2-1-2016

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

Peter Deford
Boise State University

Kasey Brown
Indiana State University

Rae Lee Richards
Indiana State University

Aric King
Indiana State University

Kristin Newburn
Indiana State University

See next page for additional authors

Authors
Peter Deford, Kasey Brown, Rae Lee Richards, Aric King, Kristin Newburn, Katherine Westover, and Allan R. Albig

MAGP2 controls Notch via interactions with RGD binding integrins: Identification of a Novel ECM – Integrin – Notch Signaling Axis.

Peter Deford[2,*], Kasey Brown[3], Rae Lee Richards[3], Aric King[3], Kristin Newburn[3], Katherine Westover[2], and Allan R. Albig[1,2,#]

[1]Biomolecular Sciences PhD Program, Boise State University, Boise, ID. 83725

[2]Dept. of Biology, Boise State University, Boise, ID. 83725

[3]Dept. of Biology, Indiana State University, Terre Haute, IN. 47809

Running Title: ECM controls Notch via Integrins

Keywords: Extracellular matrix, cell signaling, Notch, Integrin

#Address correspondence to Allan R. Albig. AllanAlbig@boisestate.edu

*Present Address: Peter Deford, Dept. of Cell, Molecular, Developmental Biology, and Biophysics, Johns Hopkins University.

Abbreviations:

MAGP2 – Microfibril Associated Glycoprotein 2

N1ICD – Notch1 Intracellular domain

ECM – Extracellular Matrix

HMEC – Human Microvascular Endothelial cells

Abstract: Canonical Notch signaling involves Notch receptor activation via interaction with cell surface bound Notch ligand. Recent findings also indicate that Notch signaling may be modulated by cross-talk with other signaling mechanisms. The ECM protein MAGP2 was previously shown to regulate Notch in a cell type dependent manner, although the molecular details of this interaction have not been dissected. Here, we report that MAGP2 cell type specific control of Notch is independent of individual Notch receptor-ligand combinations but dependent on interaction with RGD binding integrins. Overexpressed MAGP2 was found to suppress transcriptional activity from the Notch responsive Hes1 promoter activity in endothelial cells, while overexpression of a RGD→RGE MAGP2 mutant increased Notch signaling in the same cell type. This effect was not unique to MAGP2 since the RGD domain of the ECM protein EGFL7 was also found to be an important modulator of Hes1 promoter activity. Independently of MAGP2 or EGFL7, inhibition of RGD-binding integrins with soluble RGD peptides also increased accumulation of active N1ICD fragments and Notch responsive promoter activity independently of changes in Notch1, Jag1, or Dll4 expression. Finally, β1 or β3 integrin blocking antibodies also enhanced Notch signaling. Collectively, these results answer the question of how MAGP2 controls cell type dependent Notch signaling, but more importantly uncover a new mechanism to understand how extracellular matricies and cellular environments impact Notch signaling.

Introduction:

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

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

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

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

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

Results:

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

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

which MAGP2 had previously been shown to reduce or increase Notch signaling respectively [11]. As shown in figure 1A, both cell lines expressed Notch receptors 1, 3, and 4 and also shared expression of Notch ligands Jagged1 and 2 (JAG1, 2). However, expression of Notch ligands Delta-like 1 and 3 (Dll1, 3) was restricted to B16F0 cells while expression of the Delta-like 4 (Dll4) Notch ligand was restricted to SVEC cells. Therefore, we transiently transfected 293T cells with combinations of Notch1 together with MAGP2 and either Dll1, Dll3, or Dll4 cDNAs and monitored Notch activation by western blot analysis of whole cell lysates with anti-VAL1744 antibodies that only recognize the activated N1ICD domain cleaved at the VAL1744 position by gamma-secretase. As shown in figure 1B, regardless of combination, co-transfected MAGP2 cDNA decreased Notch activation independently of Notch receptor – ligand combination.

