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MAGP2 Controls Notch via Interactions with RGD Binding Integrins: Identification of a Novel ECM-Integrin-Notch Signaling Axis

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2 Novel ECM – Integrin – Notch Signaling Axis.

3

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6

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10

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12

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14

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18

19 **Abbreviations:**

20 MAGP2 – Microfibril Associated Glycoprotein 2

21 N1ICD – Notch1 Intracellular domain

22 ECM – Extracellular Matrix

23 HMEC – Human Microvascular Endothelial cells

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28 **Abstract:** Canonical Notch signaling involves Notch receptor activation via interaction
29 with cell surface bound Notch ligand. Recent findings also indicate that Notch signaling
30 may be modulated by cross-talk with other signaling mechanisms. The ECM protein
31 MAGP2 was previously shown to regulate Notch in a cell type dependent manner,
32 although the molecular details of this interaction have not been dissected. Here, we
33 report that MAGP2 cell type specific control of Notch is independent of individual Notch
34 receptor-ligand combinations but dependent on interaction with RGD binding integrins.
35 Overexpressed MAGP2 was found to suppress transcriptional activity from the Notch
36 responsive Hes1 promoter activity in endothelial cells, while overexpression of a
37 RGD→RGE MAGP2 mutant increased Notch signaling in the same cell type. This effect
38 was not unique to MAGP2 since the RGD domain of the ECM protein EGFL7 was also
39 found to be an important modulator of Hes1 promoter activity. Independently of MAGP2
40 or EGFL7, inhibition of RGD-binding integrins with soluble RGD peptides also
41 increased accumulation of active N1ICD fragments and Notch responsive promoter
42 activity independently of changes in Notch1, Jag1, or Dll4 expression. Finally, β 1 or β 3
43 integrin blocking antibodies also enhanced Notch signaling. Collectively, these results
44 answer the question of how MAGP2 controls cell type dependent Notch signaling, but
45 more importantly uncover a new mechanism to understand how extracellular matrices
46 and cellular environments impact Notch signaling.

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54 **Introduction:**

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56 Extracellular matrices within cellular microenvironments play an integral role in the
57 regulation of a wide variety of normal cellular physiological responses. Alternatively,
58 abnormal extracellular microenvironments contribute to the pathogenesis of many
59 vascular diseases of humans such as atherosclerosis, arteriosclerosis, and cancer.
60 Therefore understanding how ECM molecules in these diverse microenvironments
61 impact cell physiology is an important step towards to understanding the pathophysiology
62 of these diseases.

63

64 There are numerous receptor mechanisms whereby cells detect and interact with ECM
65 molecules within cellular microenvironments. The best understood of these cellular
66 ECM receptor systems are integrins which are heterodimeric transmembrane proteins
67 consisting of one α -subunit and one β -subunit. Collectively, there are 18 known α -
68 subunits and 8 known β -subunits that can combine in various combinations to form up to
69 24 functional integrins [1]. Integrin heterodimers have diverse ligand specificities
70 including the arginine-glycine-aspartic acid (RGD) domain [2]. Once bound to specific
71 ECM ligands, integrins initiate a wide variety of signaling cascades that are mediated by
72 activation of several downstream kinases including focal adhesion kinase (FAK), Src,
73 and the integrin-linked kinase (ILK) pathways that collectively have broad impacts on
74 cellular physiology [3].

75

76 Microfibril associated glycoprotein-2 (MAGP2) is an extracellular matrix protein that
77 interacts with microfibril/elastin networks [4, 5] and mediates cell adhesion via it's N-
78 terminal RGD domain [6]. In addition to a role in building elastin networks, MAGP2 is
79 also a pro-angiogenic component of vascular microenvironments [7] and increased
80 expression of MAGP2 has been associated with increased vascular densities and poor
81 prognosis in ovarian cancers [8]. Beyond it's structural role in the ECM, MAGP2 also
82 functions as a matricellular protein by interacting with the Notch signaling cascade.
83 Specifically, the C-terminal of MAGP2 interacts with the Notch ligand Jagged1 [9], and
84 ultimately increases Notch signaling in COS-1 cells [10]. MAGP2 does not equally

85 impact Notch signaling in all cell types however. MAGP2 increases Notch signaling in a
86 variety of non-endothelial cell lines, but consistently decreases Notch activation in
87 several varieties of human and mouse endothelial cell lines [11]. It is the ability of
88 MAGP2 to suppress Notch signaling in endothelial cells that imparts pro-angiogenic
89 activity to MAGP2 [11]. However, the exact mechanism whereby MAGP2 promotes
90 Notch signaling in some cell types, but blocks Notch signaling in endothelial cells has
91 remained a mystery.

92
93 Herein we show that the cell type-specific effect of MAGP2 on Notch signaling is
94 independent of individual Notch receptor-ligand combinations but dependent on MAGP2
95 interaction with RGD binding integrins. MAGP2 is not unique in this function however
96 since we also found that the RGD domain of EGFL7 also controls Notch signaling. On a
97 larger scale, inhibition of integrin function with blocking antibodies or soluble RGD
98 peptides also impacted Notch signaling activity. Collectively, our results lead us to
99 believe that MAGP2 and EGFL7 are just two of many ECM proteins that may indirectly
100 control Notch via interactions with RGD binding integrins since. Therefore, the broad
101 implication of our results is the identification of a general signaling axis connecting
102 cellular microenvironments (and the ECM proteins within these microenvironments) to
103 Notch via integrin signaling.

