A role for Fibroblast Growth Factor Receptor 1 in the pathogenesis of *Neisseria meningitidis*

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Summary

Neisseria meningitidis remains an important cause of human disease. It is highly adapted to the human host – its only known reservoir. Adaptations to the host environment include many specific interactions with human molecules including iron-binding proteins, components of the innate and adaptive immune systems, and cell surface receptors such as the Epidermal Growth Factor Receptor (EGFR). Interaction of the meningococcus with EGFR has been elucidated in some detail and leads to intracellular signalling and cytoskeletal changes contributing to the pathogenesis of the organism. Here, we show that the meningococcus also recruits Fibroblast Growth Factor Receptor 1 (FGFR1) onto the surface of human blood microvascular epithelial cells (HBMECs). Furthermore, meningococci internalised into these cells recruit the activated form of this receptor, and that expression and activation of FGFR1 is necessary for efficient internalisation of meningococci into HBMECs. We show that *Neisseria meningitidis* interacts specifically with the IIIc isoform of FGFR1.

Introduction

Neisseria meningitidis, or the meningococcus, while normally a harmless commensal of the human oropharynx, can occasionally cause devastating disease including meningitis, sepsis, disseminated intravascular coagulation (DIC) and multiple organ failure (Takada *et al.*, 2016). Penetration of meningococci through the oropharyngeal epithelial mucosa and into the blood is a crucial step in the development of sepsis and other systemic diseases, while penetration of the blood-brain barrier (BBB) is a prerequisite for the development of meningitis (Nassif, 1999, Virji, 2009). Attachment to endothelial cells induces membrane protrusions at the bacterial binding site and leads to the formation of specific protein complexes known as cortical plaques underneath the bacterial colonies (Merz *et al.*, 1999, Eugene *et al.*, 2002). The process by which these steps occur is incompletely understood (Sokolova *et al.*, 2004, Yazdankhah *et al.*, 2004, Hill *et al.*, 2010), but identified meningococcal adhesins include components of the type IV pilus (PilC1, PilC2 and PilQ), the outer membrane proteins Opa, Opc, Factor H-binding protein, PorA, PorB, HrpA, NadA, App and MspA, as well as lipooligosaccharide (LOS) (Merz *et al.*, 2000, Hadi *et al.*, 2001, Turner *et al.*, 2006, Morand *et al.*, 2009). Host cell receptors that have been identified include alpha actinin, integrins, CEACAMS, CD46, Complement receptor 3, GP96 scavenger receptor, laminin, platelet-activating factor, mannose receptor, Transferrin receptor 1, Laminin receptor and Galectin-3 (Merz *et al.*, 2000, Morand *et al.*, 2009, Orihuela *et al.*, 2009, Quattroni *et al.*, 2012, Alqahtani *et al.*, 2014, Khairalla *et al.*, 2015).

Fibroblast Growth Factor Receptors (FGFRs) are transmembrane proteins that belong to the Receptor Tyrosine Kinase (RTK) family of signalling molecules. This family consists of four members that are responsible for recognising all 22

42 Fibroblast Growth Factor molecules (FGFs) found in humans (Ornitz *et al.*, 2001,
43 Turner *et al.*, 2010, Guillemot *et al.*, 2011). FGFs are involved in cell
44 differentiation, migration and proliferation during early embryogenesis and play
45 an important role in tissue repair, wound healing (Ortega *et al.*, 1998) and tumour
46 angiogenesis in adulthood (Gerwins *et al.*, 2000, Eswarakumar *et al.*, 2005, Presta
47 *et al.*, 2005). Splicing of FGFR transcripts generates a variety of specific isoforms
48 in various types of cells and tissues recognising specific types of FGF molecules
49 (Miki *et al.*, 1992, Groth *et al.*, 2002, Eswarakumar *et al.*, 2005, Turner *et al.*,
50 2010).

51 FGFRs are single transmembrane receptors; the extracellular N-terminal
52 region consists of three IgG-like domains that form a ligand-binding domain with
53 an acidic box which interacts with heparin sulphate proteoglycans (HSPGs) and
54 Cell Adhesion Molecules (CAMs). This is followed by a transmembrane region and
55 a C-terminal cytoplasmic region containing 7 specific tyrosine residues (Ornitz *et*
56 *al.*, 1992, Reiland *et al.*, 1993, Cavallaro *et al.*, 2004, Francavilla *et al.*, 2009).
57 Binding of FGFs to FGFR leads to dimerisation of the receptor and activation of
58 tyrosine autophosphorylation, which in turn activates the receptor, leading to
59 activation of downstream signalling pathways (Mohammadi *et al.*, 1992,
60 Mohammadi *et al.*, 1996, Lundin *et al.*, 2003, Zhang *et al.*, 2006). Previous studies
61 confirmed the importance of FGFR1 signalling and expression in maintaining the
62 integrity and differentiation of endothelial cells forming the microvasculature
63 (Kanda *et al.*, 1996, Gerwins *et al.*, 2000, Murakami *et al.*, 2008).

64 The possible role of FGFRs in infectious diseases has not been excessively
65 investigated, although FGF2 expression enhance *Chlamydia trachomatis* binding
66 and internalisation into epithelial cells (Kim *et al.*, 2011). *C. trachomatis* facilitates
67 entry by binding directly to FGF2, which results in binding of FGF2-bacteria-

68 heperan sulfate proteoglycan (HSPG) complexes to FGFR and internalisation of
69 the elementary bodies of these bacteria into the epithelial cells. The study also
70 showed higher levels of FGFR substrate 2 (FRS2) activation as a results of FGF2/*C.*
71 *trachomatis* treatment in Hela cells, and higher level of FGF2 expression via
72 activation of ERK1, 2. More recently, HSPG-associated FGFR1 has been implicated
73 in internalisation of *Rickettsia rickettsii* into cultured human microvascular
74 endothelial cells and inhibition of FGFR1 in a *R. conorii* murine model of
75 endothelial-target spotted fever rickettsiosis reduced the rickettsial burden in
76 infected mice (Sahni *et al.*, 2017).

77 In a study of role of RTKs, and specifically EGFRs, in meningococcal infection
78 higher levels of FGFR1 activation were observed in endothelial cells in response to
79 infection (Slanina *et al.*, 2014). Here we investigated the possible direct
80 interaction of FGFRs expressed in HBMECs with meningococci and the influence of
81 such an interaction on the ability of *N. meningitidis* to invade these cells.

82

83 **Results**

84 *Meningococci recruit FGFR1 on the apical surface of Human Brain Microvascular*
85 *Endothelial Cells (HBMECs).* To study the possible interaction of FGFR1 by
86 meningococal colonies, HBMECs were infected with *N. meningitidis* for 4 h, fixed,
87 and prepared for immunofluoresce microscopy. FGFR1 was recruited by
88 meningococcal colonies on the apical surface of HBMECs. FGFR1 coincided with
89 recruitment of both 67LR and 37LRP isoforms of laminin receptor (Figure 1). To
90 address whether FGFR1 recruited by meningococci is activated, HBMECs were
91 labelled with primary antibodies specific for phosphorylated tyrosine 766 (p-
92 Y766), 67LR and 37LRP. In all cases the phosphorylated FGFR1 co-localised with
93 meningococcal cells (Figure 1).