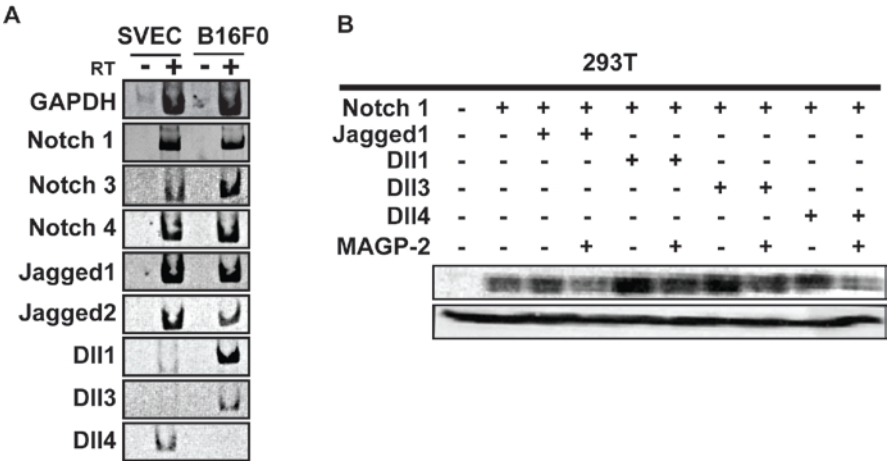

Figure 1: MAGP2 suppresses Notch activation independently of ligand type. (A) Expression patterns of Notch receptors and ligands in murine SVEC endothelial cells and B16F0 melanoma cells. Expression of Notch receptors and ligands was screened by RT-PCR with transcript specific oligos. Non-reverse transcribed RNA (RT-) was used as a negative control to control for amplification from contaminating genomic DNA. Shown are the results of a single experiment that was performed twice with identical results. (B) Effect of MAGP2 on Notch activation by various Notch ligands. 293T cells were transfected with combinations of cDNA encoding Notch1, MAGP2, and various Notch ligands (JAG1, Dll1, Dll3, Dll4). Notch activation was monitored by western blot analysis with anti-N1ICD (VAL-1744) specific antibodies. Equivalent protein loading was monitored by stripping and re-blotting with anti-β-actin antibodies. Shown is a representative result from a single experiment that was performed 6 times in it's entirety.

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

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

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

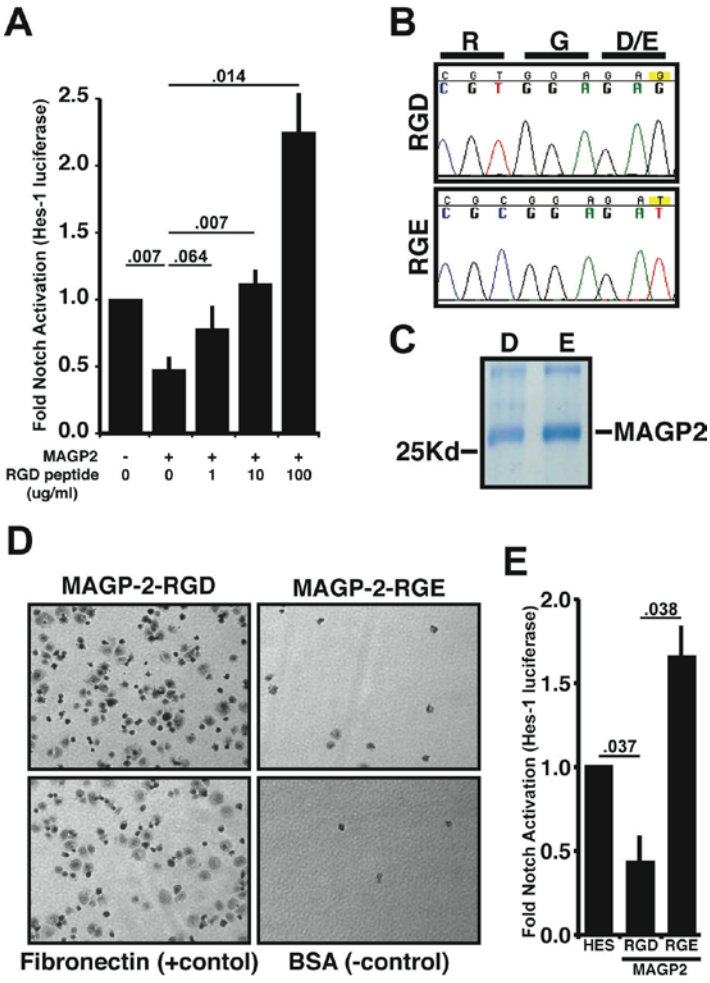

Figure 2: MAGP2 suppresses Notch activation in a RGD dependent manner. (A) Effect of MAGP2 and soluble RGD peptides on Hes-1 promoter activity. Human HMEC endothelial cells were transfected with a Notch responsive Hes-1 luciferase reporter construct +/- MAGP2 cDNA then treated with increasing concentrations of soluble RGD peptide. Hes-1 promoter activity was monitored by luciferase expression in solubilized cell lysates. The bar graph depicts data from n=5 independent experiments. P-values (compared to -MAGP2, -RGD control) were calculated by student's t-test. (B) The RGD integrin binding domain of MAGP2 was mutated to a non-integrin binding RGE domain and verified by sequence analysis. Note that the CGT to CGC change present in the R codon of the RGE mutant is silent. (C) C-terminally FLAG tagged RGD (D) and RGE (E) versions of MAGP2 were expressed in BL21-DE3 cells and purified by anti-FLAG affinity chromatography. Protein isolation was monitored by SDS-PAGE and coomassie staining. (D) Effect of MAGP2-RGE mutation on cell adhesion. Recombinantly produced and purified RGD and RGE versions of MAGP2 were coated onto cell culture plates and cell adhesion was compared to positive control fibronectin, or negative control BSA. (E) Effect of RGD and RGE versions of MAGP2 on Hes-1 promoter activity. HMEC cells were transfected with either Hes-1 luciferase reporter alone, or in combination with either MAGP2-RGD or MAGP2-RGE and Notch activity was monitored in solubilized cell lysates. The bar graph depicts data from n=4 experiments. The student's t-test was used to calculate p-values compared to cells transfected with Hes1-luciferase alone and are indicated above their corresponding bars.

Integrin function couples to Notch signaling activity.