104

105 **Results:**

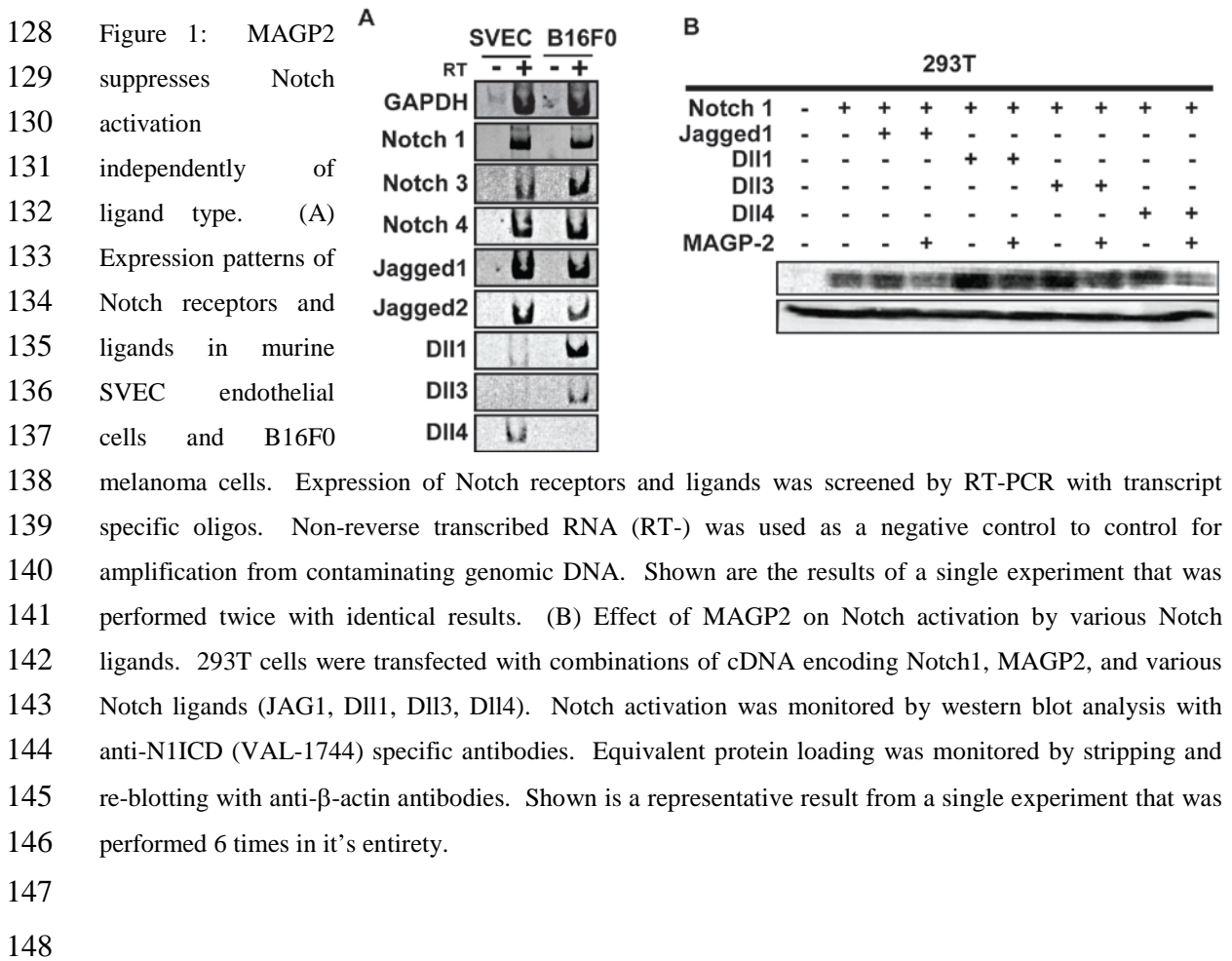
106

107 *MAGP2 suppresses Notch signaling in endothelial cells via interactions with RGD*
108 *binding integrins*

109

110 We previously demonstrated that MAGP2 inhibits Notch signaling in endothelial cells
111 but increases Notch signaling in non-endothelial cell lines [11]. Our first hypothesis to
112 explain this observation was that MAGP2 may specifically inhibit receptor – ligand
113 combinations present in endothelial cells, but promote receptor – ligand combinations
114 present in non-endothelial cells. Therefore, we used RT-PCR to compare expression of
115 Notch receptors and ligands in SVEC endothelial cells and B16F0 melanoma cells in

116 which MAGP2 had previously been shown to reduce or increase Notch signaling
 117 respectively [11]. As shown in figure 1A, both cell lines expressed Notch receptors 1, 3,
 118 and 4 and also shared expression of Notch ligands Jagged1 and 2 (JAG1, 2). However,
 119 expression of Notch ligands Delta-like 1 and 3 (Dll1, 3) was restricted to B16F0 cells
 120 while expression of the Delta-like 4 (Dll4) Notch ligand was restricted to SVEC cells.
 121 Therefore, we transiently transfected 293T cells with combinations of Notch1 together
 122 with MAGP2 and either Dll1, Dll3, or Dll4 cDNAs and monitored Notch activation by
 123 western blot analysis of whole cell lysates with anti-VAL1744 antibodies that only
 124 recognize the activated NIICD domain cleaved at the VAL1744 position by gamma-
 125 secretase. As shown in figure 1B, regardless of combination, co-transfected MAGP2
 126 cDNA decreased Notch activation independently of Notch receptor – ligand combination.
 127



149 An alternative hypothesis to explain the cell type-specific regulation of Notch by MAGP2
150 involved an unknown receptor protein for MAGP2 expressed in endothelial cells but not
151 in non-endothelial cells. Since MAGP2 contains an integrin binding RGD domain, we
152 hypothesized that MAGP2 might bind to integrins present in endothelial cells but not
153 non-endothelial cells and trigger a differential impact on Notch signaling. To test this
154 hypothesis, we transfected HMEC endothelial cells with a Notch responsive Hes-1
155 luciferase construct plus MAGP2 cDNA and added increasing amounts of soluble RGD
156 peptide to transfected cells to block activation of RGD binding integrins. As shown in
157 figure 2A and as previously observed [11], transfection of MAGP2 cDNA alone
158 decreased Hes-1 promoter activity in HMEC cells. The addition of soluble RGD peptides
159 completely blocked the ability of MAGP2 to suppress Hes-1 promoter activity suggesting
160 that MAGP2 decreases Hes-1 promoter activity by interacting with RGD binding
161 integrins.