94 Meningococcal colonies co-localised with activated FGFR1 and 37LRP, up to 60%
95 of which was co-localised with FGFR1. This was significantly higher than co-
96 localisation of the microcolonies with the 67LR isoform of the receptor (Figure 1-
97 B, C).

98 *Internalised N. meningitidis associated with activated FGFR1.* To determine
99 whether internalised bacteria are associated with activated FGFR1, HBMECs were
100 infected for 4 h with *N. meningitidis* and non-internalised bacterial cells were killed
101 by gentamicin. Immunofluorescent staining for actin and activated FGFR1 (p-
102 Y766) confirmed that meningococci recruited activated FGFR1 in the cytoplasm of
103 HBMECs, and that the receptor was trafficked inside the cells alongside with
104 meningococcal cells (Figure 2). To confirm that the bacterial cells in gentamycin-
105 treated monolayers were internalised, a Z-stack image was constructed. Bacterial
106 cells could be observed beneath the membrane of the endothelial cells (Figure 2-

107 B). Furthermore, when cells were permeabilised prior to gentamycin treatment no
108 bacterial cells were observed (Figure 2-C).

109 *FGFR1 expression and activation is required for meningococcal invasion into*
110 *HBMECs*. To study the role of FGFR1 in interaction of meningococci with HBMECs,
111 FGFR1 expression was knocked down in HBMECs using siRNA treatment. Sixty
112 hours post-siRNA treatment, cells were infected with meningococci and cell
113 association and invasion assays were performed. The chemical inhibitor of FGFR1
114 (SU5402) (Mohammadi *et al.*, 1997) was also used to examine the effect of
115 inhibiting the activation of FGFR1 during meningococcal infection.

116 The numbers of meningococcal cells associated with HBMECs was significantly and
117 dramatically reduced in response to either FGFR1 knock-down or SU5402-
118 treatment (Figure 3-A). Treatment with scrambled siRNA under the same
119 conditions did not result in a significant reduction in association of meningococci
120 with HBMEC cells. There was also a significant decrease in the number of
121 internalised meningococcal cells recovered from FGFR1 siRNA-transfected
122 HBMECs, as well as SU5402-treated HBMECs (Figure 3-B). Again, treatment with
123 a scrambled siRNA did not significantly affect binding of meningococci to the
124 HBMEC cells. This demonstrates that FGFR1 expression and activation plays an
125 important and specific role in meningococcal adhesion to and invasion into
126 HBMECs (Supporting material, S3).

127 *There is a direct and specific interaction between FGFR1 IIIc and N. meningitidis.*
128 To determine which isoforms of FGFR1 are expressed in HBMEC cells we performed
129 RT-PCR using cDNA generated from total RNA extracted from HBMECs. FGFR1 IIIc
130 and FGFR3 IIIb isoforms were both expressed in these cells (Supporting
131 material, S1). To determine whether meningococci could interact directly with the

132 extracellular domain of FGFR1 IIIc this protein was cloned and expressed as an
133 Fc-tagged fusion protein (Supporting material). Two other proteins: Fc-FGFR2 IIIa
134 TM, comprising the trans-membrane region of FGFR2 IIIa fused to the
135 immunoglobulin Fc domain, and the Fc portion of immunoglobulin alone (Fc-stop)
136 were used as controls for possible interaction with *N. meningitidis* that was not
137 specific to the FGFR1 IIIc (extracellular) domain. Both control proteins were
138 derived from clones employing the same vector and purified by the same method
139 as Fc-FGFR1 IIIc. The purified proteins were employed as an immobilised ligand
140 in ELISA experiments.

141 ELISA plates were coated with Fc-tagged purified proteins then binding of DIG-
142 labelled *N. meningitidis* (MC58) was assessed (Figure 4, A and B). *N. meningitidis*
143 (MC58) bound Fc-FGFR1 to a significantly greater degree than either Fc-FGFR2
144 IIIa TM or Fc-stop (Figure 4 A). This indicates that the observed interaction of Fc-
145 FGFR1 was not due to meningococci binding to the Fc-tag of the expressed
146 extracellular domain of FGFR1 and thus indicates a direct interaction between the
147 receptor present on the apical surface of HBMECs and surface structures of *N.*
148 *meningitidis*.

149

150 *Interaction between N. meningitidis and FGFR1 is also seen in the pathogen N.*
151 *gonorrhoea but not commensal Neisseria species, nor other bacterial pathogens*
152 *targeting the meninges.* Having shown that Fc-FGFR1 IIIc interacts with
153 meningococci, we sought to establish whether other *Neisseria* species could
154 interact with this receptor. Representative strains belonging to several *Neisseria*
155 species including the normally commensal species *N. polysaccharea* and *N.*
156 *lactamica*, and the pathogenic *N. gonorrhoeae* were tested for interaction with Fc-
157 FGFR1 in ELISA assays. *N. gonorrhoeae* cells bound to wells containing Fc-FGFR1

158 at similar levels to *N. meningitidis* MC58, and in both cases to a significantly higher
159 degree than either of the two commensal species, which did not bind to a
160 significantly higher degree to Fc-FGFR1 than to control wells containing only Fc-
161 FGFR2 TM (Figure 4 B). We previously showed that, like the meningococcus, the
162 meningeal pathogens *S. pneumoniae* and *H. influenzae* each targeted the laminin
163 receptor on HBMEC cells as a prerequisite for internalisation (Orihuela *et al.*,
164 2009). We examined the possible interaction between these two other major
165 causes of bacterial meningitis and the extracellular domain of FGFR1. Neither
166 representative *S. pneumoniae* nor *H. influenzae* strains bound significantly to Fc-
167 FGFR1 (Figure 4 C), demonstrating that the observed interaction between the
168 pathogenic *Neisseria* species was specific.
169

170 ## Discussion

171 The requirement for Focal Adhesion Kinases and activation of Src in the
172 internalization of meningococci via interaction with integrins has been reported
173 previously (Slanina *et al.*, 2012). Considering the role of FGFR1 in maintaining the
174 integrity of the BBB and angiogenesis (van Hinsbergh *et al.*, 2005), we examined
175 the possible role of FGFR1 in interactions with meningococci. Confocal microscopy
176 studies on cells infected with meningococci confirmed that these bacteria recruit
177 FGFR1 to the apical surface of HBMECs. Recently, we showed that meningococci
178 bind to both 37LRP and Galectin-3 on the surface of HBMECs (Alqahtani *et al.*,
179 2014). Here, we showed that FGFR1 recruitment coincided with recruitment of
180 both isoforms (37LRP and 67LR) of the laminin receptor; molecules already
181 implicated in Neisserial-HBMEC interactions (Orihuela *et al.*, 2009). Ligation of the
182 extracellular domain of FGFRs by their ligands leads to auto-phosphorylation of
183 tyrosine residues in the cytoplasmic domain of the receptor; these phosphorylated
184 residues subsequently serve as docking sites for a number of adaptor proteins
185 responsible for regulation of various downstream signalling cascades (Turner *et*
186 *al.*, 2010). The FGFR1 molecules recruited by meningococci were shown to be
187 activated and activated receptors and meningococcal cells also co-localised with
188 α-actinin. This is in agreement with previous studies on the trafficking of FGFR1
189 into early endosomes inside the cytoplasm, and the regulation of its trafficking by
190 Syndecan 4 in a clathrin-independent manner (Elfenbein *et al.*, 2012). We also
191 showed that the whole receptor is internalised into the cytoplasm along with
192 invading meningococci; this was also observed by confocal microscopy in HBMECs
193 treated with gentamicin after 4 h of meningococcal infection. Interestingly, FGFR1
194 engagement by basic fibroblast growth factor receptor has recently been shown
195 to protect the integrity of HBMEC monolayers preventing the downregulation of