We found that the RGD domain of MAGP2 was essential for suppression of Notch in HMEC cells. RGD domains are common throughout the extracellular matrix where they serve as binding sites for several types of integrins [2]. Therefore, it was important to determine if the RGD domain of MAGP2 was unique in it's ability to control Notch. EGF-like domain-containing protein 7 (EGFL7) also contains an RGD domain that interacts with αvβ3 integrin [12] and has previously been shown to control Notch [13, 14]. To determine if the RGD domain of EGFL7 also controls Notch signaling we compared Hes-1 promoter activity in HMEC cells transfected with RGD → RGE EGFL7 mutants. As shown in figure 3A, EGFL7-RGD significantly enhanced Hes-1 promoter activity compared to non-transfected cells. RGD → RGE mutation of EGFL7 further increased Hes1 promoter activity suggesting that integrin ligation by EGFL7 decreases Notch1 signaling activity.

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

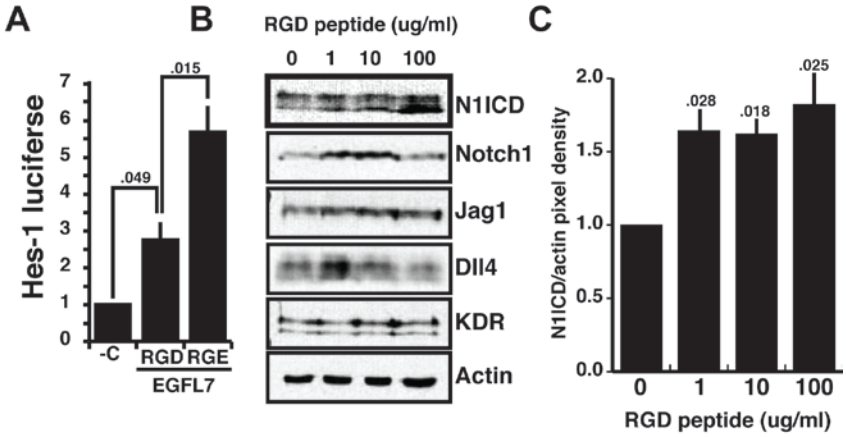

Figure 3: RGD binding integrins control Notch. (A) The effect of EGFL7 on Hes-1 promoter activity. Empty vector (-C), RGD or RGE versions of EGFL7 were co-transfected with Hes-1 luciferase plasmid into HMEC cells and luciferase activity was monitored in whole cell lysates. The data depict the average +/- SE of n=4 experiments. P-values are indicated above their corresponding bars. (B) The effect of soluble RGD peptides on N1ICD accumulation in HMEC cells. HMEC cells were treated with increasing concentrations of soluble RGD peptides and N1ICD accumulation was monitored by western blot in fractionated whole cell lysates with anti-VAL1744 antibodies. Expression of full-length Notch1, Jagged1, Dll-4, and VEGFR2 (KDR), was monitored by subsequent stripping and re-blotting with specific antibodies. Equivalent protein loading was monitored by blotting with anti-β-actin antibodies. Shown are representative blots from a single experiment that was performed n=5 independent times. (C) Image-J quantitation of N1ICD western blot data presented in panel B. Bar graph depicts data from n=5 experiments. P-values were calculated with the student's t-test compared to untreated control cells and are indicated above their corresponding bars.

β3 and β1 integrins control Notch signaling.

At least eight of the 24 known integrin heterodimers have affinity for RGD motifs [2]. Therefore, we used RT-PCR to compare expression of α and β integrin subunits known to heterodimerize into RGD binding integrins in HMEC cells [2]. HMEC cells expressed α2, α5, αV, β1, β3, and β6 subunits (Fig. 4A). Both MAGP2 and EGFL7 had previously been shown to interact with αvβ3 integrins but not with β1 integrins [6, 12] leading to the hypothesis that β3 but not β1 integrins would interact with Notch signaling. To test this hypothesis, we cultured HMEC endothelial cells in the presence of 0.5 to 2.0 μg/ml of β3 or β1 blocking antibodies and used western blot analysis to monitor Notch activation via N1ICD fragment accumulation in whole cell lysates. As shown in figure 4B and 4C, 7H2 β3 blocking antibodies that had previously been shown to block β3 integrin mediated adhesion [16] dose-dependently enhanced N1ICD accumulation. In contrast, P5D2 β1

blocking antibodies that had previously been shown to block β1 integrin mediated adhesion [17] induced N1ICD accumulation at low dose (0.5 μg/ml), although higher concentrations of β1 blocking antibodies failed to significantly affect N1ICD accumulation. We next transfected HMEC cells with the Hes-1 luciferase reporter and monitored Hes-1 promoter activity in the presence or absence of blocking antibodies directed against β3 or β1 integrins. Interestingly, application of both β3 and β1 blocking antibodies dose-dependently increased Hes-1 promoter activity across all tested antibody concentrations (0.5 to 2.0 μg/ml) (Fig 4D). Moreover, this activity was not restricted to the Hes-1 promoter since both β3 and β1 blocking antibodies also enhanced promoter activity from the Notch responsive Hes-5 and synthetic 4X-CSL promoters at 2.0 μg/ml (Fig 4E). Since HMEC cells also expressed β6 integrin, we also examined HES-1 promoter activity in the presence of 10D5 αvβ6 blocking antibodies but did not observe a significant change in reporter activity (data not shown). Collectively these results confirmed our hypothesis that β3 integrins couple to the Notch signaling pathway, and also suggested that β1 integrin couples to Notch signaling via a mechanism that has similarities, but may also have distinctions compared to β3 – Notch signaling.