162

163 To directly test if MAGP2 suppresses Hes-1 promoter activity in an RGD dependent
164 manner, we used site directed mutagenesis to induce an RGD→RGE mutation in
165 MAGP2 and compared Hes-1 promoter activity in the presence of RGD and RGE
166 versions of MAGP2. The mutation was confirmed by sequence analysis (Fig 2B) and
167 recombinant proteins were purified from bacterial cells by anti-FLAG chromatography
168 (Fig 2C). The functional outcome of the mutation was confirmed by comparing
169 endothelial cell adhesion to RGD or RGE versions of the purified proteins. Purified
170 proteins were coated onto cell culture plates and remaining binding sites were blocked
171 with BSA. As anticipated, HMEC endothelial cells successfully adhered to both
172 MAGP2-RGD and positive control fibronectin, but failed to adhere to MAGP2-RGE or
173 BSA negative control indicating that the RGD domain is the sole binding site for HMEC
174 endothelial cells on MAGP2 (Fig 2D). HMEC cells were subsequently transfected with
175 the Hes-1 luciferase reporter and either RGD or RGE versions of MAGP2 cDNAs to
176 monitor Notch signaling activity. As previously shown, MAGP2-RGD suppressed Hes-1
177 promoter activity. Surprisingly, MAGP2-RGE had a completely opposite effect and
178 increased Notch signaling (Fig 2E). This result demonstrated that ligation of RGD

179 binding integrins by MAGP2 decreased Hes-1 promoter activity and that MAGP2 likely
 180 has both positive and negative Notch regulatory activities.

181

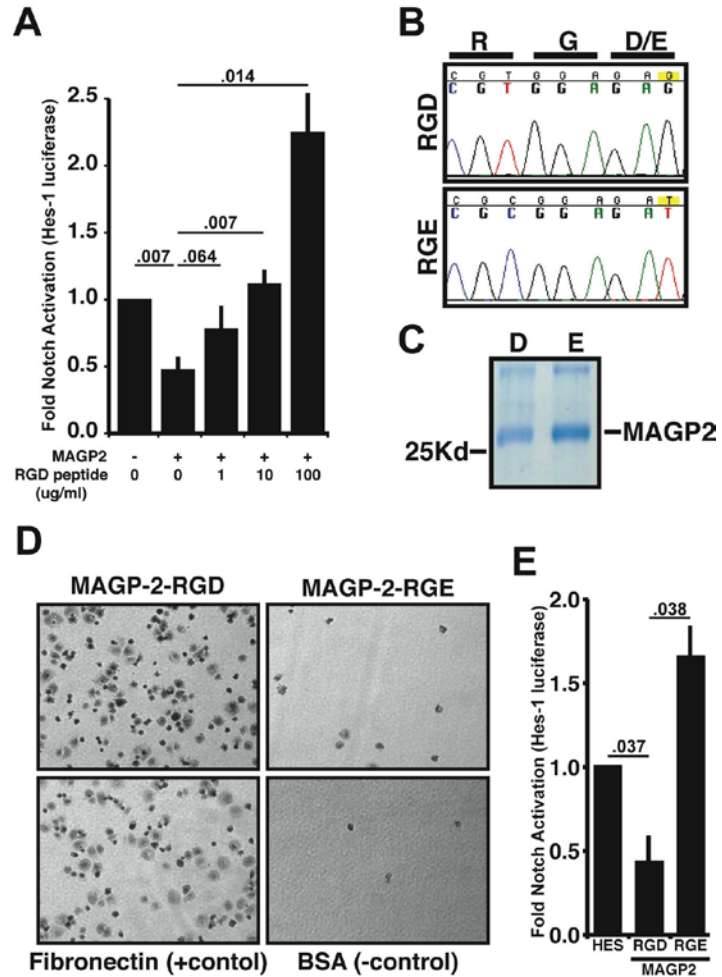
182 Figure 2: MAGP2 suppresses Notch
 183 activation in a RGD dependent manner.

184 (A) Effect of MAGP2 and soluble
 185 RGD peptides on Hes-1 promoter
 186 activity. Human HMEC endothelial
 187 cells were transfected with a Notch
 188 responsive Hes-1 luciferase reporter
 189 construct +/- MAGP2 cDNA then
 190 treated with increasing concentrations
 191 of soluble RGD peptide. Hes-1
 192 promoter activity was monitored by
 193 luciferase expression in solubilized cell
 194 lysates. The bar graph depicts data

195 from n=5 independent experiments. P-
 196 values (compared to -MAGP2, -RGD
 197 control) were calculated by student's t-
 198 test. (B) The RGD integrin binding
 199 domain of MAGP2 was mutated to a
 200 non-integrin binding RGE domain and
 201 verified by sequence analysis. Note
 202 that the CGT to CGC change present in
 203 the R codon of the RGE mutant is

204 silent. (C) C-terminally FLAG tagged RGD (D) and RGE (E) versions of MAGP2 were expressed in
 205 BL21-DE3 cells and purified by anti-FLAG affinity chromatography. Protein isolation was monitored by
 206 SDS-PAGE and coomassie staining. (D) Effect of MAGP2-RGE mutation on cell adhesion.
 207 Recombinantly produced and purified RGD and RGE versions of MAGP2 were coated onto cell culture
 208 plates and cell adhesion was compared to positive control fibronectin, or negative control BSA. (E) Effect
 209 of RGD and RGE versions of MAGP2 on Hes-1 promoter activity. HMEC cells were transfected with
 210 either Hes-1 luciferase reporter alone, or in combination with either MAGP2-RGD or MAGP2-RGE and
 211 Notch activity was monitored in solubilized cell lysates. The bar graph depicts data from n=4 experiments.
 212 The student's t-test was used to calculate p-values compared to cells transfected with Hes1-luciferase alone
 213 and are indicated above their corresponding bars.