196 the junction proteins zO-1, occludin and VE-cadherin in response to oxygen-
197 glucose deprivation and deoxygenation (Lin *et al.*, 2017). This might have
198 implications for the route of entry of meningococci via a trans-cellular pathway
199 through HBMECs rather than a para-cellular pathway in which the integrity of the
200 monolayer would have to be compromised.

201 To determine whether FGFR1 was required for meningococcal-HBMECs
202 interactions, FGFR1 expression was transiently inhibited by using FGFR1 siRNA
203 transfection and in other experiments its activity was inhibited by the specific
204 chemical inhibitor SU5402. Both FGFR1 knock-down and SU5402 treatment of
205 HBMECs resulted in a dramatic reduction in both association and internalisation of
206 meningococci into HBMECs. Our data confirm that direct interaction between the
207 extracellular domain of FGFR1 and meningococci is required for consequent
208 activation of the receptor and internalisation of bacteria into the HBMECs.

209 The mechanisms by which recruitment of FGFR1 by meningococcal colonies leads
210 to their internalisation is unknown. Interaction of meningococci with HBMECs has
211 previously been shown to lead to higher levels of activation of ERK 1, 2 due to
212 activation of ErbB2 in these cells (Hoffmann *et al.*, 2001). However, levels of ERK
213 1,2 activation in cells in which FGFR1 was knocked down by siRNA transfection
214 were unaffected, demonstrating that FGFR1 does not regulate the levels of ERK1,
215 2 activation (Estes *et al.*, 2006).Several studies on meningococcal infection of
216 endothelial cells showed that invasion of bacteria requires activation of Src,
217 phosphorylation of cortactin via the Src pathway and activation of focal adhesion
218 kinases (FAKs) (Hoffmann *et al.*, 2001, Miller *et al.*, 2012, Slanina *et al.*, 2012).
219 Also, it has been shown that meningococcal cells hijack the β-arrestin/β 2-
220 adrenoreceptor pathway to invade endothelial cells and cross the BBB: inhibition
221 of β-arrestin mediated activation of Src, prevents the invasion of meningococcal

222 cells (Coureuil *et al.*, 2010). Src is required for cortactin phosphorylation by FGF1
223 which can provide an alternate downstream pathway of FGFR1 from PLCγ and can
224 be involved in cytoskeletal rearrangement (Zhan *et al.*, 1994, Liu *et al.*, 1999).
225 However it was reported that mutation of Y766 in FGFR1 leads to higher level
226 activations of PLCγ which inhibits Src activation (Landgren *et al.*, 1995). These
227 observations suggest that FGFR1 siRNA transfection of HBMECs and chemical
228 inhibition of FGFR1 (SU5402 treatment) may have led to the same effect on
229 inhibition of Src activation which consequently inhibited meningococcal invasion
230 into HBMECs. It is likely that FGFR1 plays an important role in meningococcal
231 interaction with the BBB during infection. This effect appears to be specific to the
232 meningococcus as the bacterial pathogens *H. influenzae* and *S. pneumoniae*,
233 which also cross the BBB and can cause meningitis, do not interact with FGFR1
234 IIIc on the surface of endothelial cells. On the other hand, *N. gonorrhoea*, which
235 does not usually interact with the BBB is able to bind this receptor. The significance
236 of this is unknown. Further investigations are required to understand the role of
237 FGFR1 signalling in meningococcal invasion into HBMECs.
238

239 **Experimental Procedures**

240 *Bacterial growth and culture*

241 *N. meningitidis* serogroup B strain MC58 was obtained from the American Type
242 Culture Collection (ATCC) (Tettelin *et al.*, 2000) and routinely cultured on
243 chocolated horse blood agar (Chocolate agar; Oxoid). *H. influenzae* Rd KW20
244 (ATCC 51097) (Fleischmann *et al.*, 1995) and *S. pneumoniae* T4R
245 (unencapsulated) (Fernebro *et al.*, 2004) were also cultured on chocolated horse
246 blood agar (Oxoid). All three bacteria were grown at 37°C, in an atmosphere of
247 air plus 5% CO_2.

248 *Cell association assay and cell invasion (Gentamicin protection) assay*

249 To quantify cell association and cell invasion of HBMECs with *N. meningitidis*
250 HBMECs were seeded and grown overnight or for 48 h after siRNA transfection
251 until 100% confluent in 24-well plates. Cells were infected with 1×10^7 CFU
252 bacteria for 4 h in ECM-b media without any supplements. Cell association and
253 invasion were then determined as described previously (Oldfield *et al.*, 2007).

254 *Confocal Immunofluorescent Microscopy*

255 HBMECs were seeded onto fibronectin-coated coverslips (1-10 × 10^5 cells) and
256 grown overnight to reach a confluency of 70-80%. Cells were infected for 2-4 h
257 (MOI 200-300). Coverslips were washed with PBS and fixed with 4%
258 paraformaldehyde (w/v) in PBS for 5 min. Coverslips were then washed with PBS
259 and blocked in 4% (w/v) BSA/PBS at 4°C overnight. For intracellular staining, cells
260 were permeabilised by treatment with 0.1% Saponin, 20 mM glycine in 4%
261 BSA/TBS at 4°C. Subsequent staining procedures were carried out in r 4%
262 (w/v)BSA/TBS. Briefly, coverslips incubated with primary antibody for 1 h were
263 washed with PBS followed by one wash with dH_2O. Coverslips were then incubated

264 with secondary antibody for 1 h in the dark followed by washes with PBS-Tween
265 (0.05% v/v; PBS-T), PBS and then dH_2O. Coverslips were then mounted on glass
266 slides with ProLong® Gold and SlowFade® Gold Antifade Reagents with DAPI
267 (Invitrogen). Coverslips were analysed using a Zeiss LSM-700 confocal
268 microscope. Images were processed with ImageJ, Adobe Photoshop and LSM
269 Image Browser software.