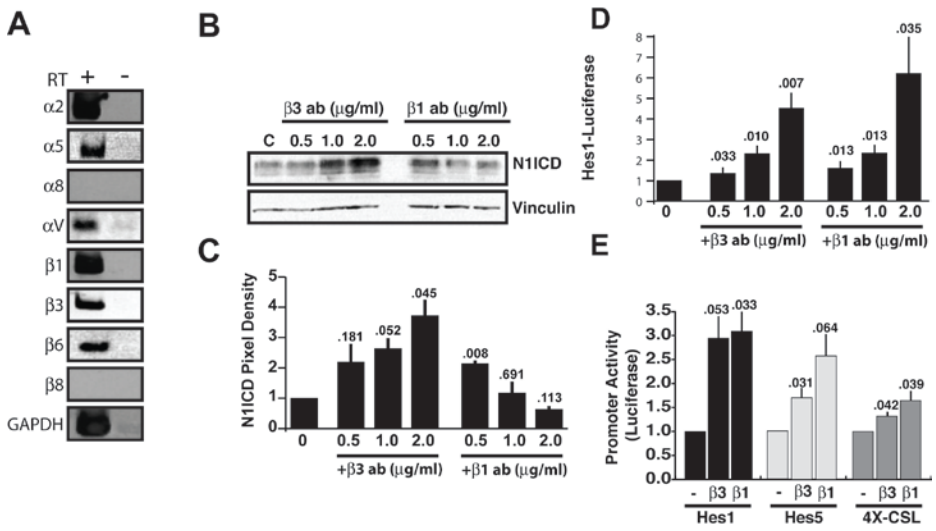

Figure 4: β3 and β1 integrins couple to Notch signaling. (A) Analysis of RGD binding α and β integrin subunits in HMEC endothelial cells. PCR analysis of reverse transcribed (RT+) or non-reverse transcribed (RT-) RNA with sequence specific oligos was used to detect expression of various RGD binding integrin subunits or GAPDH as a control. PCR products were resolved in PAGE gels and detected with ethidium bromide. Shown are the results of a representative experiment that was performed twice with identical results. (B) Effect of β3 and β1 blocking antibodies on N1ICD fragment accumulation in HMEC cells.

HMEC endothelial cells were cultured in the presence of increasing concentrations of β3 or β1 blocking antibodies and N1ICD accumulation was monitored by western blot analysis of whole cell lysates with anti-VAL1744 specific antibodies. Protein loading was monitored by stripping and subsequent re-blotting with anti-vinculin antibodies. Shown are the results of a single experiment from n=4 independent experiments. (C) Image-J quantitation of data presented in panel B. The bar graph depicts N1ICD pixel density from n=4 experiments. The student's t-test was used to calculate p-values compared to untreated HMEC cells and are indicated above their corresponding bars. (D) Effect of β3 or β1 blocking antibodies on Hes-1 promoter activity. HMEC endothelial cells were transfected with Hes-1 luciferase constructs and incubated in increasing concentrations of β3 or β1 blocking antibodies. Notch signaling was monitored by measuring luciferase activity in solubilized cell lysates. The bar graph depicts data from n=4 experiments. P-values compared to untreated cells were calculated using the student's t-test and are indicated above corresponding bars. (E) Comparison of β3 or β1 blocking antibodies effect on Hes-1, Hes-5, and 4X-CSL promoters. HMEC cells were transfected with luciferase reporter vectors containing either Hes-1, Hes-5, or 4X-CSL promoters and treated with 0 or 2μg/ml of β3 or β1 blocking antibodies. The bar graph depicts data from n=4 experiments. P-values compared to untreated cells were calculated with the student's t-test and are indicated above their corresponding bars.

Discussion:

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

αvβ3), MAGP2 acts in a dominant negative fashion negating the pro-Notch signaling conferred by the MAGP2 C-terminal. However in cells lacking MAGP2 binding integrins, the C-terminal of MAGP2 increases Notch through induced dissociation of the Notch extracellular domain as previously demonstrated [10]. Interestingly, MAGP2 is subject to cleavage by proprotein convertase near the C-terminal [18] raising the intriguing possibility that cleavage of MAGP2 (or other ECM proteins) may act as an additional level of regulatory activity. A similar mechanism can also be envisioned for EGFL7. In this case however, we found that EGFL7 increased Notch signaling and mutation of the RGD domain further increase Notch signaling. These results suggest that EGFL7 may also contain both pro- and anti-Notch regulatory activity although it is not known if EGFL7 is subject to cleavage in the ECM.