214



215 *Integrin function couples to Notch signaling activity.*

216 We found that the RGD domain of MAGP2 was essential for suppression of Notch in
217 HMEC cells. RGD domains are common throughout the extracellular matrix where they
218 serve as binding sites for several types of integrins [2]. Therefore, it was important to
219 determine if the RGD domain of MAGP2 was unique in its ability to control Notch.
220 EGF-like domain-containing protein 7 (EGFL7) also contains an RGD domain that
221 interacts with $\alpha v\beta 3$ integrin [12] and has previously been shown to control Notch [13,
222 14]. To determine if the RGD domain of EGFL7 also controls Notch signaling we
223 compared Hes-1 promoter activity in HMEC cells transfected with RGD \rightarrow RGE EGFL7
224 mutants. As shown in figure 3A, EGFL7-RGD significantly enhanced Hes-1 promoter
225 activity compared to non-transfected cells. RGD \rightarrow RGE mutation of EGFL7 further
226 increased Hes1 promoter activity suggesting that integrin ligation by EGFL7 decreases
227 Notch1 signaling activity.

228 To more broadly examine the role of integrin ligation in Notch signaling, we
229 treated HMEC cells with soluble RGD peptides that bind RGD binding integrins but
230 prevent integrin activation [15]. HMEC endothelial cells were incubated with increasing
231 concentrations of soluble RGD peptides and accumulation of cleaved Notch1 NICD
232 fragments was monitored in cell lysates by western blot with anti-VAL1744 antibodies.
233 As shown in figures 3B and 3C, soluble RGD peptides dose-dependently caused a
234 significant accumulation of N1ICD fragments. Further western blot analysis suggested
235 that activation of Notch signaling did not appear to obviously correlate with increased
236 expression of either the full length Notch1 receptor, the Notch ligands Jagged1 or Dll4, or
237 the VEGF receptor KDR. Collectively, these findings demonstrated that generation of
238 the N1ICD domain is regulated by RGD binding integrins and thus supported our
239 hypothesis that the ECM may regulate Notch via interactions with RGD binding
240 integrins.

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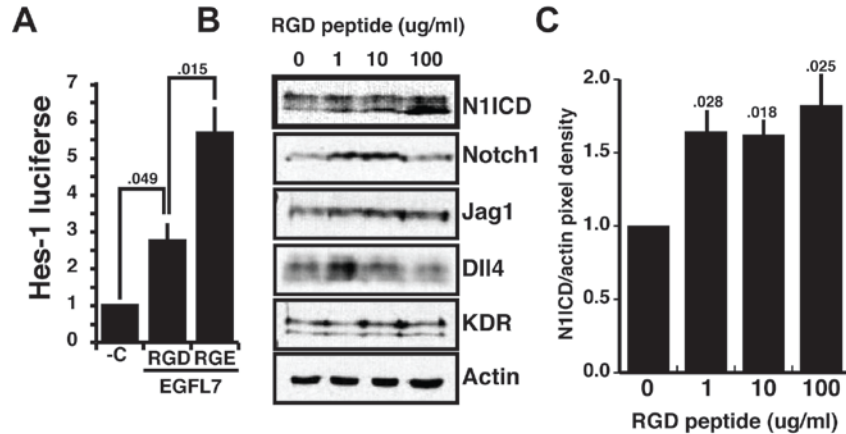
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246 Figure 3: RGD binding
 247 integrins control Notch.
 248 (A) The effect of EGFL7
 249 on Hes-1 promoter
 250 activity. Empty vector (-
 251 C), RGD or RGE
 252 versions of EGFL7 were
 253 co-transfected with Hes-
 254 1 luciferase plasmid into
 255 HMEC cells and
 256 luciferase activity was



257 monitored in whole cell lysates. The data depict the average +/- SE of n=4 experiments. P-values are
 258 indicated above their corresponding bars. (B) The effect of soluble RGD peptides on N1ICD accumulation
 259 in HMEC cells. HMEC cells were treated with increasing concentrations of soluble RGD peptides and
 260 N1ICD accumulation was monitored by western blot in fractionated whole cell lysates with anti-VAL1744
 261 antibodies. Expression of full-length Notch1, Jagged1, Dll-4, and VEGFR2 (KDR), was monitored by
 262 subsequent stripping and re-blotting with specific antibodies. Equivalent protein loading was monitored by
 263 blotting with anti- β -actin antibodies. Shown are representative blots from a single experiment that was
 264 performed n=5 independent times. (C) Image-J quantitation of N1ICD western blot data presented in panel
 265 B. Bar graph depicts data from n=5 experiments. P-values were calculated with the student's t-test
 266 compared to untreated control cells and are indicated above their corresponding bars.

267

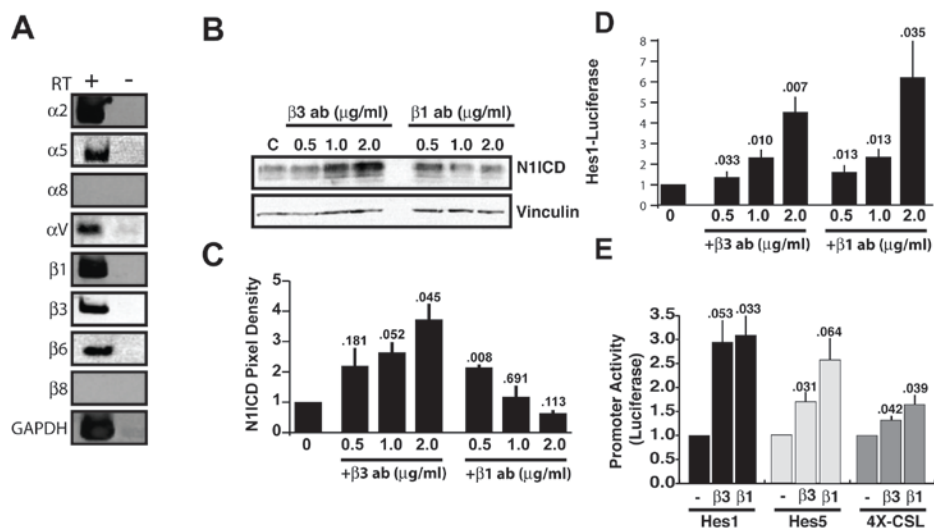
268 *β 3 and β 1 integrins control Notch signaling.*

