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"Efecto de la sobrexpresión del péptido ReNeg-AID derivado de la proteína Hairless de *Drosophila melagonaster* en células de cáncer de mama"

Tesis

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Contenido:

Contenido

I. RESUMEN GENERAL........1

II. SUMMARY........2

III. INTRODUCCIÓN GENERAL........3

ORÍGENES........3
MECANISMOS Y CIRCUITOS DE COMUNICACIÓN DE LA VÍA NOTCH........6
COMPONENTES DE LA VÍA NOTCH........8
ACTIVACIÓN Y REPRESIÓN DE LA VÍA NOTCH........11
CAPÍTULO 1: NOTCH Y CÁNCER DE MAMA........15

IV. HIPÓTESIS........28

V. OBJETIVOS........29

VI. RESULTADOS........30

CAPÍTULO 2: RENEG-AID Y LA INDUCCIÓN DE LA REGULACIÓN NEGATIVA DE VN........30
CAPÍTULO 3: VÍA NO CANÓNICA DE NOTCH........63

VII. CONCLUSIONES Y DISCUSIÓN GENERAL........81

VIII. PERSPECTIVAS Y RECOMENDACIONES........83

IX. BIBLIOGRAFÍA COMPLEMENTARIA........85

I. Resumen General

La vía de señalización Notch (vN) es un circuito molecular de comunicación ancestral entre célula-célula que controla procesos clave para mantener la homeostasis de los metazoarios; como la diferenciación y organogénesis en etapas tempranas del desarrollo embrionario, la especialización e identidad celular en organismos adultos, regula procesos críticos en la transición del ciclo celular en la fase G1/S, participa en la comunicación cruzada con otras vías de señalización celular como Wingless (WNT) y Hedgehog (Hh) y participa en la regulación de eventos apoptóticos. En mamíferos la vía Notch es mediada por la interacción de 4 receptores (Notch1-4) y 4 ligandos (Delta1, 4; Jagged1, 2) y varias proteínas reguladoras que pueden ser específicas en cada especie. Dentro de estas proteínas se encuentra Hairless (H), un regulador negativo de la vN presente en insectos y moluscos. Por experimentos de afinidad y unión proteína-proteína se ha comprobado que la proteína H es capaz de regular negativamente el complejo activador Notch/CSL (CBF-1, Su[H], Lag1) en mamíferos. La desregularización o sobrexpresión constitutiva de la vN se ha relacionado con la aparición y mantenimiento de varios tipos de cánceres humanos. En el cáncer de mama la activación constitutiva de la vN juega un rol principal en la progresión aberrante del ciclo celular, promueve una deficiente e incompleta diferenciación celular e inhibe los mecanismos de apoptosis. Dada la gran conservación evolutiva entre las proteínas de la vN en metazoarios; se diseñó un plásmido que expresa un péptido derivado del dominio que usa la proteína H de *Drosophila melanogaster* para unirse a la proteína CSL y regular negativamente la vN. Este péptido (llamado, ReNeg-AID), fue usado para regular de manera negativa la sobrexpresión de los receptores Notch en células epiteliales de cáncer de mama humano. Los principales resultados mostraron una disminución en la proliferación y un arresto del ciclo celular entre la transición de la fase G1 a S, una regulación negativa directa del receptor principal Notch-1, un intercambio en la comunicación cruzada entre la vía WNT y Hh y un mecanismo que inhibe la iniciación diferencial de la transición de epitelio a mesénquima en tejido altamente diferenciado.

Palabras clave: Regulación genética, Regulación negativa, Notch, Cáncer de mama, Notch-1.

II. Summary

The Notch signaling pathway (NSP) is an ancestral cell-cell communication circuit that controls key processes to maintain metazoan homeostasis such as differentiation and organogenesis in early stages of embryonic development, cell identity and specialization in adult organisms. NSP regulates critical processes in the transition of the cell cycle in the G1 / S phase, participates in cross-communication with other cell signaling pathways such as Wingless (WNT) and Hedgehog (Hh) and participates in the regulation of apoptotic events. In mammals the NSP is mediated by the interaction of 4 receptors (Notch1-4) and 4 ligands (Delta1, 4; Jagged1, 2) and several regulatory proteins that may be species-specific. Among these proteins is Hairless (H), a negative regulator of NSP present in insects and mollusks. Hairless protein has been shown to be capable of downregulating the Notch/CSL activator complex in mammals by protein-protein binding and affinity experiments.

The deregulation or constitutive overexpression of the NSP has been related to the appearance and maintenance of various types of human cancers. In breast cancer, constitutive activation of the NSP plays a major role in aberrant cell cycle progression, promotes poor and incomplete cell differentiation, and inhibits apoptosis mechanisms. Given the great evolutionary conservation among NSP proteins in metazoans; a plasmid expressing a peptide derived from the *Drosophila melanogaster* Hairless protein domain was designed and used to bind to the CSL protein and downregulate NSP. This peptide (called ReNeg-AID) was used to negatively regulate the overexpression of Notch receptors in human breast cancer epithelial cells. The main results showed a decrease in proliferation and an arrest of the cell cycle between the transition from the G1 to S phase, a direct negative regulation of the main Notch-1 receptor, an interchange in the cross communication between the WNT and Hh pathway and a mechanism that inhibits the differential initiation of the epithelium to mesenchyme transition (EMT) in highly differentiated tissue.

Keywords: Genetic regulation, negative regulation, Notch, Breast cancer, Notch-1

III. Introducción General

Orígenes

La comunicación puede ser entendida como un proceso que transmite, procesa y comparte información que será empleada en la respuesta a estímulos, censar el ambiente o simplemente estar en constante equilibrio con el entorno. Este flujo de información está presente en todos los niveles de la naturaleza, desde las fuerzas elementales, la interacción de los átomos y moléculas y por supuesto con las redes complejas de convivencia que existen entre las entidades biológicas como virus, células y organismos multicelulares. Esta transferencia de información necesita de dos componentes fundamentales, una fuente emisora y un receptor de la información. Con el paso del tiempo la naturaleza, con la ayuda de los mecanismos evolutivos inherentes a ella, ha desarrollado intrincadas formas de transmitir y permutar la información de los seres vivos, desarrollando las vías de señalización celular.