270 *Antibodies and reagents*

271 Antibodies detecting FGFR1 (Flg S-16 and Flg C-15), phosphorylated FGFR1
272 (p-Y766) were purchased from Santa Cruz Biotechnology. Secondary antibodies
273 conjugated to various fluorochromes, and Phalloidin conjugated to fluorochrome
274 488 were obtained from Life Technologies-Invitrogen.
275 Antibody detecting 37LRP (A-7) was purchased from Santa Cruz Biotechnology
276 and antibody against 67LR (Mluc-5) was purchased from Thermo Scientific.
277 FGFR1-specific inhibitor SU5402 (Mohammadi *et al.*, 1997) was purchased from
278 Calbiochem.
279 Fc-tagged FGFR1 IIIc (*ca.* 80 kDa), Fc-tagged FGFR2 IIIa TM (*ca.* 61.9 kDa) and
280 Fc-tag (*ca.* 29.26 kDa) were expressed and purified using a protein A column (S1).

281 *FGFR1 siRNA transfection in HBMECs*

282 Human FGFR1 siRNA (siGENOME SMART pool) and control scrambled siRNA were
283 obtained from Dharmachon/Thermo Scientific and reconstituted following the
284 manufacturer guidance. FGFR1 siRNA was resuspended in 1 ml of 1 × siRNA buffer
285 to a final concentration of 5 μM (stock). HBMECs were seeded into 24-well plates
286 pre-coated with fibronectin, as previously described, and grown overnight to reach
287 a confluency of 70-80%. Transfection media was prepared by mixing serum- and
288 antibiotic-free media with siRNA from a 50μM stock to a final concentration of

289 5μM; in a separate tube Transfection reagent number 1 (Thermo Scientific-
290 Dharmacon) was added to serum and antibiotic-free media. Both tubes were
291 incubated for 5 min at room temperature and then mixed together by pipetting
292 and incubated at room temperature for 20-30 min. Cells were washed with serum
293 and antibiotic-free medium and 240 μl of complete media without antibiotics were
294 added to each well and then the transfection mixture was added drop-wise to each
295 well to a final concentration of 50 nM siRNA/ well. Cells were then incubated for 6
296 h and then the media was replaced with complete media (Endothelial cell medium
297 (ECM-b) (ScienCell) supplemented with ECGS (containing EGF, VEGF) (ScienCell)
298 (1% v/v) and FBS (5% v/v) and penicillin/streptomycin (ScienCell; 1% v/v). The
299 level of FGFR1 expression was examined at RNA and protein levels at 24, 48 and
300 72 h post transfection.

301 *FGFR1 inhibition in HBMECs*

302 HBMECs were serum-starved and treated with SU5402 (0.5 μM) 1 h prior to
303 infection. Approximately 10×10^{6} / well (MOI: 10) of bacteria were added to each
304 well and infected cells were incubated for 4 h at 37°C/5% CO2 in medium
305 containing 0.5 μM SU5402. Cells were then washed twice with PBS and lysed in
306 1% saponin/PBS. Cell lysates were homogenised and appropriate dilutions plated
307 out to calculate the levels of association. For invasion assays, cells were treated
308 with gentamicin for 1 h to kill non-internalised bacteria.

309 *ELISA*

310 96-well plates (NUNC Immobilizer Amino) were coated with 100 μl of protein A
311 (Pierce; 1 μg ml^{-1}) in PBS for 1 h, washed once with PBS-T and then 86.5×10^{-15}
312 M of Fc-tagged recombinant proteins added to each well in carbonate buffer (pH
313 9.6). Plates were incubated for 1 h and then washed three times with PBS-T.

314 Plates were blocked with 1% BSA/PBS (w/v) for 1 h, then washed once with PBS-
315 T. Bacterial cells harvested from overnight plates and resuspended in PBS-T,
316 washed with the same buffer three times and finally resuspended in sodium
317 carbonate buffer (44 mM $NaHCO_3$, 6.0 mM Na_2CO_3; pH: 9.6). The OD_{600} was
318 measured and 20 ng (2 µl of 10 ng μl^{-1}) of digoxigenin (DIG; Roche) was added
319 to 1 ml of bacterial suspension with OD_{600}:1. The bacterial suspensions were
320 incubated for 2 h in the dark at room temperature on the shaker. Bacteria then
321 were washed three times with PBS-T by centrifugation (13000 × *g* for 1 minute)
322 and resuspended in 1% BSA/PBS (w/v). OD_{600} in 1% BSA/PBS was adjusted to
323 0.02 for ELISA. For each experiment fresh labelled bacterial strains were used.
324 100 µl of DIG-labelled bacteria were added to each well and plates were incubated
325 at 4°C overnight then washed five times with PBS-T. 100 µl of anti-DIG-alkaline
326 phosphatase antibody (Roche; 0.0002 v/v) in 1% BSA/PBS was then added to
327 each well and incubated for 1 h then washed three times with PBS-T. 200 µl of
328 alkaline phosphatase substrate (SIGMA) was added and plates were incubated for
329 1 h. The OD_{405} was measured for each sample and values obtained subtracted
330 from the binding of the same DIG-labelled strain to 1% BSA/PBS.
331

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538 ## Figure Legends

539 **Figure 1. Activated FGFR1 is recruited by *N. meningitidis* colonies**. (A)
540 HBMECs were infected with meningococcal cells (MOI: 200) for 2-4 h. *N.*
541 *meningitidis* colonies were visualized with DAPI (blue), FGFR1 was probed with
542 anti-FGFR1 primary antibody and detected with anti-goat Alexa Fluor 680 antibody
543 (red). 37LRP and 67LR were probed with primary antibodies and detected with
544 anti-mouse Alexa Fluor 488 antibodies (green). FGFR1 was recruited by
545 meningococcal colonies, which coincides with recruitment of 37LRP and, to a less
546 extent, 67LR. Co-localisation area is shown by arrows (images are representative
547 of 10 infected cells). (B) FGFR1 phosphorylated at Tyrosine 766 (p-Y766) was
548 labelled with Alexa Fluor 680 (red) and both isoforms of Laminin receptor (67LR
549 and 37LRP) with Alexa Fluor 488 (green). Recruitment of activated FGFR1 (p-
550 Y766) coincided with recruitment of 37LRP and, to a less extent, 67LR. Levels of
551 co-localization of MC58 with 37LRP, 67LR and p-Y766 (activated FGFR1) were
552 quantified by measuring the percentage of co-localisation of each receptor with
553 MC58 in 30 fields. There was a significant difference between recruitment of
554 activated FGFR1 and 37LRP with recruitment of 67LR (p= 0.0009 and p= 0.0028
555 respectively; two tailed unpaired t-test).