269 At least eight of the 24 known integrin heterodimers have affinity for RGD motifs [2].
 270 Therefore, we used RT-PCR to compare expression of α and β integrin subunits known
 271 to heterodimerize into RGD binding integrins in HMEC cells [2]. HMEC cells expressed
 272 α 2, α 5, α V, β 1, β 3, and β 6 subunits (Fig. 4A). Both MAGP2 and EGFL7 had previously
 273 been shown to interact with α v β 3 integrins but not with β 1 integrins [6, 12] leading to the
 274 hypothesis that β 3 but not β 1 integrins would interact with Notch signaling. To test this
 275 hypothesis, we cultured HMEC endothelial cells in the presence of 0.5 to 2.0 μ g/ml of β 3
 276 or β 1 blocking antibodies and used western blot analysis to monitor Notch activation via
 277 N1ICD fragment accumulation in whole cell lysates. As shown in figure 4B and 4C, 7H2
 278 β 3 blocking antibodies that had previously been shown to block β 3 integrin mediated
 279 adhesion [16] dose-dependently enhanced N1ICD accumulation. In contrast, P5D2 β 1

280 blocking antibodies that had previously been shown to block $\beta 1$ integrin mediated
 281 adhesion [17] induced N1ICD accumulation at low dose (0.5 $\mu\text{g/ml}$), although higher
 282 concentrations of $\beta 1$ blocking antibodies failed to significantly affect N1ICD
 283 accumulation. We next transfected HMEC cells with the Hes-1 luciferase reporter and
 284 monitored Hes-1 promoter activity in the presence or absence of blocking antibodies
 285 directed against $\beta 3$ or $\beta 1$ integrins. Interestingly, application of both $\beta 3$ and $\beta 1$ blocking
 286 antibodies dose-dependently increased Hes-1 promoter activity across all tested antibody
 287 concentrations (0.5 to 2.0 $\mu\text{g/ml}$) (Fig 4D). Moreover, this activity was not restricted to
 288 the Hes-1 promoter since both $\beta 3$ and $\beta 1$ blocking antibodies also enhanced promoter
 289 activity from the Notch responsive Hes-5 and synthetic 4X-CSL promoters at 2.0 $\mu\text{g/ml}$
 290 (Fig 4E). Since HMEC cells also expressed $\beta 6$ integrin, we also examined HES-1
 291 promoter activity in the presence of 10D5 $\alpha v\beta 6$ blocking antibodies but did not observe a
 292 significant change in reporter activity (data not shown). Collectively these results
 293 confirmed our hypothesis that $\beta 3$ integrins couple to the Notch signaling pathway, and
 294 also suggested that $\beta 1$ integrin couples to Notch signaling via a mechanism that has
 295 similarities, but may also have distinctions compared to $\beta 3$ – Notch signaling.

296

297 Figure 4: $\beta 3$ and
 298 $\beta 1$ integrins couple
 299 to Notch signaling.
 300 (A) Analysis of
 301 RGD binding α
 302 and β integrin
 303 subunits in HMEC
 304 endothelial cells.
 305 PCR analysis of
 306 reverse transcribed
 307 (RT+) or non-
 308 reverse transcribed



309 (RT-) RNA with sequence specific oligos was used to detect expression of various RGD binding integrin
 310 subunits or GAPDH as a control. PCR products were resolved in PAGE gels and detected with ethidium
 311 bromide. Shown are the results of a representative experiment that was performed twice with identical
 312 results. (B) Effect of $\beta 3$ and $\beta 1$ blocking antibodies on N1ICD fragment accumulation in HMEC cells.

313 HMEC endothelial cells were cultured in the presence of increasing concentrations of $\beta 3$ or $\beta 1$ blocking
314 antibodies and N1ICD accumulation was monitored by western blot analysis of whole cell lysates with
315 anti-VAL1744 specific antibodies. Protein loading was monitored by stripping and subsequent re-blotting
316 with anti-vinculin antibodies. Shown are the results of a single experiment from n=4 independent
317 experiments. (C) Image-J quantitation of data presented in panel B. The bar graph depicts N1ICD pixel
318 density from n=4 experiments. The student's t-test was used to calculate p-values compared to untreated
319 HMEC cells and are indicated above their corresponding bars. (D) Effect of $\beta 3$ or $\beta 1$ blocking antibodies
320 on Hes-1 promoter activity. HMEC endothelial cells were transfected with Hes-1 luciferase constructs and
321 incubated in increasing concentrations of $\beta 3$ or $\beta 1$ blocking antibodies. Notch signaling was monitored by
322 measuring luciferase activity in solubilized cell lysates. The bar graph depicts data from n=4 experiments.
323 P-values compared to untreated cells were calculated using the student's t-test and are indicated above
324 corresponding bars. (E) Comparison of $\beta 3$ or $\beta 1$ blocking antibodies effect on Hes-1, Hes-5, and 4X-CSL
325 promoters. HMEC cells were transfected with luciferase reporter vectors containing either Hes-1, Hes-5, or
326 4X-CSL promoters and treated with 0 or 2 μ g/ml of $\beta 3$ or $\beta 1$ blocking antibodies. The bar graph depicts
327 data from n=4 experiments. P-values compared to untreated cells were calculated with the student's t-test
328 and are indicated above their corresponding bars.