Although our original intent was to explore the mechanism by which MAGP2 controls Notch, our results have uncovered a mechanism that may be broadly applied to many ECM proteins that interact with integrins. As such these results add a new dimension to the emerging idea that the cellular microenvironment via specific extracellular matrices is capable of controlling Notch signaling activity. Other reports have also hinted at this possibility. Weijers et al., [19] described an effect of low molecular weight fibronectin fragments on the expression of the Notch ligand Dll4 and subsequent Notch activation in endothelial cells. More recently, Estrach et al., [20] and Stenzel et al., [21] demonstrated that laminin 111 and laminin α4 increase Dll4 expression in endothelial cells via α2β1 and α6β1 integrins. Stenzel et al., continued to show that disruption of this signaling system had dramatic complications for normal angiogenesis thus hinting at the biological significance of this signaling system [21]. While similar in some ways, our results are distinct since treatment of HMEC cells with soluble RGD peptides increased Notch signaling activity independently of Notch1, Jagged1, or Dll4 expression (Fig 3). Therefore, instead of controlling Notch signaling via increased Notch receptor or ligand expression, our results suggest that integrin ligation directly engages in cross-talk with Notch. Support for this mechanism has been published elsewhere. Suh et al., [22] demonstrated that collagen1 increases NICD accumulation via interactions with α2b1 integrins, Mo et al., [23] observed that the downstream integrin regulator ILK (Integrin

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

Our results not only suggest that integrins control Notch signaling, but that signaling through β1 and β3 integrins differentially controls Notch. We found that blocking antibodies against β3 and β1 integrins both increased Hes-1, Hes-5, and 4X-CSL promoter activity while β3 but not β1 blocking antibodies dose-dependently increased N1ICD accumulation (Fig 4). While we don't know how β3 and β1 integrins differentially control Notch, this observation is consistent with previous work showing that β1 and β3 integrins have both overlapping and independent mechanotransduction activities in cells [26-28]. Building on this idea is the fact that β1 and β3 ligands often have distinct spatiotemporal distributions in tissues. For instance, β1 ligands such as laminins and collagen 4 are enriched in angiostatic vascular basement membranes [29], while β3 ligands such as vitronectin, fibronectin, and fibrin are enriched in pro-angiogenic provisional matrices [30]. Therefore, we speculate that diverse microenvironments differentially regulate Notch in response to cellular integrin expression profiles and the local extracellular matrix composition.

Future experiments will need to determine the scope to which ECM proteins in the microenvironment influence angiogenesis through Notch signaling, but it is noteworthy that a number of ECM proteins have been shown to regulate Notch signaling and to interact with either β3 integrins (*e.g.* EGFL7 [12, 13] and MAGP2 [6, 7, 11] or with β1 integrins (*e.g.* CCN3 [31, 32] and Reelin [33, 34]). Finally, additional observations have demonstrated that Notch1 and β1 integrin co-localize in neural stem cells [35] and that

activation of Notch signaling can control β1 integrin affinity [36, 37] suggesting the existence of a feedback loop that coordinates Notch and integrin function. Collectively, our observations combined with other results suggest the presence of an ECM – integrin – Notch signaling axis that may represent an important mechanism enabling cells to respond to their microenvironment.

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

Materials and Methods:

Plasmids

The pcDNA3.1 myc-his tagged MAGP2 expression construct was previously described [7] and was subjected to site-directed mutagenesis with mutagenic oligos to produce the MAGP2-RGE construct. The EGFL7 expression plasmid was constructed by gateway cloning a human EGFL7 cDNA (clone ID# 30400137) that had been amplified by PCR with oligos that added 5' Kozak sequence and 3'FLAG tag, cloned into pcDNA-DEST40, and sequenced in its entirety. Mutagenesis of the EGFL7 expression construct was performed by site-directed mutagenesis with mutagenic oligos and mutants were identified by sequence analysis. The Myc-tagged mammalian expression vectors encoding murine Notch1 (pCS2+mN1FL6MT) and Jagged-1 (pCS2+Jag1-6MT) were kindly provided by Dr. Raphael Kopan (Washington University, St. Louis, MO). The Delta-like 1 (Dll1) and Delta-like 3 (Dll3) expression constructs were kindly provided by Dr. Geraldine Weinmaster (UCLA, Los Angles, CA). The Delta-like 4 (Dll4) expression construct was cloned by PCR amplification of murine Dll4 cDNA (clone ID# 86280 with oligos that introduced 5' kozak and EcoR1 sequences, and 3' SacII sequence. The PCR product was ligated into pcDNA3.1 Myc-his and sequenced in it's entirety. The Hes1 and Hes5-luciferase reporters were purchased from Addgene and consist of nucleotides -