556 **Figure 2. Activated FGFR1 is recruited by internalised meningococci**
557 **within HBMECs.** (A) In infected HBMECs α-actinin and actin were labelled with
558 Alexa Fluor 680 (Red) and the activated form of FGFR1 (p-Y766) labelled with
559 Alexa Fluor 488 (Green). MC58 was labelled with DAPI (Blue). Internalised
560 bacteria co-localised with both α-actinin and activated FGFR1 (p-Y766) (arrow
561 heads). (B and C) Z-stack image of meningococcal colonies showes that
562 internalised bacteria (co-localising with α-actinin) recruit activated FGFR1 (p-
563 Y766) inside the cells.
564
565

566 **Figure 3. Expression and activation of FGFR1 plays an important role in**
567 **the invasion of meningococci into HBMECs.** (A) HBMECs were infected with
568 *N. meningitidis* MC58 for 4 h (MOI: 10). Cells were washed and lysed in 500 µl of
569 1% saponin in PBS and 100 µl of homogenized lysates used for serial dilution
570 preparation (up to 10 fold). 10 µl of each dilution was plated onto chocolate agar
571 and CFUs were calculated for each sample. There was a significant reduction in
572 the number of meningococci associated with HMBECs after FGFR1 knockdown
573 (FGFR1 siRNA; p=0.0031, two tailed unpaired t-test, n=8) or in SU5402-treated
574 cells in which activation of FGFR1 is chemically inhibited (p=0.0054, two tailed
575 unpaired t-test, n=8; experiments were performed in triplicate wells and means
576 shown represent 8 independent experiments). (B) For invasion assays, gentamicin
577 was added after 4 h of infection and plates were incubated for a further 1 h. Cells
578 were then washed, lysed, homogenised and dilutions plated onto chocolate blood
579 agar plates. There was a significant difference between the number of internalised
580 meningococci in FGFR1 siRNA-transfected cells compared to untreated cells, or
581 cells treated with scrambled siRNA (p=0.0003 two tailed unpaired t-test, n=7).
582 Chemical inhibition of FGFR1 activation also inhibited internalization of
583 meningococcal cells into HBMECs (p=0.0005 two-tailed unpaired t-test, n=7, the
584 error bars represent standard deviation of mean).
585

586 **Figure 4. *N. meningitides* and *N. gonorrhoeae* interact directly with the**
587 **extracellular domain of FGFR1.** Fc-tagged purified proteins were used as the
588 immobilised ligand in ELISA experiments. Levels of interaction with DIG-labelled
589 MC58 were measured; the values shown are those following subtraction of binding
590 to 1% BSA/PBS. (A) *N. meningitidis* MC58 interacts directly with the extracellular
591 domain of Fc-FGFR1 (two tailed t-test; each experiment was performed in 6
592 technical replicates and the data shown is derived from 6 independent
593 experiments). (B) Binding of commensal *Neisseria,* but not *N. gonorrhoeae,* to
594 FGFR1 IIIc was significantly lower than that shown by *N. meningitidis* MC58. (C)
595 The meningeal pathogens *H. influenzae* and *S. pneumoniae* bound FGFR1 IIIc to
596 a negligible degree that was significantly lower than cells of *N. meningitidis* MC58
597 ($p<0.002$).
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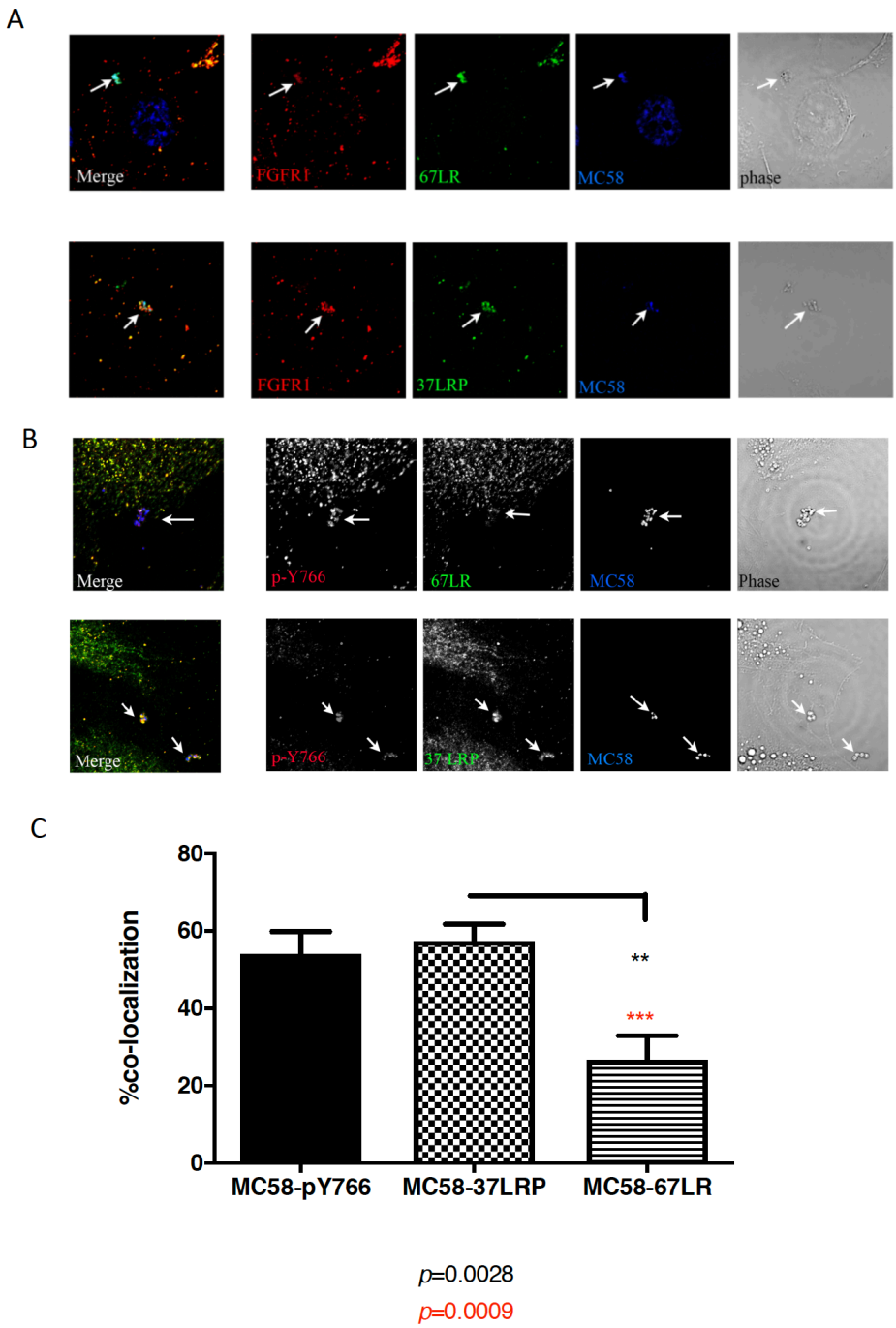
613 # Figures