329

330 **Discussion:**

331

332 The original intent of this project was to explore the mechanistic basis by which MAGP2
333 suppresses Notch signaling in endothelial cells but promotes Notch signaling in non-
334 endothelial cells. The capacity of MAGP2 to differentially control Notch was originally
335 hypothesized to be based on MAGP2 interactions with specific Notch receptor – ligand
336 combinations present in endothelial cells but not in non-endothelial cells. In testing this
337 hypothesis, we observed differential expression of Notch receptors in endothelial (SVEC)
338 and non-endothelial (B16F0) cells, but transplantation of these ligands and MAGP2 into
339 293T cells did not suggest a differential ability of MAGP2 to regulate Notch1 activation
340 by individual ligands (Fig 1). Instead, mutation of the MAGP2 RGD domain to a non-
341 integrin binding RGE domain not only eliminated the ability of MAGP2 to suppress
342 Notch signaling in endothelial cells, but also imbued MAGP2 with the ability to promote
343 Notch signaling in endothelial cells (Fig 2). Combining these results and the results of
344 Miyamoto et al [10] which demonstrated that the C-terminal of MAGP2 is necessary to
345 promote Notch signaling in 3T3 cells, we now hypothesize that MAGP2 controls Notch
346 signaling with a two-part mechanism. In cells expressing MAGP2 binding integrins (*i.e.*

347 $\alpha v\beta 3$), MAGP2 acts in a dominant negative fashion negating the pro-Notch signaling
348 conferred by the MAGP2 C-terminal. However in cells lacking MAGP2 binding
349 integrins, the C-terminal of MAGP2 increases Notch through induced dissociation of the
350 Notch extracellular domain as previously demonstrated [10]. Interestingly, MAGP2 is
351 subject to cleavage by proprotein convertase near the C-terminal [18] raising the
352 intriguing possibility that cleavage of MAGP2 (or other ECM proteins) may act as an
353 additional level of regulatory activity. A similar mechanism can also be envisioned for
354 EGFL7. In this case however, we found that EGFL7 increased Notch signaling and
355 mutation of the RGD domain further increase Notch signaling. These results suggest that
356 EGFL7 may also contain both pro- and anti-Notch regulatory activity although it is not
357 known if EGFL7 is subject to cleavage in the ECM.

358

359 Although our original intent was to explore the mechanism by which MAGP2 controls
360 Notch, our results have uncovered a mechanism that may be broadly applied to many
361 ECM proteins that interact with integrins. As such these results add a new dimension to
362 the emerging idea that the cellular microenvironment via specific extracellular matrices is
363 capable of controlling Notch signaling activity. Other reports have also hinted at this
364 possibility. Weijers et al., [19] described an effect of low molecular weight fibronectin
365 fragments on the expression of the Notch ligand Dll4 and subsequent Notch activation in
366 endothelial cells. More recently, Estrach et al., [20] and Stenzel et al., [21] demonstrated
367 that laminin 111 and laminin $\alpha 4$ increase Dll4 expression in endothelial cells via $\alpha 2\beta 1$
368 and $\alpha 6\beta 1$ integrins. Stenzel et al., continued to show that disruption of this signaling
369 system had dramatic complications for normal angiogenesis thus hinting at the biological
370 significance of this signaling system [21]. While similar in some ways, our results are
371 distinct since treatment of HMEC cells with soluble RGD peptides increased Notch
372 signaling activity independently of Notch1, Jagged1, or Dll4 expression (Fig 3).
373 Therefore, instead of controlling Notch signaling via increased Notch receptor or ligand
374 expression, our results suggest that integrin ligation directly engages in cross-talk with
375 Notch. Support for this mechanism has been published elsewhere. Suh et al., [22]
376 demonstrated that collagen1 increases NICD accumulation via interactions with $\alpha 2\beta 1$
377 integrins, Mo et al., [23] observed that the downstream integrin regulator ILK (Integrin

378 linked Kinase) decreases Notch signaling by stimulating ubiquitination and rapid
379 degradation of the active Notch1 NICD fragment, and Ma et al., [24] found that the
380 kinase domain of SRC binds to the ankyrin domain of active NICD. Finally, a recent
381 screen to find genetic interactions with Notch identified a signaling mechanism involving
382 Notch, SRC, and JNK that was important for normal eye development in drosophila [25].
383 Further investigation will be required to determine the mechanism by which integrins
384 couple to Notch signaling, however it is worth noting that SRC and ILK are well known
385 downstream effectors of integrins [3].

386

387 Our results not only suggest that integrins control Notch signaling, but that signaling
388 through $\beta 1$ and $\beta 3$ integrins differentially controls Notch. We found that blocking
389 antibodies against $\beta 3$ and $\beta 1$ integrins both increased Hes-1, Hes-5, and 4X-CSL
390 promoter activity while $\beta 3$ but not $\beta 1$ blocking antibodies dose-dependently increased
391 N1ICD accumulation (Fig 4). While we don't know how $\beta 3$ and $\beta 1$ integrins
392 differentially control Notch, this observation is consistent with previous work showing
393 that $\beta 1$ and $\beta 3$ integrins have both overlapping and independent mechanotransduction
394 activities in cells [26-28]. Building on this idea is the fact that $\beta 1$ and $\beta 3$ ligands often
395 have distinct spatiotemporal distributions in tissues. For instance, $\beta 1$ ligands such as
396 laminins and collagen 4 are enriched in angiostatic vascular basement membranes [29],
397 while $\beta 3$ ligands such as vitronectin, fibronectin, and fibrin are enriched in pro-
398 angiogenic provisional matrices [30]. Therefore, we speculate that diverse
399 microenvironments differentially regulate Notch in response to cellular integrin
400 expression profiles and the local extracellular matrix composition.