Las vías de señalización celular tienen como objetivo la regulación positiva o negativa de genes, además, mantienen un equilibrio en esta expresión genética respondiendo a los estímulos interno y/o externos de la célula. Varias vías de señalización presentan una comunicación cruzada entre ellas, como resultado, esto les confiere un mayor control entre la regulación positiva o negativa de los genes; mediada por este complejo flujo de información

En el reino animal existen alrededor de 20 vías de señalización que se encargan de llevar acabo la mayoría de los procesos críticos de comunicación célula-célula, pero solo 7 actúan en la diferenciación celular, decisión de linajes celulares y los patrones estructurales que forman los tejidos en un organismo; estas vías son: Factor transformante beta (TGF-β), Receptores Tirosina Quinasa (RTK), Jak/STAT, Receptor Nuclear Hormonal (NHR), Wnt, Hh y vN. (1,2).

Todas estas vías de señalización funcionan de forma dependiente de sus factores de transcripción bajo una regulación retroalimentada; esto quiere decir que existen circuitos comunicativos entre ellas para regular la expresión directa e indirecta (vía canónica y no canónica, respectivamente) de sus propios genes blanco. Estas vías de

señalización tambien participan fuera del estadio embrionario y de diferenciación en procesos vitales para la célula, por ejemplo, controlando el progreso de las fases del ciclo celular, intervienen en el crecimiento y apoptosis celular, migración y polarización celular. El resultado final de esta interconexión a nivel de la maquinaria molecular que caracteriza a las vías de señalización celular se basa en mantener el estado de homeostasis el mayor tiempo posible y recuperarlo si ha sido perturbado por algun agente externo o interno en el contexto celular (3).

La vN es un circuito de comunicación celular donde es necesario el contacto físico entre membranas celulares adyacentes (por medio de un ligando y un receptor) para efectuar su actividad con resultados distintos dependiendo del estado de desarrollo de la célula y de la configuración espaciotemporal de la misma; repercutiendo directamente en la homeostasis del organismo (1-3).

El estudio de la vN comenzó hace más de un siglo con los trabajos realizados por Morgan y Bridgeds en 1916 y por Mohr en 1919; autores que encontraron una relación directa entre malformaciones en las alas, torsos, ojos y cabeza de *D. melanogaster* y la mutación de ciertas zonas en específico del cromosoma sexual de la mosca de la fruta. Morgan y Mohr nombraron a estas zonas como “Factores X” y concluyeron que su ausencia total o parcial podía provocar estos efectos morfológicos en el insecto, en algunos casos ciertos factores X resultaban letales en un estadio larvario, abortando el plan de la organogénesis de la mosca de la fruta. Durante los años consecuentes cesó la investigación en este campo (2).

Poulson, en el año 1940, estableció una de las primeras relaciones entre las capas germinales del desarrollo embrionario y los factores X que describieron Morgan y Mohr. Encontró que estos factores X, cuando eran silenciados o mutados, provocaban que la capa germinativa primitiva, el blastodermo, no lograra ser biológicamente funcional en ciertos momentos del desarrollo e impedía que las siguientes capas germinales embrionarias emergieran, y en algunos casos el nuevo plan para crear un organismo fuera abortado (4).

En los años consecuentes la investigación en el campo de la embriogénesis retomó fuerza y aun se siguen encontrando puntos clave para su compresión. Durante este

periodo se ha logrado un mayor entendimiento de como estos factores X (vías de señalización celular) relacionados con la embriogénesis y la organogénesis, son capaces controlar la expresión y represión de genes para forjar y crear complejas redes de información que darán forma a los patrones fisiológicos de un organismo para que sea completamente funcional (5).

El origen de los mecanismos de acción de las vías de transducción de señales es aún incierto, sin embargo, se ha esclarecido el origen de ciertos funcionamientos moleculares relacionados directamente con los dominios estructurales que conforman a las proteínas de las vías de señalización. Un dominio proteico es una secuencia de aminoácidos relacionados con una función física estructural que se conserva durante generaciones o clados de especies. Se ha especulado que los primeros dominios proteicos de la vN ya existían en los urmetazoarios (último ancestro de los metazoarios), sin embargo, el proceso evolutivo de esta vía se dio gracias a una diversidad de mecanismos moleculares donde se conservaron, eliminaron y agregaron dominios proteicos durante la evolución del reino eucariota (5,6).

La vN es considerada una sinapomorfía que dio origen a los metazoarios y eumetazoarios. Aunque varios componentes de la vN han sido encontrados en otras ramas evolutivas como plantas, levaduras y algunos hongos, los receptores y ligandos de la vN son exclusivos de los organismos bilaterales. Organismos que pueden ser seccionados anatómicamente en tres planos anatómicos; sagital, transversal y coronal y que dan origen a los ejes fisiológicos; anterior, posterior, dorsal, ventral, proximal y distal (7,8).

Mecanismos y circuitos de comunicación de la vía Notch

En los metazoarios y eumetazoarios la vN es un componente clave para el desarrollo correcto de un organismo en estadio embrionario y adulto. La vN es la encargada de dar origen a la primera oleada de diferenciación celular cuando los gametos fusionados han alcanzado la octava división celular, promoviendo un patrón de expresión y represión de genes que darán origen a proteínas de la familia bHLH (basic Helix-Loop-Helix, por sus siglas en inglés); estas proteínas bHLH son factores de transcripción que activarán o regularán negativamente otras vías de señalización involucradas en establecer los patrones celulares entre diferentes sistemas y órganos, como es el caso de la familia de genes *HOX*, *HES* y *HER* (9,10).

La vN posee 3 formas principales para dar origen a los patrones estructurales en un organismo, orquestando la polarización de ejes (anterior, posterior, izquierda, derecha, dorsal y ventral) de los organismos bilaterales:

I) La señalización de inductiva, que da origen a las células que limitan y comunican diferentes órganos o sistemas, p. ej. el tejido epitelial o endotelial.

II) La señalización inhibitoria lateral, que da origen a los linajes celulares que pertenecen a un mismo órgano o sistema, p. ej. la red neuronal.