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

Luciferase assays

For experiments examining the effect of RGD peptides, or WT vs RGE MAGP2/EGFL7 cDNAs on Hes-1 promoter activity, HMEC cells were seeded into 24-well plates at a density of 25,000 cells/well and transfected the following day with LT-1 liposomes containing various combinations of Hes-1 luciferase (200ng/well), MAGP2/EGFL7 cDNAs (WT or RGE) (100ng/well), and CMV-β-gal control plasmid (10ng/well). Where appropriate, cells were treated with 1, 10, or 100 μg/ml of soluble RGD (GCGYGRGDSPG) peptide (GenScript, Piscataway, NJ). 48 hours after transfection, cells were lysed in passive lysis buffer (Promega, Madison, WI) and luciferase and β-gal activities were measured on a Glo-Max luminometer. In experiments with β3 and β1 blocking antibodies, HMEC cells were transfected by electroporating 2,000,000 cells in PBS with 1.9 μg of luciferase reporter (Hes-1, Hes-5, or 4X-CSL luciferase) and 0.1 μg of CMV-β-gal reporter. Cells were pulsed in a nucleofector 2b (Lonza, Walkersville, MD) electroporator (2mm gap) set for "HUVEC", diluted into EGM2 growth media, and plated into 12 wells of a 24-well plate (250 μl/well) to which 0, 0.5, 1, or 2 μg/ml of β3 (7H2) or β1 (P5D2) blocking antibodies (Developmental Studies Hybridoma Bank, Iowa City, IA) were immediately added. Electroporated cells were collected 24 hours later and luciferase activity was measured as previously described [38].

Reverse transcription PCR

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

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

Recombinant protein and adhesion assay

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

Antibodies

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

Acknowledgements

We would like to thank Drs. Geraldine Weinmaster and Raphael Kopan for the donation of cDNAs encoding Notch receptors and ligands. This work was supported by funding from the NCI to ARA (1R15CA133829-01), the NIGMS to ARA (1R15GM102852-01), and the INBRE program (NIH Grant # P20 RR016454 (National Center for Research Resources) and # P20 GM103408 (National Institute of General Medical Sciences).

Author contributions were as follows. Conceived and designed the experiments: ARA. Performed the experiments: PD, KNB, RLR, AK, KL, KW, and ARA. Analyzed the data: PD, KNB, RLR, AK, KL, KW, and ARA. Contributed reagents/materials/analysis tools: ARA. Wrote the paper ARA.

References:

1. Giancotti, F.G. and E. Ruoslahti, Integrin signaling. Science, 1999. 285(5430): p. 1028-32.
2. Ruoslahti, E., RGD and other recognition sequences for integrins. Annu Rev Cell Dev Biol, 1996. 12: p. 697-715.
3. Harburger, D.S. and D.A. Calderwood, Integrin signalling at a glance. J Cell Sci, 2009. 122(Pt 2): p. 159-63.
4. Gibson, M.A., M.L. Finnis, J.S. Kumaratilake, and E.G. Cleary, Microfibril-associated glycoprotein-2 (MAGP-2) is specifically associated with fibrillin-containing microfibrils but exhibits more restricted patterns of tissue localization and developmental expression than its structural relative MAGP-1. J Histochem Cytochem, 1998. 46(8): p. 871-86.
5. Hanssen, E., F.H. Hew, E. Moore, and M.A. Gibson, MAGP-2 has multiple binding regions on fibrillins and has covalent periodic association with fibrillin-containing microfibrils. J Biol Chem, 2004. 279(28): p. 29185-94.
6. Gibson, M.A., D.I. Leavesley, and L.K. Ashman, Microfibril-associated glycoprotein-2 specifically interacts with a range of bovine and human cell types via alphaVbeta3 integrin. J Biol Chem, 1999. 274(19): p. 13060-5.
7. Albig, A.R., T.G. Roy, D.J. Becenti, and W.P. Schiemann, Transcriptome analysis of endothelial cell gene expression induced by growth on matrigel matrices: identification and characterization of MAGP-2 and lumican as novel regulators of angiogenesis. Angiogenesis, 2007. 10(3): p. 197-216.
8. Mok, S.C., T. Bonome, V. Vathipadiekal, A. Bell, M.E. Johnson, K.K. Wong, D.C. Park, K. Hao, D.K. Yip, H. Donninger, L. Ozbun, G. Samimi, J. Brady, M. Randonovich, C.A. Pise-Masison, J.C. Barrett, W.H. Wong, W.R. Welch, R.S. Berkowitz, and M.J. Birrer, A gene signature predictive for outcome in advanced ovarian cancer identifies a survival factor: microfibril-associated glycoprotein 2. Cancer Cell, 2009. 16(6): p. 521-32.
9. Nehring, L.C., A. Miyamoto, P.W. Hein, G. Weinmaster, and J.M. Shipley, The extracellular matrix protein MAGP-2 interacts with Jagged1 and induces its shedding from the cell surface. J Biol Chem, 2005. 280(21): p. 20349-55.