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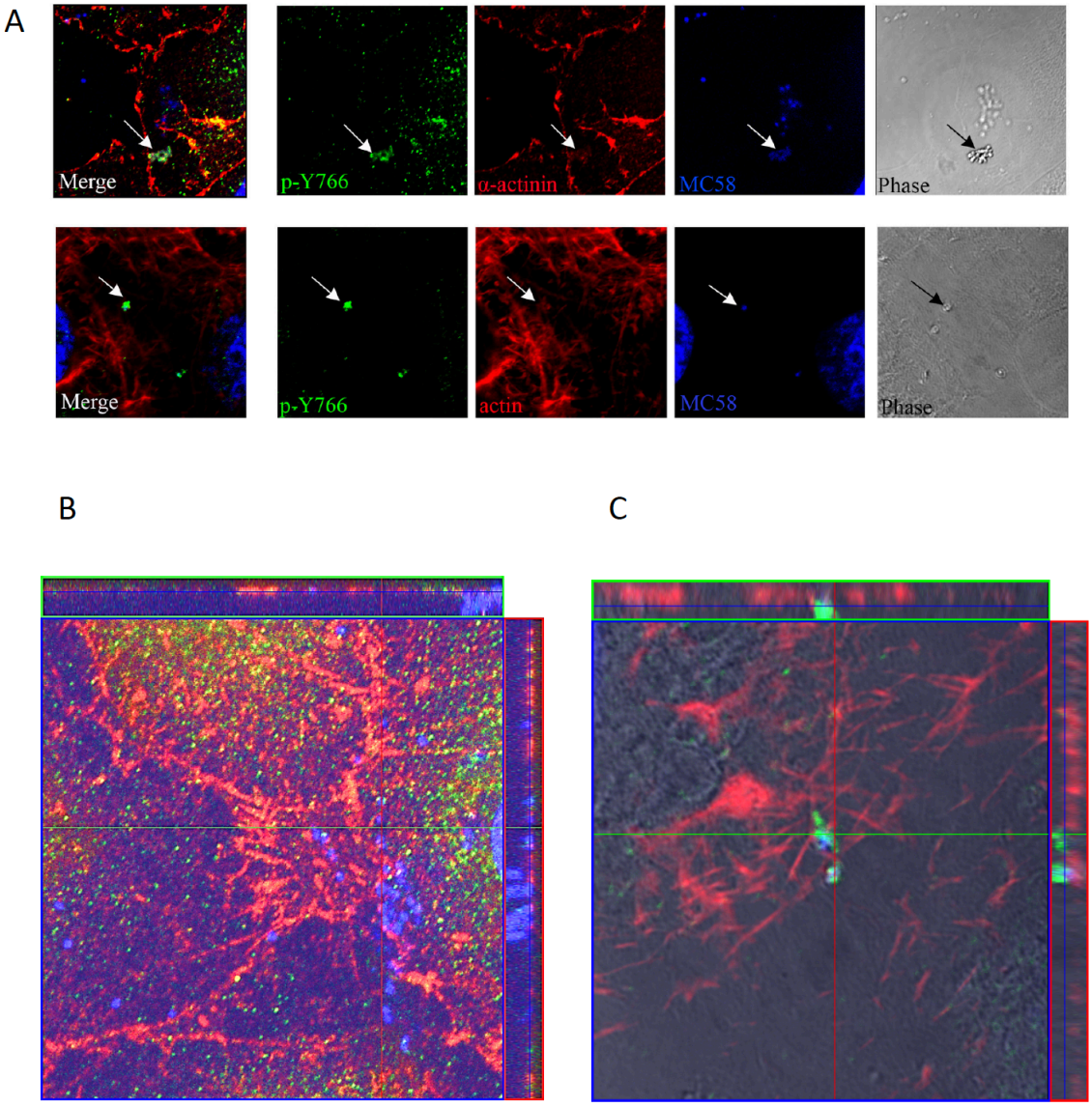

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617 Figure 2.

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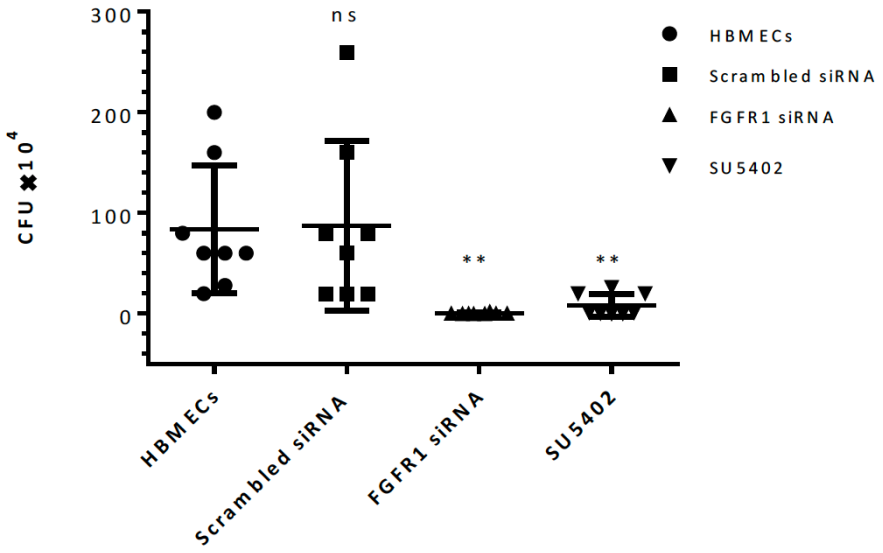

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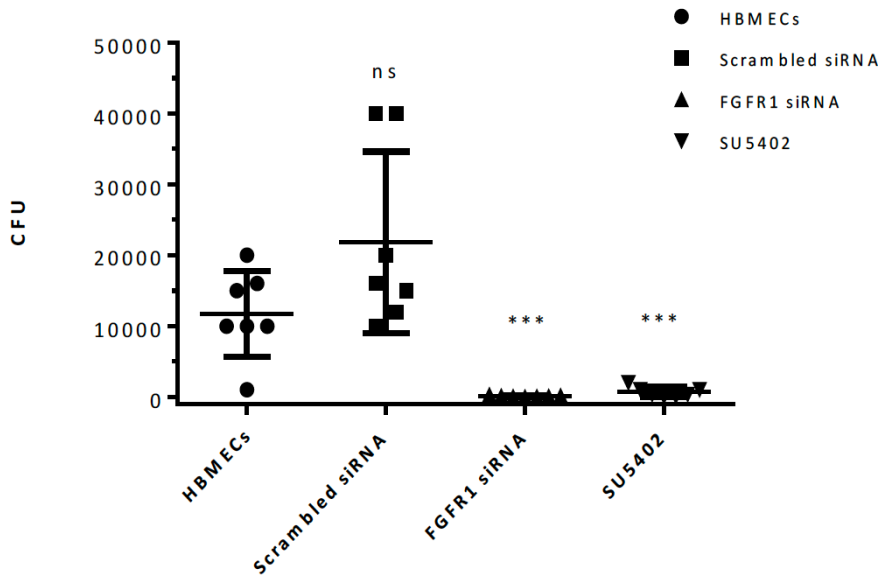