III) La señalización por decisión de linajes que intercambia entre la activación y represión de la vía Notch en cada una de las nuevas células hijas, p. ej. el sistema inmune.

En mamíferos se sabe que la vN participa puntualmente en procesos de diferenciación como la somitogénesis, neurogénesis, gliogénesis, angiogénesis, diferenciación cardiovascular, diferenciación en páncreas, estómago, intestinos, huesos y sistema respiratorio. No obstante, y debido a que regula y participa en varios procesos vitales de la célula, cuando esta vía presenta alteraciones en sus patrones de expresión de genes y comunicación cruzada con otras vías, puede ocurrir la aparición de enfermedades degenerativas como el Alzheimer o Parkinson, mal funcionamiento de órganos, pérdida de la identidad celular y, en los casos más severos, la aparición y mantenimiento de varios tipos de cáncer (11,12).

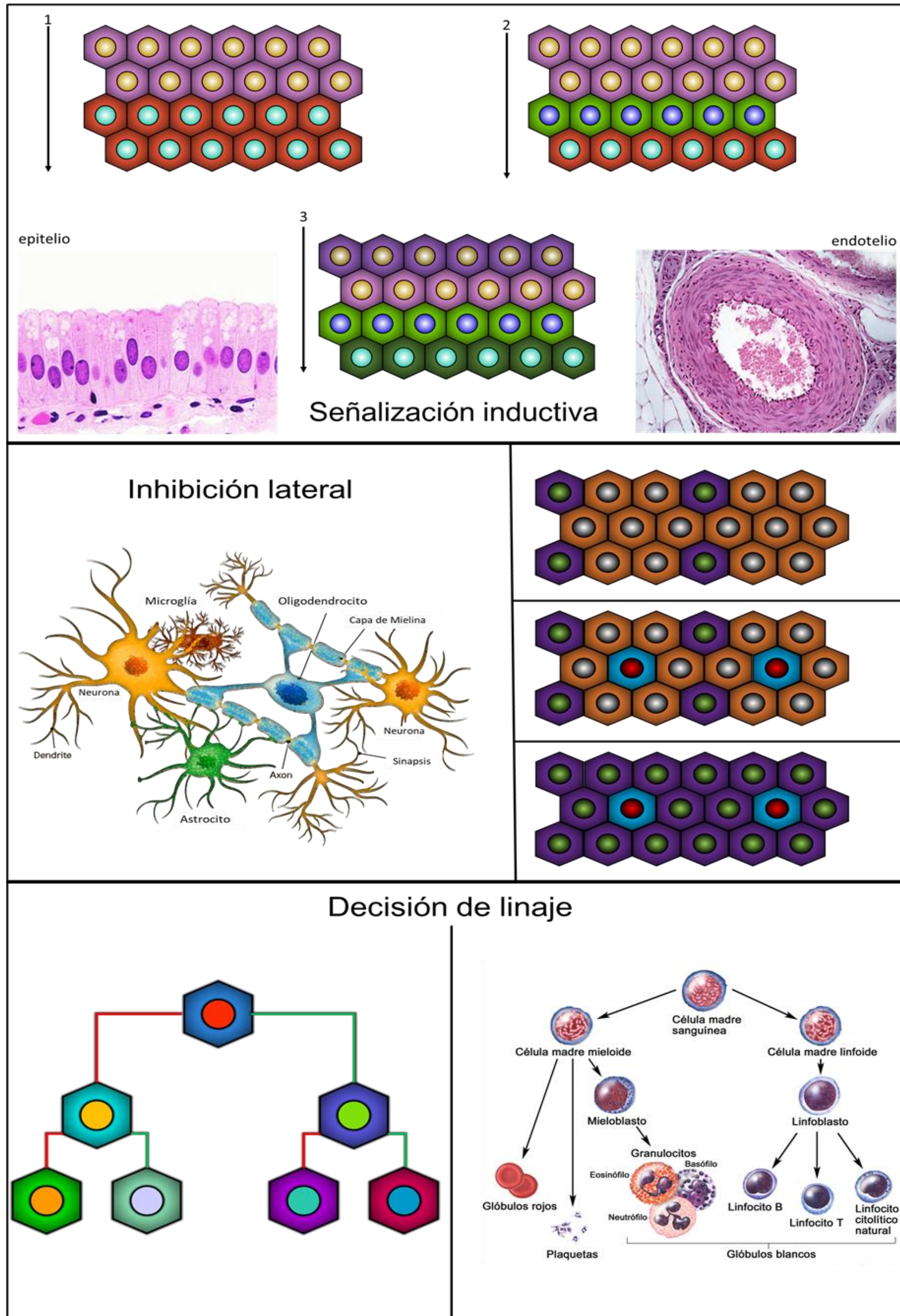

Figura 1. Patrones mecanísticos de la vN. ***Señalización inductiva***: las flechas indican la dirección de la activación de la vN. **Inhibición lateral**: la diferenciación se da entre células adyacentes en una dirección radial. **Decisión de linaje**: la diferenciación se alterna entre activación de la vN (línea verde) y represión de la vN (línea roja).

Componentes de la vía Notch

La vN está constituida por proteínas altamente conservadas a nivel de módulos de dominios proteicos, y ocurre tanto en los metazoarios como en los eumetazoarios. Las proteínas que componen la vN se pueden separar en dos grupos. En el primer grupo se incluyen las proteínas que funcionan como ligandos, receptores, represores, co-represores y factores de transcripción, las cuales constituyen el núcleo de la vía y son las encargadas de transferir la información de la señal (Tabla 1) (13,14).

En el segundo grupo se incluyen a las proteínas reguladoras que modulan la duración e intensidad de la señalización y pueden ser proteasas y metaloproteasas, ubiquitin-ligasas, endoproteasas y O-fucosilasas (Tabla 2); haciendo modificaciones directas a las proteínas integrantes del primer grupo. Las proteínas Notch (familia LIN-12/Notch) funcionan como receptores para la familia de ligandos **D**elta, **S**errate y **L**ag-1 (familia DSL). En mamíferos se conocen cuatro diferentes receptores de la vN (Notch1, Notch2, Notch3 y Notch4) y cinco ligandos (Delta-like1, Delta-like3 y Delta-like4, Jagged1 y Jagged2) y un solo factor de transcripción llamado CSL [**C**BF-1/**S**u(H)/**L**ag-2; mamíferos/insectos/nemátodos, respectivamente]. La comunicación entre ligandos y receptores debe ser precisa, ordenada y controlada para que la célula responda adecuadamente al contexto en el que se encuentra y así asegurar su correcto funcionamiento al censar el medio que la rodea (15,16).