10. Miyamoto, A., R. Lau, P.W. Hein, J.M. Shipley, and G. Weinmaster, Microfibrillar proteins MAGP-1 and MAGP-2 induce Notch1 extracellular domain dissociation and receptor activation. J Biol Chem, 2006. 281(15): p. 10089-97.
11. Albig, A.R., D.J. Becenti, T.G. Roy, and W.P. Schiemann, Microfibril-associate glycoprotein-2 (MAGP-2) promotes angiogenic cell sprouting by blocking notch signaling in endothelial cells. Microvasc Res, 2008.
12. Nikolic, I., N.D. Stankovic, F. Bicker, J. Meister, H. Braun, K. Awwad, J. Baumgart, K. Simon, S.C. Thal, C. Patra, P.N. Harter, K.H. Plate, F.B. Engel, S. Dimmeler, J.A. Eble, M. Mittelbronn, M.K. Schafer, B. Jungblut, E. Chavakis, I. Fleming, and M.H. Schmidt, EGFL7 ligates alphavbeta3 integrin to enhance vessel formation. Blood, 2013. 121(15): p. 3041-50.
13. Schmidt, M.H., F. Bicker, I. Nikolic, J. Meister, T. Babuke, S. Picuric, W. Muller-Esterl, K.H. Plate, and I. Dikic, Epidermal growth factor-like domain 7 (EGFL7) modulates Notch signalling and affects neural stem cell renewal. Nat Cell Biol, 2009. 11(7): p. 873-80.
14. Nichol, D., C. Shawber, M.J. Fitch, K. Bambino, A. Sharma, J. Kitajewski, and H. Stuhlmann, Impaired angiogenesis and altered Notch signaling in mice overexpressing endothelial Egfl7. Blood, 2010. 116(26): p. 6133-43.
15. Pierschbacher, M.D. and E. Ruoslahti, Variants of the cell recognition site of fibronectin that retain attachment-promoting activity. Proc Natl Acad Sci U S A, 1984. 81(19): p. 5985-8.
16. Kutok, J.L. and B.S. Coller, Partial inhibition of platelet aggregation and fibrinogen binding by a murine monoclonal antibody to GPIIIa: requirement for antibody bivalency. Thromb Haemost, 1994. 72(6): p. 964-72.
17. Wayner, E.A., S.G. Gil, G.F. Murphy, M.S. Wilke, and W.G. Carter, Epiligrin, a component of epithelial basement membranes, is an adhesive ligand for alpha 3 beta 1 positive T lymphocytes. J Cell Biol, 1993. 121(5): p. 1141-52.
18. Donovan, L.J., S.E. Cha, A.R. Yale, S. Dreikorn, and A. Miyamoto, Identification of a functional proprotein convertase cleavage site in microfibril-associated glycoprotein 2. Matrix Biol, 2013. 32(2): p. 117-22.
19. Weijers, E.M., M.H. van Wijhe, L. Joosten, A.J. Horrevoets, M.P. de Maat, V.W. van Hinsbergh, and P. Koolwijk, Molecular weight fibrinogen variants alter gene expression and functional characteristics of human endothelial cells. J Thromb Haemost, 2010. 8(12): p. 2800-9.
20. Estrach, S., L. Cailleteau, C.A. Franco, H. Gerhardt, C. Stefani, E. Lemichez, L. Gagnoux-Palacios, G. Meneguzzi, and A. Mettouchi, Laminin-binding integrins induce Dll4 expression and Notch signaling in endothelial cells. Circ Res, 2011. 109(2): p. 172-82.
21. Stenzel, D., C.A. Franco, S. Estrach, A. Mettouchi, D. Sauvaget, I. Rosewell, A. Schertel, H. Armer, A. Domogatskaya, S. Rodin, K. Tryggvason, L. Collinson, L. Sorokin, and H. Gerhardt, Endothelial basement membrane limits tip cell formation by inducing Dll4/Notch signalling in vivo. EMBO Rep, 2011.
22. Suh, H.N. and H.J. Han, Collagen I regulates the self-renewal of mouse embryonic stem cells through alpha2beta1 integrin- and DDR1-dependent Bmi-1. J Cell Physiol, 2011. 226(12): p. 3422-32.