401

402 Future experiments will need to determine the scope to which ECM proteins in the
403 microenvironment influence angiogenesis through Notch signaling, but it is noteworthy
404 that a number of ECM proteins have been shown to regulate Notch signaling and to
405 interact with either $\beta 3$ integrins (*e.g.* EGFL7 [12, 13] and MAGP2 [6, 7, 11] or with $\beta 1$
406 integrins (*e.g.* CCN3 [31, 32] and Reelin [33, 34]). Finally, additional observations have
407 demonstrated that Notch1 and $\beta 1$ integrin co-localize in neural stem cells [35] and that

408 activation of Notch signaling can control β 1 integrin affinity [36, 37] suggesting the
409 existence of a feedback loop that coordinates Notch and integrin function. Collectively,
410 our observations combined with other results suggest the presence of an ECM – integrin
411 – Notch signaling axis that may represent an important mechanism enabling cells to
412 respond to their microenvironment.

413

414 In conclusion, through basic research aimed at understanding how MAGP2 controls
415 Notch signaling, we have arrived at a more universal understanding of how ECM
416 molecules in the cellular microenvironment impact cell physiology via integrin ligation
417 and subsequent manipulation of the Notch signaling pathway.

418

419 **Materials and Methods:**

420

421 *Plasmids*

422

423 The pcDNA3.1 myc-his tagged MAGP2 expression construct was previously described
424 [7] and was subjected to site-directed mutagenesis with mutagenic oligos to produce the
425 MAGP2-RGE construct. The EGFL7 expression plasmid was constructed by gateway
426 cloning a human EGFL7 cDNA (clone ID# 30400137) that had been amplified by PCR
427 with oligos that added 5' Kozak sequence and 3'FLAG tag, cloned into pcDNA-DEST40,
428 and sequenced in its entirety. Mutagenesis of the EGFL7 expression construct was
429 performed by site-directed mutagenesis with mutagenic oligos and mutants were
430 identified by sequence analysis. The Myc-tagged mammalian expression vectors
431 encoding murine Notch1 (pCS2+mN1FL6MT) and Jagged-1 (pCS2+Jag1-6MT) were
432 kindly provided by Dr. Raphael Kopan (Washington University, St. Louis, MO). The
433 Delta-like 1 (Dl1) and Delta-like 3 (Dl3) expression constructs were kindly provided by
434 Dr. Geraldine Weinmaster (UCLA, Los Angeles, CA). The Delta-like 4 (Dl4) expression
435 construct was cloned by PCR amplification of murine Dl4 cDNA (clone ID# 86280 with
436 oligos that introduced 5' kozak and EcoR1 sequences, and 3' SacII sequence. The PCR
437 product was ligated into pcDNA3.1 Myc-his and sequenced in its entirety. The Hes1
438 and Hes5-luciferase reporters were purchased from Addgene and consist of nucleotides -

439 467 to +46 and -800 to +73 relative to the transcriptional start sites respectively. The 4X-
440 CSL luciferase construct was also purchased from Addgene and consists of 4 tandem
441 repeats of the high affinity CSL binding sites (5'CGTGGGAA3').

442

443 *Luciferase assays*

444

445 For experiments examining the effect of RGD peptides, or WT vs RGE MAGP2/EGFL7
446 cDNAs on Hes-1 promoter activity, HMEC cells were seeded into 24-well plates at a
447 density of 25,000 cells/well and transfected the following day with LT-1 liposomes
448 containing various combinations of Hes-1 luciferase (200ng/well), MAGP2/EGFL7
449 cDNAs (WT or RGE) (100ng/well), and CMV- β -gal control plasmid (10ng/well). Where
450 appropriate, cells were treated with 1, 10, or 100 μ g/ml of soluble RGD
451 (GCGYGRGDSPG) peptide (GenScript, Piscataway, NJ). 48 hours after transfection,
452 cells were lysed in passive lysis buffer (Promega, Madison, WI) and luciferase and β -gal
453 activities were measured on a Glo-Max luminometer. In experiments with β 3 and β 1
454 blocking antibodies, HMEC cells were transfected by electroporating 2,000,000 cells in
455 PBS with 1.9 μ g of luciferase reporter (Hes-1, Hes-5, or 4X-CSL luciferase) and 0.1 μ g
456 of CMV- β -gal reporter. Cells were pulsed in a nucleofector 2b (Lonza, Walkersville,
457 MD) electroporator (2mm gap) set for "HUVEC", diluted into EGM2 growth media, and
458 plated into 12 wells of a 24-well plate (250 μ l/well) to which 0, 0.5, 1, or 2 μ g/ml of β 3
459 (7H2) or β 1 (P5D2) blocking antibodies (Developmental Studies Hybridoma Bank, Iowa
460 City, IA) were immediately added. Electroporated cells were collected 24 hours later and
461 luciferase activity was measured as previously described [38].

462

463 *Reverse transcription PCR*

464

465 Total RNA was extracted from cultured cells using Ribosol (Amresco, Solon, OH) and
466 iScript reverse transcriptase (Bio-Rad, Hercules, CA) was used to generate cDNA pools
467 from 1 μ g of total RNA. RT-PCR reactions were performed using 12.5ng of cDNA,
468 0.8 μ M each oligo, 200 μ M dNTP, 1x standard buffer, and 2 units Taq Polymerase in a
469 total reaction volume of 25 μ l. Cycling parameters were as follows: 1 cycle at 94 $^{\circ}$ C for 2

470 min; 25 cycles at 94°C for 45 sec, 55°C for 30 sec, and 72°C for 30 sec. Oligonucleotide
471 sequences are reported in table 1.

472

473 *Recombinant protein and adhesion assay*

474

475 The bacterial pSBET MAGP2 expression vector was previously described [7]. The
476 MAGP2 RGE mutant vector was constructed by site-directed mutagenesis of wild-type
477 MAGP2 as described above. Recombinant MAGP2 proteins were expressed in BL21-
478 DE3 *E. coli* cells and purified from sonicated cell lysates by affinity chromatography on
479 FLAG-M2 monoclonal antibody columns (Sigma, St. Louis, MO). Bound proteins were
480 washed initially with 10 column volumes of TBS/0.1% Triton X-100, followed by an
481 additional 20 column volumes of TBS. Afterward, recombinant proteins were eluted by
482 addition of 2.5 column volumes of FLAG M2 peptide (100 µg/ml), which s
483 was concentrated by centrifugation in 5 kD centricon devices (Sartorius, Goettingen,
484 Germany).