Las proteínas clave que conforman a la vN involucradas en la regulación positiva tienen un alto nivel de conservación de dominios funcionales entre las diferentes especies en donde han sido descrita, lo cual posibilita que algunas proteínas puedan interaccionar de forma interespecie. Una excepción a esta característica es la proteína **H**, que solo ha sido descrita en insectos y moluscos y es indispensable en la regulación negativa de la vN en *D. melanogaster*. La proteína H actúa uniéndose al factor transcripcional Supresor de Hairless [Su(H)], gracias a su dominio de unión ubicado entre las posiciones de aminoácidos 1021 a 2444 del gen que codifica para la proteína H, tambien se une a los co-represores Groucho (Gro) y la proteína de unión a C-terminal (CtBP, por sus siglas en inglés). Hairless forma complejos de represión que impiden la activación de genes bajo el control de la vN, haciendo posible un cambio en el patrón de expresión de genes en sincronía con el contexto celular. La expresión

selectiva de dichos genes estimula y promueve que la célula receptora se dirija hacia un destino celular distinto al de la célula que envía la señal durante el funcionamiento canónico de la vN (15,17).

Tabla 1. Proteínas núcleo de la vía Notch			
Gen	**Proteina**	**Función**	**Dominios conservados**
DLL1	**Delta 1**	**Ligando**	**PS - MNLL - DSL - EGF - TM**
DLL2	**Delta 2**		
DLL3	**Delta 3**		
JAG1	**Jagged 1**		**PS - MNLL - DSL - EGF - VWC - TM**
JAG2	**Jagged 2**		
Notch1	**Notch-1**	**Receptor**	**PS - EGF - LNR - HD - TM - NLS - ANK - NLS - NCR - TAD - PEST**
Notch2	**Notch-2**		
Notch3	**Notch-3**		
Notch4	**Notch-4**		**PS - EGF - LNR - HD - TM - NLS - ANK - PEST**
MAML	**Mastermind-like**	**co-activador**	**MAML-1**
ctbp	**CtBP**	**co-regulador**	**NAD-P - D-isomer**
CBF1	**CSL**	**Factor Transcripcional**	**NTD - BTD - CTD**
SuH			
LAG1			
SHARP/SPEN like	**SPEN - SHARP**	**Represor**	**RBD - RRM - SPOC - SHARP**
Hairless D.m.	**Hairless *D.m.***		**PRD - CID**

Tabla 1. Proteínas núcleo de la vN. Los ligandos Delta conservan los dominios **PS** (Peptido señal N-terminal), **MNLL** (dominio de ligando-Notch), **DSL** (dominio Delta-Serrate-Lag), **EGF** (dominio de repetidos de factor de crecimiento epidérmico) y **TM** (dominio transmembranal). Los ligandos Jagged conservan los mismos dominios presentes en las proteínas Delta, pero contienen el modulo **VWL** (dominio tipo C de Von Willebrand). Los receptores de la vN conservan dominios entre si como **PS**, **EGF**, **LNR** (dominio regulador Lin-Notch), **HD** (dominio Heterodimérico), **TM**, **NLS** (dominio de secuencia de localización nuclear), **ANK** (dominio de repetido de ankirinas), **NCR** (dominio de represión Notch rico en cisteínas), **TAD** (dominio Trans-activador) y **PEST** (dominio de degradación). El coactivador Mastermind conserva el módulo **MAML-1** (dominio Mastermind-1) y el co-regulador CtBP conserva dos dominios; **NAD-P** (dominio de unión Oxidorreductasa NAD) y **D-isomer** (dominio de isomerazión D). Los factores de transcripción conservan los dominios **NTD** (dominio N-terminal) **BTD** (dominio de 3 láminas beta-plegadas) y **CTD** (dominio C-terminal). Los represores de la vN aunque evolutivamente tomaron rutas distintas siguen conservando dominios entre ellos como en la familia SHARP/SPEN que conservan **RBD** (dominio de unión a ARN), **RRM** (dominio de reconocimiento a ARN), **SPOC** (dominio parálogo/ortólogo de SPEN C-terminal), **SHARP** (dominio regulador de HDAC’s).

El represor Hairless *D.m.* (*Drosophila melanogaster*) contiene dos dominios que se han logrado reconocer, **PRD** (dominio de repetidos de motivos de histidina-cisteína-prolina) y **CID** (dominio de reconocimiento a CSL).

<table>
<tr><th colspan="4">Tabla 2. Proteínas moduladoras de la vía Notch</th></tr>
<tr><th>Gen</th><th>Proteína</th><th>Función</th><th>Dominios modulados</th></tr>
<tr><td>POFUT1</td><td>O-Fucosiltransferasa 1</td><td>O-fucosilación</td><td>Receptor Notch/EGF</td></tr>
<tr><td>MFNG</td><td>Manic Fringe</td><td rowspan="3">N-acetilglucosaminiltransferasa</td><td rowspan="3">Receptor Notch/EGF-HD</td></tr>
<tr><td>LFNG</td><td>Lunatic Fringe</td></tr>
<tr><td>RFNG</td><td>Radical Fringe</td></tr>
<tr><td>ADAM10</td><td>ADAM Metalopeptidasa domain 10</td><td rowspan="2">Metaloproteasa</td><td rowspan="2">Receptor Notch/NCR-HD</td></tr>
<tr><td>ADAM17</td><td>ADAM Metalopeptidasa domain 17</td></tr>
<tr><td>PSENEN</td><td rowspan="5">Complejo proteico γ-secretasa</td><td rowspan="5">Endoproteasa</td><td rowspan="3">Receptor Notch/HD-TM</td></tr>
<tr><td>PSEN1</td></tr>
<tr><td>PSEN2</td></tr>
<tr><td>APH1A</td><td rowspan="2">Ligando DSL/TM</td></tr>
<tr><td>NCSTN</td></tr>
</table>

Tabla 2. Proteínas moduladoras de la vN. La mayoría de los moduladores de la vN actúan sobre los receptores en los dominios EGF que aportan la especificidad de unión a sus ligandos y sobre los dominios que exponen los sitios proteolíticos del mismo receptor Notch.

