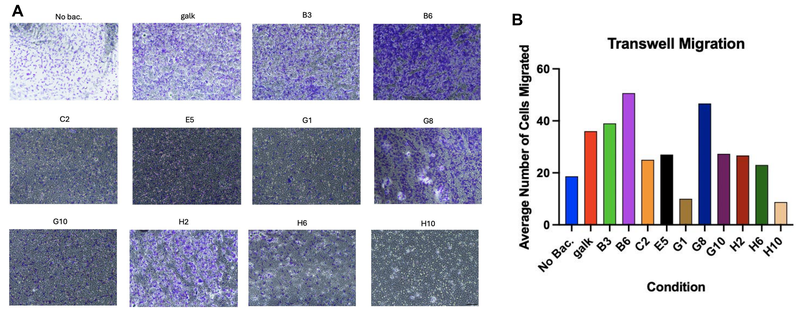

Fig. 6: Infection of OSCC cells using 8 µm pore semi-permeable membrane. $1x10^5$ OSCC cells infected at MOI 50. **(A)** Infection with individual mutants resulted in promoted, normal, or attenuated transwell migration compared to parental control one week post infection. Scale: 100 µm. **(B)** Quantification of transwell migration.

Discussion

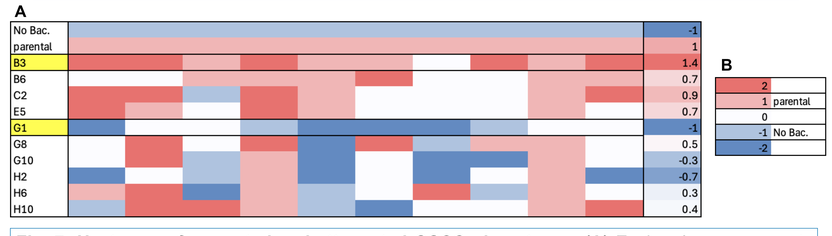

Fig. 7: Heat map of promoted and attenuated OSCC phenotypes. (A) Each column represents the results of one cancerous assay. The OSCC phenotype resulting from infection with each individual *Fn* mutant was scored from -2 to 2 based on quantitative results. The average score for each mutant shown in the rightmost column. **(B)** Ranking system. A score of 1 represents an OSCC phenotype comparable to that of the parental control, and -1 representing a phenotype comparable to the negative control.

Identified two potential key virulence factors in *Fn*-promoted OSCC.

Mutant B3: TRAP transporter substrate-binding protein DctP.

Mutant G1: s-methyl-5-thioribose-1-phosphate isomerase.

Future Aims

- **Markerless deletion mutants of B3::Tn5 and G1::Tn5 are currently being generated.**
- **Deletion mutants will be used for conformation of promotional and attenuated phenotypes in cancerous assays.**
- **Following conformation, we will further characterize the gene mechanisms by employing RNAseq along with proteomics and structural biology approaches.**

References

1.Han YW (2015). *Fusobacterium nucleatum*: a commensal-turned pathogen. Curr Opin Microbiol 23:141- 7. PMC4323942

2.Geng F, Zhang Y, Lu Z, Zhang S, Pan Y. (2020). Fusobacterium nucleatum Caused DNA Damage and Promoted Cell Proliferation by the Ku70/p53 Pathway in Oral Cancer Cells. *DNA Cell Biol* 39:144-151. PMC6978777

3.C. Wu, Y. Chen, M. Scheible, C. Chang, M. Wittchen, J.H. Lee, T.T. Luong, B.L. Tiner, A. Tauch, A. Das, & H. Ton-That. (2021). Genetic and molecular determinants of polymicrobial interactions in Fusobacterium nucleatum, *Proc. Natl. Acad. Sci. U.S.A.* 118

4.Brennan, C.A., Garrett, W.S. *Fusobacterium nucleatum* — symbiont, opportunist and oncobacterium. *Nat Rev Microbiol* **17**, 156–166 (2019). https://doi.org/10.1038/s41579-018-0129-6