23. Mo, J.S., M.Y. Kim, S.O. Han, I.S. Kim, E.J. Ann, K.S. Lee, M.S. Seo, J.Y. Kim, S.C. Lee, J.W. Park, E.J. Choi, J.Y. Seong, C.O. Joe, R. Faessler, and H.S. Park, Integrin-linked kinase controls Notch1 signaling by down-regulation of protein stability through Fbw7 ubiquitin ligase. Mol Cell Biol, 2007. 27(15): p. 5565-74.
24. Ma, Y.C., C. Shi, Y.N. Zhang, L.G. Wang, H. Liu, H.T. Jia, Y.X. Zhang, F.H. Sarkar, and Z.S. Wang, The tyrosine kinase c-Src directly mediates growth factor-induced Notch-1 and Furin interaction and Notch-1 activation in pancreatic cancer cells. PLoS One, 2012. 7(3): p. e33414.
25. Ho, D.M., S.K. Pallavi, and S. Artavanis-Tsakonas, The Notch-mediated hyperplasia circuitry in Drosophila reveals a Src-JNK signaling axis. Elife, 2015. 4: p. e05996.
26. Roca-Cusachs, P., N.C. Gauthier, A. Del Rio, and M.P. Sheetz, Clustering of alpha(5)beta(1) integrins determines adhesion strength whereas alpha(v)beta(3) and talin enable mechanotransduction. Proc Natl Acad Sci U S A, 2009. 106(38): p. 16245-50.
27. Lin, G.L., D.M. Cohen, R.A. Desai, M.T. Breckenridge, L. Gao, M.J. Humphries, and C.S. Chen, Activation of beta 1 but not beta 3 integrin increases cell traction forces. FEBS Lett, 2013. 587(6): p. 763-9.
28. Leavesley, D.I., M.A. Schwartz, M. Rosenfeld, and D.A. Cheresh, Integrin beta 1- and beta 3-mediated endothelial cell migration is triggered through distinct signaling mechanisms. J Cell Biol, 1993. 121(1): p. 163-70.
29. LeBleu, V.S., B. Macdonald, and R. Kalluri, Structure and function of basement membranes. Exp Biol Med (Maywood), 2007. 232(9): p. 1121-9.
30. Midwood, K.S., L.V. Williams, and J.E. Schwarzbauer, Tissue repair and the dynamics of the extracellular matrix. Int J Biochem Cell Biol, 2004. 36(6): p. 1031-7.
31. Sakamoto, K., S. Yamaguchi, R. Ando, A. Miyawaki, Y. Kabasawa, M. Takagi, C.L. Li, B. Perbal, and K. Katsube, The nephroblastoma overexpressed gene (NOV/ccn3) protein associates with Notch1 extracellular domain and inhibits myoblast differentiation via Notch signaling pathway. J Biol Chem, 2002. 277(33): p. 29399-405.
32. Lin, C.G., C.C. Chen, S.J. Leu, T.M. Grzeszkiewicz, and L.F. Lau, Integrin-dependent functions of the angiogenic inducer NOV (CCN3): implication in wound healing. J Biol Chem, 2005. 280(9): p. 8229-37.
33. Keilani, S. and K. Sugaya, Reelin induces a radial glial phenotype in human neural progenitor cells by activation of Notch-1. BMC Dev Biol, 2008. 8: p. 69.
34. Dulabon, L., E.C. Olson, M.G. Taglienti, S. Eisenhuth, B. McGrath, C.A. Walsh, J.A. Kreidberg, and E.S. Anton, Reelin binds alpha3beta1 integrin and inhibits neuronal migration. Neuron, 2000. 27(1): p. 33-44.
35. Campos, L.S., L. Decker, V. Taylor, and W. Skarnes, Notch, epidermal growth factor receptor, and beta1-integrin pathways are coordinated in neural stem cells. J Biol Chem, 2006. 281(8): p. 5300-9.
36. Leong, K.G., X. Hu, L. Li, M. Noseda, B. Larrivee, C. Hull, L. Hood, F. Wong, and A. Karsan, Activated Notch4 inhibits angiogenesis: role of beta 1-integrin activation. Mol Cell Biol, 2002. 22(8): p. 2830-41.

633 37. Hodkinson, P.S., P.A. Elliott, Y. Lad, B.J. McHugh, A.C. MacKinnon, C. Haslett,
634 and T. Sethi, Mammalian NOTCH-1 activates beta1 integrins via the small
635 GTPase R-Ras. J Biol Chem, 2007. 282(39): p. 28991-9001.
636 38. Sharma, B. and A.R. Albig, Matrix Gla protein reinforces angiogenic resolution.
637 Microvasc Res, 2012.
638

639

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	TATGCTCACAGTGCTGGCCATAGT
AA21	Mouse Dll1 fwd RT-PCR	AATCTGTCTGCCAGGGTGTGATGA
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	TCCATGACAACTTTGGTATTCGT
AA104	Human GAPDH rev RT-PCR	AGTAGAGGCAGGGATGATGTT
KW181	Human Int α2 fwd RT-PCR	TCTCAGAAGTCTGTTGCCTGCGAT
KW182	Human Int α2 rev RT-PCR	ACTGATGTCACCAGCCTTGTCTGT
KW183	Human Int α5 fwd RT-PCR	TCGAGACAAACTCTCGCCGATTCA
KW184	Human Int α5rev RT-PCR	TCACGGCAAAGTAGTCACAGCTCA
KW185	Human Int αV fwd RT-PCR	AAGATGTTGGGCCAGTTGTTCAGC
KW186	Human Int αV rev RT-PCR	AGCAACTCCACAACCCAAAGTGTG
KW187	Human Int β1 fwd RT-PCR	TCTGCGGACAGTGTGTTTGTAGGA
KW188	Human Int β1 rev RT-PCR	AATGGGACACAGGATCAGGTTGGA
KW189	Human Int β3 fwd RT-PCR	CCCACTTGGCATCATTCACAGCAA
KW190	Human Int β3 rev RT-PCR	AAGAGACCTTCAAGACTGGCTGCT
AK366	Human Int β8 fwd RT-PCR	AGCAAATTGGCAGGCATAGTGGTG
AK367	Human Int β8 rev RT-PCR	TCGTCACGTTTCTGCATCCTTCCA
AK368	Human Int β6 fwd RT-PCR	AGCAAATTGGCAGGCATAGTGGTG
AK369	Human Int β6 rev RT-PCR	AGACATCTCTTTGGAAAGCCGGGA

AK370	Human Int α8 fwd RT-PCR	AAGGGATTTCGACCACTGAGCTGT
AK371	Human Int α8 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

641