Activación y represión de la vía Notch

La vN comienza con pro-Notch, una proteína homodimérica que es procesada proteolíticamente por una proteína Furina-convertasa en la red del aparato de Golgi, haciendo el primer corte proteolítico en el dominio TM del receptor (S1) que convierte a pro-Notch en una proteína transmembranal de clase I heterodimérica con dos dominios [dominio extracelular de Notch (DEN) y dominio intracelular de Notch (DIN)] (Figura 2); una vez se ha realizado el primer corte proteolítico S1, la familia de proteínas Fringe y O-fut hacen modificaciones postraduccionales de glucosilación, O-glucosilación y N-acetilglucosilación en el dominio de repetidos de EGF de DEN; este procesamiento por Fringe y O-Fut le otorga a los receptores Notch la especificidad de ser reconocido por los diferentes ligandos DSL. Una vez realizadas las modificaciones postraduccionales el receptor Notch, es llevado a la membrana celular con la ayuda del transporte vesicular del aparato de Golgi (18,19).

Una vez que el receptor Notch se encuentra anclado en la membrana celular es reconocido por el ligando DSL (Figura 3). Los dominios EGF del receptor y del ligando forman una estructura proteica que ejercerán una fuerza mecánica de tensión en el dominio EGF de DEN ocasionando que el dominio HD del receptor sufra una modificación física y deje expuesto un motivo rico en cisteínas que será reconocido por las metaloproteasas ADAM10 y ADAM17; este reconocimiento promoverá el segundo corte proteolítico (S2), haciendo que el ligando DSL separe y endocite el dominio DEN en la célula que ha mandado la señalización (20,21).

El dominio DIN aún continúa anclado a la membrana celular, sin embargo, gracias a la pérdida de su dominio DEN el complejo proteico de las γ-secretasas reconocen el dominio TM expuesto del receptor haciendo un tercer corte proteolítico (S3) que separa el dominio DIN de TM y de la membrana celular; este dominio (TM) queda anclado dentro de la membrana celular y es llamado β-notch, aún se desconoce la función exacta de este péptido, pero se ha relacionado con procesos secundarios de regulación de la propia vN (22,23).

El corte proteolítico realizado por el complejo de las γ-secretasas libera el dominio DIN de la membrana celular y es un evento crucial en la señalización de la vN, ya que es

después de este paso donde ocurren la mayoría de los procesos regulatorios de la expresión. Cuando el dominio DIN del receptor Notch es liberado de la membrana celular comienza un viaje hacia el núcleo de la célula. Existen varias proteínas involucradas en el transporte proteico del receptor Notch como Deltex y FBXW7 que promueven la regulación positiva o negativa del tráfico de Notch hacia el núcleo, o Numb y Su(dx) que promueven la regulación negativa de Notch iniciando procesos de desintegración proteica vacuolar por proteasomas (12,24).

En el transcurso desde la membrana celular al núcleo el dominio DIN de queda expuesto para ser reconocido por los circuitos de otras vías de señalización, por ejemplo; el dominio DIN puede interactuar físicamente con elementos pertenecientes a la vía Hh como el factor HIIF-1α, GLI1 y GLI2 promoviendo que aumente la vida media del receptor Notch en citoplasma, asi mismo, el dominio DIN interactúa físicamente con elementos que pertenecen a la vía WNT como β-catenina que promueve positivamente que la cantidad de dominios DIN aumenten su concentración en núcleo. El dominio DIN con ayuda de sus dominios NLS de manera canónica durante su viaje hacia el núcleo forma complejos proteicos de represión con Hairless (no en mamíferos) SMRT, SHARP, MINT, SPEN y KyoT2, o forma complejos de activación con Mastermind y CtBP; ambos complejos, represión y activación se unen de manera dependiente al único factor transcripcional CSL y este a su vez se une al ADN en la secuencia conservada 5´-GTGGGAA-3´ de los genes diana. La dinámica entre la activación y represión de la vN estimula cambios epigenéticos mediados por acetilasas o deacetilasas de histonas que concluirán en la expresión o represión de genes de la familia *HES* o *HER-HERP* encargados de la diferenciación celular, genes involucrados en el ciclo celular como la *Ciclina D* y *E*, genes involucrados la autorregulación de la propia vN como sus propios receptores, ligandos y factores transcripcionales asi como varios genes que ejercen una función en la apoptosis y con la comunicación cruzada con la vía Hh y WNT (10,15,25).

Por la alta conservación entre los elementos de activación de la vN y los dominios que conforman a sus proteínas núcleo se diseñó un péptido (ReNeg-AID) derivado de la proteína Hairless de *D. melanogaster* capaz de unirse al factor transcripcional CSL de

mamíferos gracias a su dominio CID y ejercer una regulación negativa al competir contra el dominio DIN en células donde la vN sufre una expresión constitutiva como en varios tipos de cáncer; con el fin de explorar los efectos que serán promovidos por la inducción de un contexto celular que regule negativamente la expresión de genes dependientes de la sobrexpresión constitutiva de la vN (25).