485

486 *Antibodies*

487

488 Antibodies against Notch1 (#3608), Jagged1 (#2620), Dll4 (#2589), N1ICD (VAL1744,
489 #2421), and KDR (VEGFR2) (#2472) were purchased from Cell Signaling Technologies
490 (Danvers, MA). The 7H2 β3 blocking antibodies and P5D2 blocking antibodies were
491 previously described [16, 17] and purchased as monoclonal supernatants from the
492 Developmental Studies Hybridoma Bank (Iowa City, Iowa). Anti-β Actin antibodies (sc-
493 130656) and anti-Vinculin antibodies (sc-5573) were purchased from Santa Cruz (Paso
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495

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497

498

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500

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509 tools: ARA. Wrote the paper ARA.

510

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640 Table 1: Oligonucleotides used in this study.

Oligo Name	Oligo Use	Oligo sequence
AA37	Mouse GAPDH fwd RT-PCR	GACAATGAATACGGCTACAGCAAC
AA38	Mouse GAPDH rev RT-PCR	GTGCAGCGAACTTTATTGATGGTA
AA11	Mouse Notch1 fwd RT-PCR	TGCACCTGCTGTCATCTCTGACTT
AA12	Mouse Notch1 rev RT-PCR	AGGATCAGTGGAGTTGTGCCATCA
AA13	Mouse Notch3 fwd RT-PCR	AGCTGTGTCAGGAAGGTGGAAAGT
AA14	Mouse Notch3 rev RT-PCR	AACAGAGATAGCGGGCCACAAGAT
AA17	Mouse Dll3 fwd RT-PCR	TGTGAAGAGCCTGATGAATGCCGT
AA18	Mouse Dll3 rev RT-PCR	ACCTCACATCGAAGCCCGTAGAAT
AA19	Mouse Dll4 fwd RT-PCR	ACTCACCACTCTCCGTGCAAGAAT
AA20	Mouse Dll4 rev RT-PCR	TATGCTCACAGTGTGGCCATAGT
AA21	Mouse Dll1 fwd RT-PCR	AATCTGTCTGCCAGGTGTGATGA
AA22	Mouse Dll1 rev RT-PCR	TGCACGGCTTATGGTGAGTACAGT
AA23	Mouse Notch4 fwd RT-PCR	TGAAGGGCCACACTGTGAGAAAGA
AA24	Mouse Notch4 rev RT-PCR	ACACACACACAAGGATCTCTGGCA
AA25	Mouse JAG2 fwd RT-PCR	TAGCAAGGTATGGTGCGGATGGAA
AA26	Mouse JAG2 rev RT-PCR	GTCGGGCACAGTTGTTGTCCAAAT
AA28	Mouse JAG1 fwd RT-PCR	TGCTGAGCATGCTTGTCTCTCTGA
AA29	Mouse JAG1 rev RT-PCR	CAAGGTTTGGCCTCGCACTCATTT
AA103	Human GAPDH fwd RT-PCR	TCCATGACAACCTTTGGTATTCGT
AA104	Human GAPDH rev RT-PCR	AGTAGAGGCAGGGATGATGTT
KW181	Human Int $\alpha 2$ fwd RT-PCR	TCTCAGAAGTCTGTTGCCTGCGAT
KW182	Human Int $\alpha 2$ rev RT-PCR	ACTGATGTCACCAGCCTTGTCTGT
KW183	Human Int $\alpha 5$ fwd RT-PCR	TCGAGACAAACTCTCGCCGATTCA
KW184	Human Int $\alpha 5$ rev RT-PCR	TCACGGCAAAGTAGTCACAGCTCA
KW185	Human Int αV fwd RT-PCR	AAGATGTTGGGCCAGTTGTTTCAGC
KW186	Human Int αV rev RT-PCR	AGCAACTCCACAACCCAAAGTGTG
KW187	Human Int $\beta 1$ fwd RT-PCR	TCTGCGGACAGTGTGTTTGTAGGA
KW188	Human Int $\beta 1$ rev RT-PCR	AATGGGACACAGGATCAGGTGGA
KW189	Human Int $\beta 3$ fwd RT-PCR	CCCCTTGGCATCATTACAGCAA
KW190	Human Int $\beta 3$ rev RT-PCR	AAGAGACCTTCAAGACTGGCTGCT
AK366	Human Int $\beta 8$ fwd RT-PCR	AGCAAATTGGCAGGCATAGTGGTG
AK367	Human Int $\beta 8$ rev RT-PCR	TCGTCACGTTTCTGCATCCTTCCA
AK368	Human Int $\beta 6$ fwd RT-PCR	AGCAAATTGGCAGGCATAGTGGTG
AK369	Human Int $\beta 6$ rev RT-PCR	AGACATCTCTTTGGAAAGCCGGGA

AK370	Human Int α 8 fwd RT-PCR	AAGGGATTTTCGACCACTGAGCTGT
AK371	Human Int α 8 rev RT-PCR	ACTCCTCTTATTTCCACCTGCGCT
AA953	Mouse MAGP2 RGE mutagenesis	GTGAATGTCTCAGGCACATCCTCTCCA CGTTGACCACTGAC
AA952	Mouse MAGP2 RGE mutagenesis	GTCAGTGGTCAACGTGGAGAGGATGTGCCT GAGACATTCAC
PD424	hEGFL7 RGE mutagenesis	GGATGGCGGGGTGAGACTTGCCAGTCAGATG
PD425	hEGFL7 RGE mutagenesis	CATCTGACTGGCAAGTCTCACCCCGCCATCC
AA39	Mouse DLL4 fwd cloning	GGCGGCGAATTCACCATGGCGGCAG CGTCCCGG
AA6	Mouse DLL4 rev cloning	GGCGGCCCGCGGTACCTCCGTGGCAATGAC