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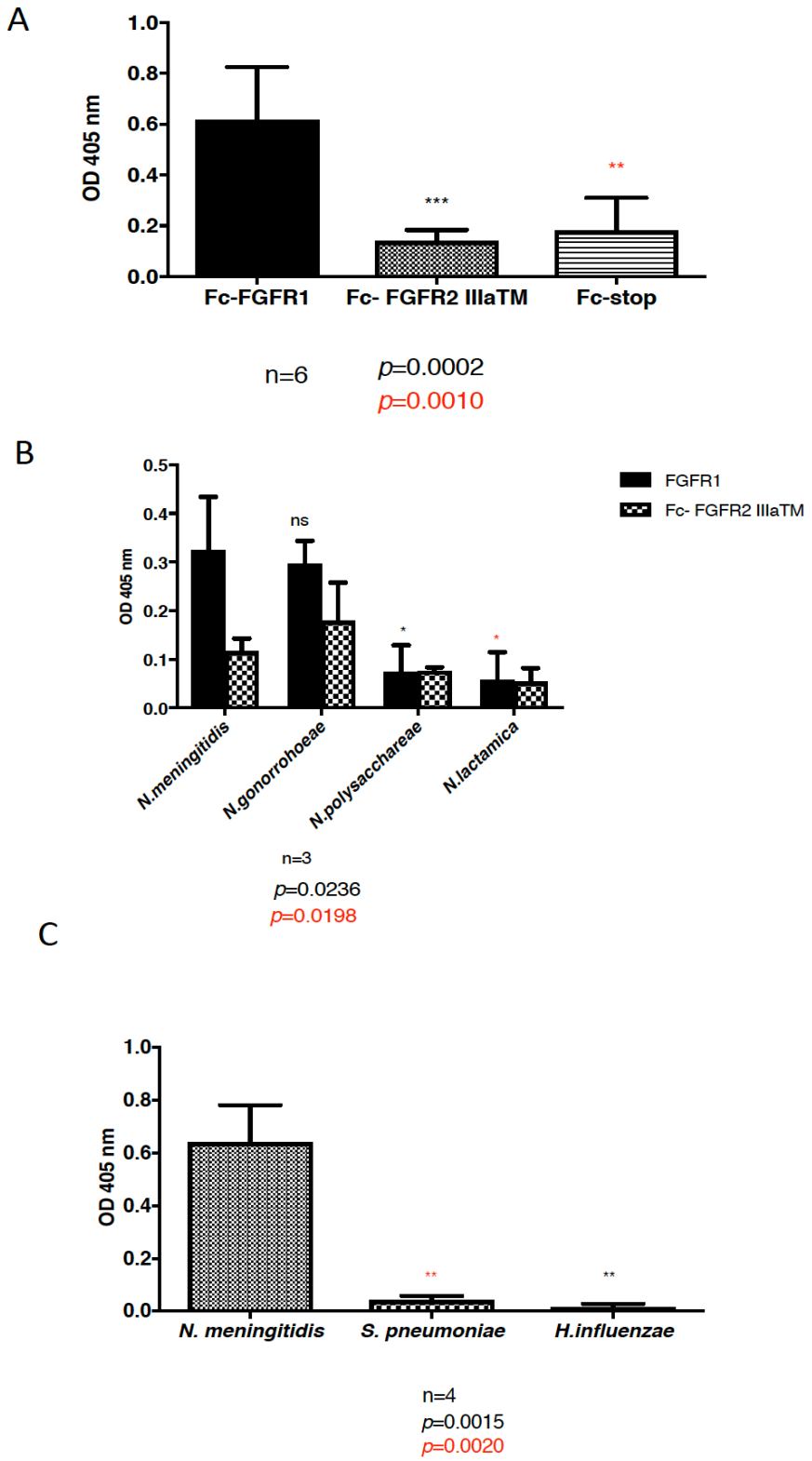
619 Figure 3.



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621 Figure 4.

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Supporting material

A role for Fibroblast Growth Factor Receptor 1 in the pathogenesis of *Neisseria meningitidis*

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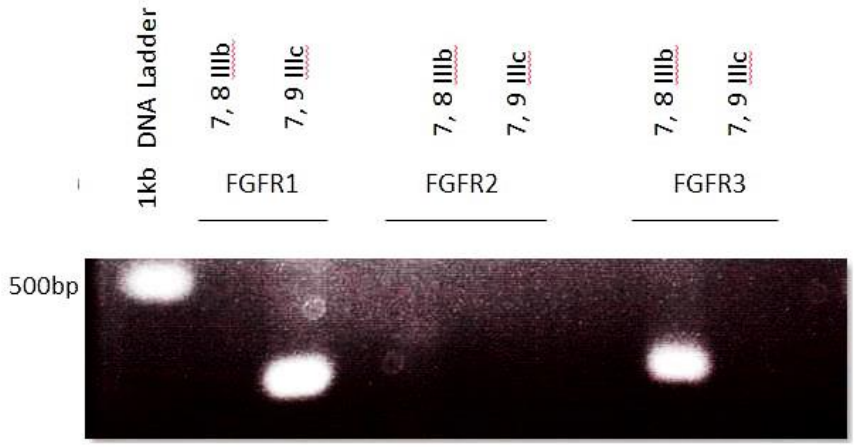
Running title: FGFR1 and meningococcal pathogenesis

Keywords: Meningococci, Neisseria, meningitidis, FGFR1, epithelial, endothelial, host-pathogen

665 *HBMECs express FGFR1 IIIc and FGFR3 IIIb isoforms*

666 HBMECs were grown overnight, RNA extracted and cDNA prepared. This was then
667 used as template to amplify the third IgG-like domain of FGFRs (exon 7, 8 IIIb)
668 and (exon 7, 9 IIIc) isoforms (Hajihosseini *et al.*, 1999). RT-PCR showed that
669 HBMECs express FGFR1 IIIc and FGFR3 IIIb isoforms.



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671 **S1. HBMECs express FGFR1 IIIc and FGFR3 IIIb isoforms**. RT-PCR
672 performed using cDNA generated from total RNA extracted from HBMECs. 100ng
673 of cDNA was used in as template in each PCR reaction. Various combinations of
674 primers specific for exon 7, 8 and 9 were used to determine the type of FGFR
675 expressed by HBMECs.
676

677 *Construction of FGFR1 IIIc into pEF-Bos-ss-Fc-ires-TPZ mammalian vector*

678 HBMECs were routinely grown in fibronectin-coated T75 flasks (BD Bioscience).
679 For RNA extraction, cells were harvested by trypsin treatment. Cell suspensions
680 were then centrifuged for 5 min at 300 × *g*, the supernatant was discarded and
681 cells were lysed in lysis buffer (SIGMA GenElute™ Mammalian Total RNA Miniprep
682 Kits) supplemented with 1% (v/v) 2-β-Mercaptoethanol and RNA extracted
683 according to the manufacturer's protocol (Sigma GenElute™ Mammalian Total RNA
684 Miniprep Kit). The concentration of extracted RNA was measured using a Nanodrop
685 spectrophotometer and adjusted to 200 ng μl^{-1}. To remove traces of genomic DNA

686 from RNA samples, DNAse treatment was performed following the manufacturers
687 protocol (Turbo DNase, Life Technologies). 10 µl of RNA was then used for cDNA
688 preparation using the High Capacity cDNA Reverse Transcription kit (Applied
689 Biosystem). HBMEC cDNA was then used as template to amplify the extracellular
690 domain of FGFR1 IIIc isoform. Primers were designed to amplify the extracellular
691 domain of FGFR1 containing restriction sites for NdeI and NotI enzymes. The
692 amplified PCR product was gel purified, digested and then ligated into NotI and
693 NdeI-digested pEF-Bos-ss-Fc-FGFR2IIIaTM-ires-TPZ (Mizushima et al., 1990,
694 Burgar et al., 2002, Wheldon et al., 2011) using the LigaFast TMRapid DNA
695 Ligation System (Promega). The ligated plasmid was used for transformation of
696 competent *E. coli* JM109 cells (Promega). Plasmids extracted from ampicillin-
697 resistant transformants were screened by restriction digestion and DNA
698 sequencing. The plasmid from one correct clone was named pEF-Bos-ss-Fc-
699 extFGFR1IIIc-ires-TPZ.