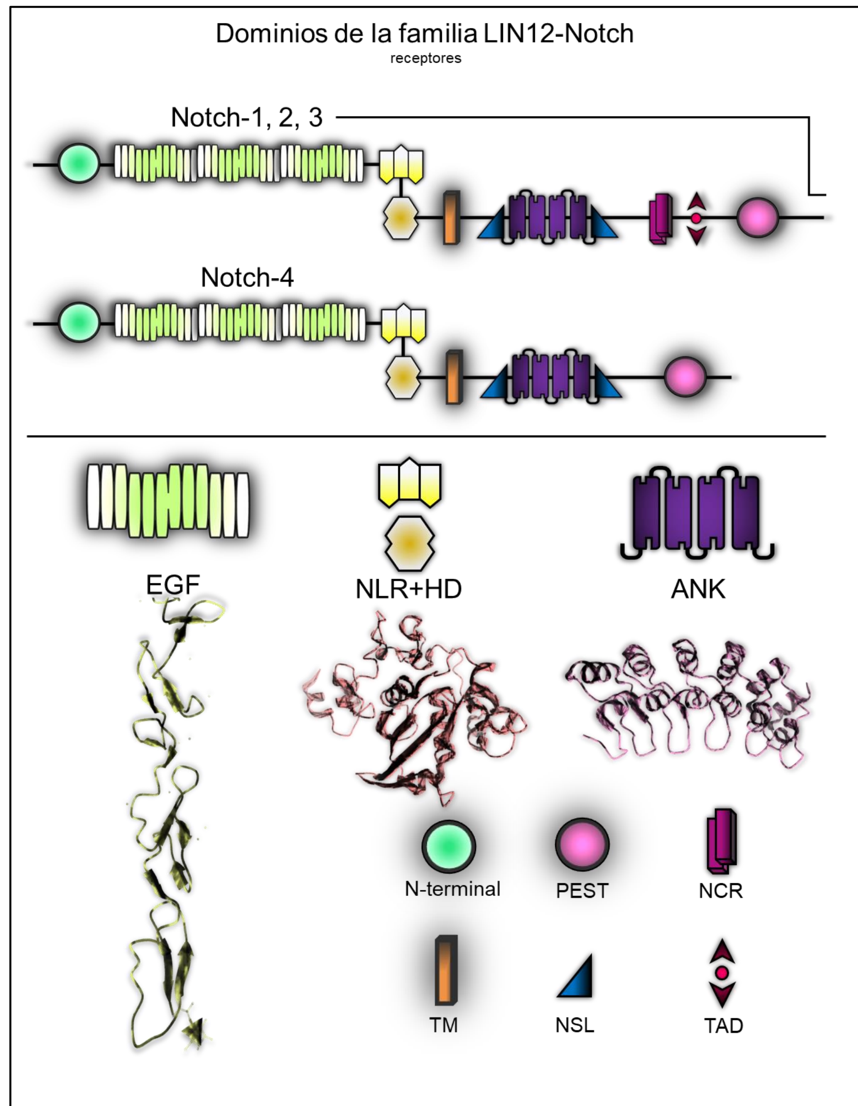

Figura 2. Dominios de los receptores de la vía Notch: Los dominios N-terminal, EGF, NLR y HD conforman el dominio DIN del receptor Notch. Los dominios TM, NLS, ANK, NCR, TAD y PEST conforman el dominio DEN del receptor.

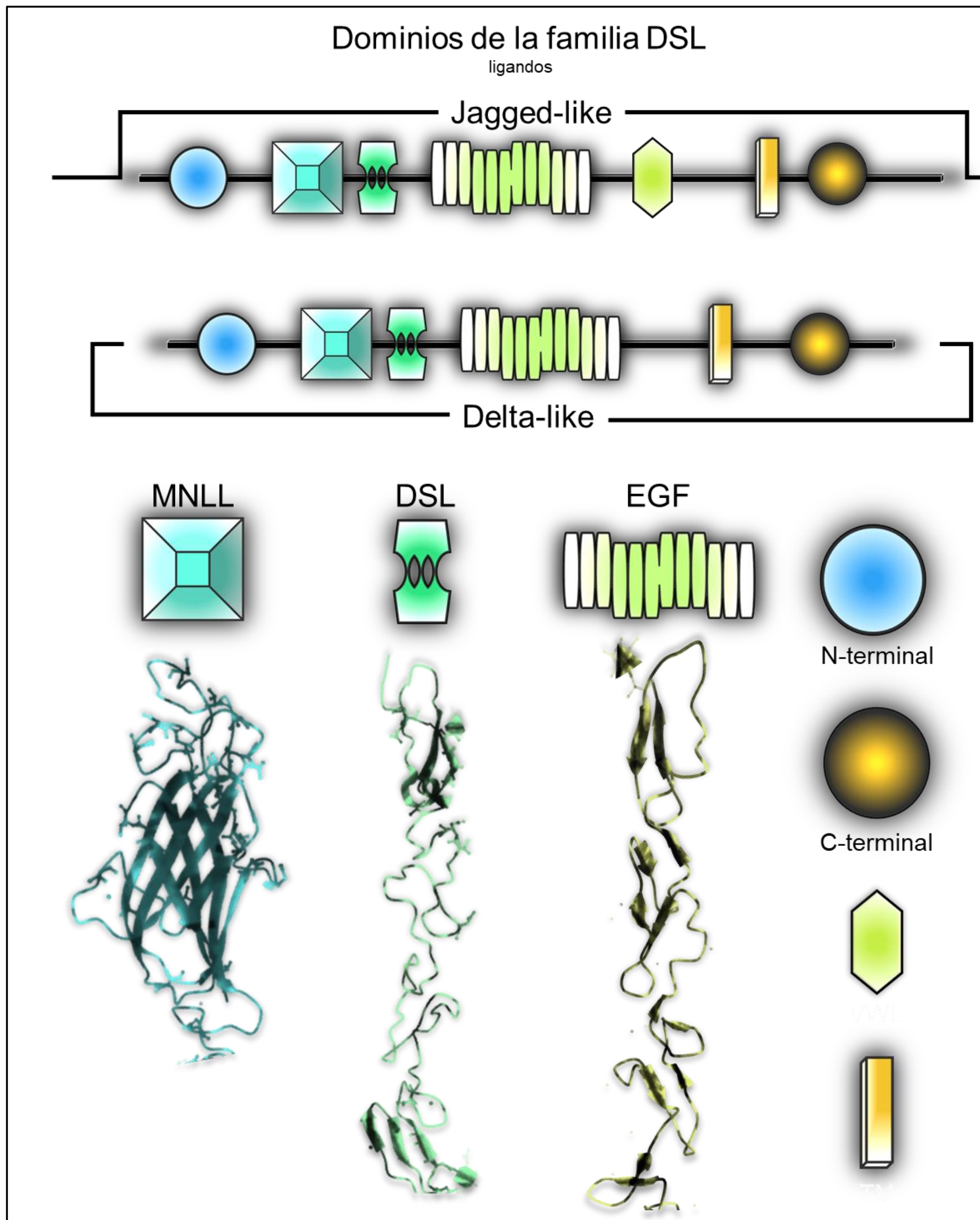

Figura 3. Dominios de los ligandos de la vía Notch: Los dominios MNLL, DSL y EGF son los módulos principales que reconocen los dominios EGF y NLR de los receptores Notch.

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REVIEW ARTICLE

The Notch Signaling Pathway and Breast Cancer: The Importance of Balance and Cellular Self-Control

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Abstract: Notch is a cell signaling pathway that is highly conserved in all metazoans and is the master responsible for cell differentiation and cross-communication with other signaling pathways such as WNT and Hh. In most cancers, the Notch signaling pathway is altered, causing normal controls of vital processes such as cell cycle, differentiation, and apoptosis to be compromised, leading the cell to a carcinogenic state. Currently, research has taken an interest in the Notch signaling pathway to support the design of strategies that regulate the activity of this pathway since it is commonly known that the Notch pathway is over-expressed or aberrant in its functioning in the cancer microenvironment. However, most of the existing strategies are focused on the systematic and complete inhibition of the pathway at the membrane level by way of the ligand-receptor binding (GSIs). There are few strategies that act at the nuclear level to inhibit activation of the complexes via the Notch receptor, the transcriptional factor CSL, and the Mastermind coactivator. Inhibition of the Notch pathway that acts at the nuclear level can provide a selective effect to inhibit the pathway, depending on each cell context and cancer cell microenvironment.

Keywords: Notch-Signaling Pathway, carcinogenesis, Signal transduction, breast cancer.

1. INTRODUCTION

The Notch Signaling pathway (NS) is made up of proteins that are highly conserved in the metazoans and eumetazoans and plays a central and fundamental role in embryonic development during lineage cell decisions. The proteins that make up the structure of the NS can be separated into two groups: the first group involves proteins that function as ligands, receptors, repressors, co-repressors, and transcription factors, which constitute the nucleus of the pathway and transduce the signal; in the second group, regulatory proteins are found to include those that modulate the cellular response and determine the duration of the received signal, modifying the proteins of the first group [1]. The LIN-12/Notch protein family are receptors for the ligands Delta, Serrate, and Lag-2 (DSL family) [2, 3]. In mammals, four different receptors are known (Notch1 – 4), in addition to five ligands (Delta-like 1, Delta-like 3, Delta-like 4, Jagged 1, and Jagged 2) [4, 5]. The ligand-receptor interaction must be accurate, ordered, and controlled in order to get a satisfactory cell response in accordance with the cell context or environment that surrounds it [6].

During embryo development and even during the life of adult organisms, NS acts on cells through any of the following three mechanisms: lateral inhibition, asymmetric division, and inductive signaling. The pathway is activated when two integral membrane proteins from two adjacent cells interact, one as a signal molecule and the other as a receptor. From this moment on, a sequence of molecular events occurs to regulate the signaling pathway in both cells, with the most notorious sign being activation and/or repression of target genes transcription or the modulation of these at the receiving signal cell. The NS lacks intermediary proteins for signal amplification, so the regulation of gene expression is carried out through the formation of transcription activation or repression complexes [7].

The aim of this review is to throw light on the participation of NS in breast cancer and how its various activation mechanisms generate different phenomena in cells through cross-communication with other signaling pathways that are closely related to NS and to present several strategies that are known so far to prevent and counteract the over-expression of NS.

1.1. The Role of Notch Signaling

The key proteins that constitute NS are highly conserved in their functional domains in the different species in which they are described. This makes it possible for these proteins to function in the different species where they have been

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identified. An exception to this feature is the Hairless protein (H) that has been described only in insects and is essential to the negative regulation of NS in *Drosophila melanogaster*. H protein acts by way of specific binding to the Suppressor of Hairless protein [Su (H)], which is the transcription factor of the NS. This interaction occurs through a binding domain located between positions 1021 to 2444 of the *Hairless* gene, which is then capable of attracting the Groucho (Gro) and C-terminal binding protein (CtBP) co-repressors. H forms repression complexes that prevent the activation of genes under the control of the NS, making a change in the pattern of gene expression possible in accordance with the cellular context. This selective expression of genes defines a cell fate at the recipient cell that is different than the cell that sends the signal during normal operation of the pathway [1,8].

In the NS context, the regulation of both positive gene expression, mediated by the intracellular domain of Notch protein (NICD), or negative gene expression mediated by H, is conditioned by space, time, type, and the cell development stage [9]. The regulation of gene expression mediated by NS is influenced by the activity of other signaling pathways such as Wingless (Wnt), Transforming Growth Factor beta (TGF-β), Hedgehog (Hh), Tyrosine Kinase Receptor (RTK), Nuclear Receptor, and the Jak / STAT factor, and its effects are seen in cell fate, metabolism, cell cycle, apoptosis, and cell survival, leading the design of a complex multicellular organism [10,11].

The ability of this signaling pathway to influence a significant number of specific aspects of cell development indicates that an error in the elements that compose it would result in a disorder of the cellular controls, producing unfavorable changes for the organism [12].

The affinity of the Notch receptors for the different ligands is regulated by glycosylation in the extracellular domain of the Notch protein (NEC). This glycosylation is catalyzed by Lunatic, Maniatic, and Radical Fringe enzymes, which show glucosyltrasferase activity [13], giving binding specificity to the ligands depending on the cellular context. The activity of the Fringe proteins has attracted a lot of attention in the cancer context and several strategies have focused on blocking the activity of these proteins in several cancers such as breast, testicular, and lung cancer. However, these strategies only make sense when the NS acts in its canonical form, which is exceeded when the intracellular fragment of the Notch receptor presents aberrations in its expression in several types of cancer [14-16].

The O-fut1 protein, another important glucosyl transferase for NS, is also one of those proteins responsible for the specificity of Notch receptors in a specific ligand; therefore, it is considered a critical control point for the proper function of the NS. However, in a cancer scenario, when one of the Notch receptors is over-expressed, the function of this protein is to give the cell a potentiated activation effect of the NICD [17].