700

Forward	**gcgGCTTAATCATATG**cagggacccggatcc**ATGTGGAGCTGGAAGTGCC**
Reverse **(extracellular domain)**	gcgcg**ATTAAGCGGCCGC**ttaCAGGGGCGAGGTCA

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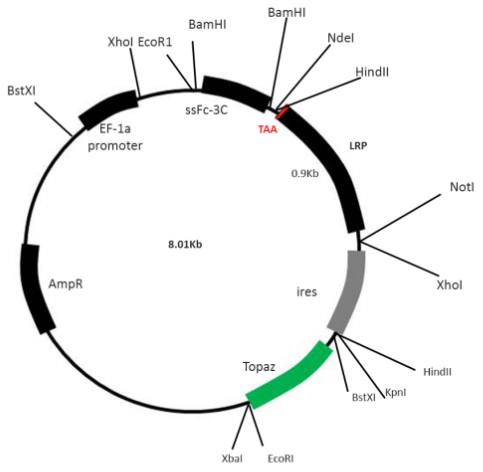

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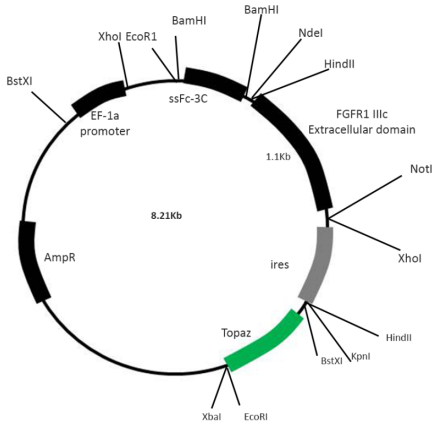

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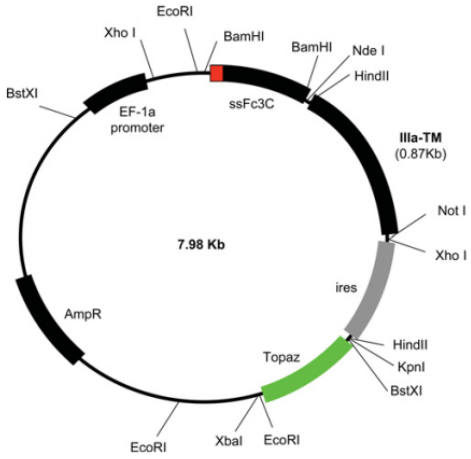

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709 **S2. Schematic view of the plasmid pEF-Bos-ss-Fc-extFGFR1IIIc-ires-TPZ. The**
710 **extracellular domain of FGFR1 IIIc (A) and LRP were replaced with FGFR2 IIIaTM**
711 **insert of the plasmid pEF-Bos-ss-Fc-FGFR2 IIIa TM-ires-TPZ (C) (Wheldon *et al.*,**
712 **2011). Stop codon (TAA in Red-B) was introduced in start of the LRP coding gene**
713 **to be able express the Fc-tag and not the LRP protein (B). The plasmid encoding Fc-**
714 **Stop-LRP was constructed in house by Dr. S. Morroll.**

715 *Purification of Fc-tagged recombinant proteins*

716 Media was collected 72 h post-transfection of 293T cells and Fc-tagged
717 recombinant proteins purified on a Protein AIG-Sepharose (Source BioScience
718 LifeSciences) column. Briefly, the media was diluted 1:1 (v/v) with binding buffer
719 (0.05 M sodium borate, 0.15 M sodium chloride; pH: 8). 100 µl of beads were
720 added to 30ml of diluted media and incubated with shaking at 4°C overnight. The
721 column was prepared and washed 4-5 × with binding buffer. The column was then
722 washed with binding buffer containing 0.5 M sodium chloride. Proteins were eluted
723 in 1 ml of 0.1 M glycine (pH: 2.5) and neutralised with 100 µl of 1 M Tris-HCl (pH:
724 9) per 1 ml of eluted samples. A total number of 9-10 protein fractions were eluted
725 and stored at -80°C. Protein concentration in each fraction was quantified using
726 the BCA kit following manufacturer's protocol (Thermo Scientific).

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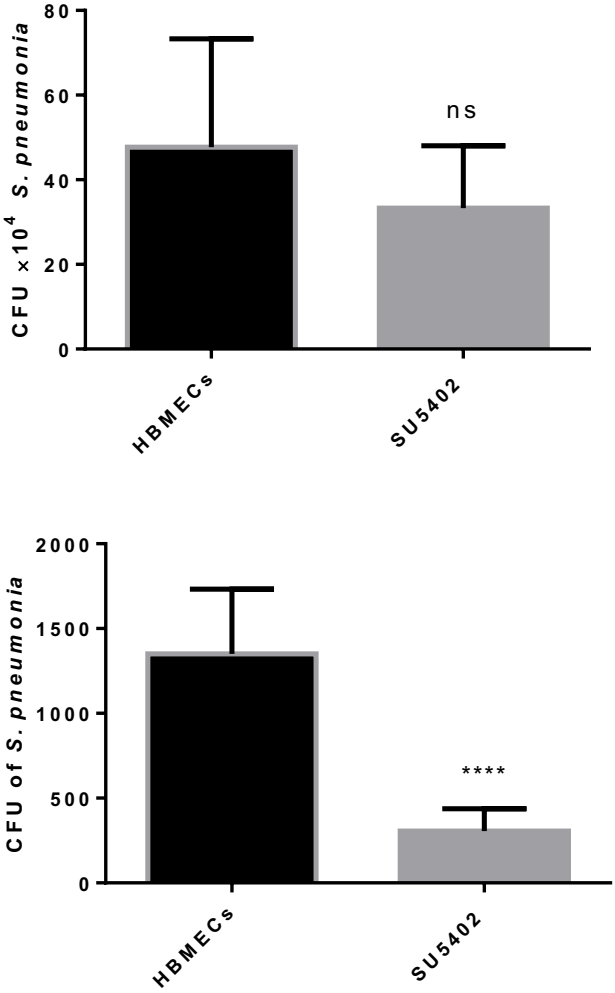

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729 **S 3. Chemical inhibition of FGFR1 does not affect the number of *S.***
730 ***pneumoniae* associated with HBMECs but reduced the number of invaded**
731 **bacteria into HBMECs.** HBMECs were infected with *S. pneumonia* at MOI: 10.
732 Cell association and invasion assays were performed and the CFUs were
733 calculated.

734

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