After the ligand-receptor interaction had been established, the Notch receptors undergo some proteolytic cuts via protein complexes such as ADAM10/TACE and γ-secretases releasing NICD, which is transported to the nucleus where it interacts with the transcriptional factor CSL. This is the main and only NS transcription factor effector that maintains *per se* a state of transcriptional repression when NICD is not present. It is known that many repressors and co-repressors act together with the CSL transcription factor in a tissue-specific manner, in vertebrates as well as *D. melanogaster* with results dependent on the degree of cellular differentiation. CSL family proteins are known as CBF-1/RBP-Jk in mammals and they all bind to a specific and conserved DNA sequence 5'-CGTGGGAA-3 at the promoter region of the NS target genes, where they act as constitutive repressors by recruiting co-repressors such as SMRT or N-coR, SKIP, CIR, and Histone Deacetylases of classes I and II, as well as SHARP, CtBP/CtIP, and MeCP2 [18, 19].

1.2. Notch and Cancer

Research shows that in a cancer context where NS is overexpressed, the function of CSL as a negative transcription regulator is overshadowed by the overexpression of NICD, promoting a decrease in the negative regulation mediated by this transcriptional factor. There are descriptions of how the repressors and co-repressors that are used by CSL also participate in other signal transduction pathways such as Hh, WNT, and TGF; however, studies also show that the initial function of CSL in primitive eukaryotes, such as yeast, is to communicate with histone deacetylases; hence, the evolutionary purpose of NS is to counteract the repression mediated by these transcriptional factors. CSL, in the cancer context, has become the target of strategies at the nuclear level, repressing NICD - CSL interaction when the NS is constitutively active in order to prevent the aberrant effects of this overexpression [20-23].

Once NICD binds to CSL and activates the NS (Fig. 1), the Notch target genes are expressed. These are mainly genes that encode proteins of the HES family (Hairy Enhancer of Split), which are found in *D. melanogaster* and their homologs in mammals known as HES, HER, HEY, and HERP. All of them are transcription factors of the bHLH type. This family of proteins is involved in the transcriptional repression of other transcriptional factors that give rise to the cell differentiation process [9, 24].

HERP1 and HERP2, like HER, function exclusively by repressing genes when NS is activated. The HERP protein family possesses specific negative regulation mechanisms and recruits the mSin3 complex with a higher affinity than the HER proteins to form a transcriptional repression complex with the HDAC1 and HDAC2. Likewise, research has shown how the HERP can form heterodimers with HER proteins and homodimers with other HERP proteins, to increase the spectrum of the negative regulation of the NS. The HERP and HES factors are not expressed simultaneously in all of the cells. The HERP protein alone shows an intrinsic repression activity of the NS; however, in several cells where HERP and HES are simultaneously expressed, the negative regulation is increased for the target genes of the NS. The HERP family of proteins seems to have stricter controls at the moment of being expressed, promoting a more efficient negative regulation than the HES gene family and its activation occurs exclusively through the canonical pathway of NS [25].

Consequently, in a constitutive expression context, the high efficiency of HERP and HES transcriptional factors that

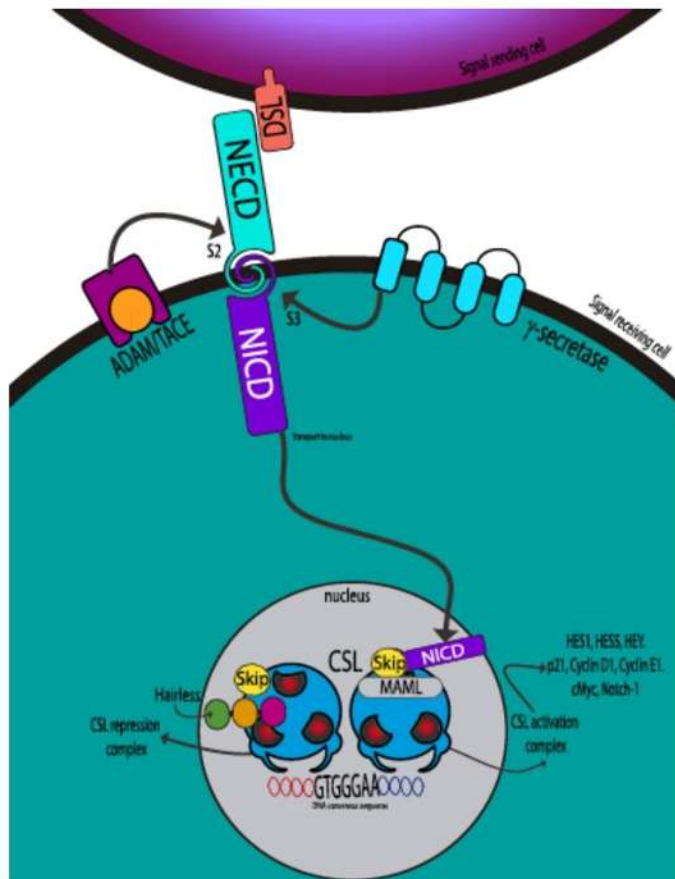

Fig. (1). Notch signaling pathway activation process. Once the Notch protein is transported to the membrane and is recognized by the DSL family, it undergoes a proteolytic cleavage via the ADAM/TACE metalloprotease family, exposing a recognition site for the γ-secretases complex to make another proteolytic cut that releases the intracellular domain of Notch (NICD) from the membrane and is translocated to the nucleus. Once the NICD reaches the nucleus, it is able to remove the repression complexes that are linked to CSL and thus transform them into activation complexes by recruiting the MAML and SKIP coactivators. The activation of the NS promotes the expression of the HES and HEY family genes that are transcriptional repressors, as well as target genes such as p21, Cyclin D1, Cyclin E1, and cMyc. The expression of these genes influences the regulation of crucial processes such as apoptosis, differentiation, and the cell cycle.

negatively modulate other bHLH proteins that control the cell differentiation processes could mean that the correct cell fate is never reached and, therefore, cell malfunctions could occur in the regulation of vital processes, such as the cell cycle and apoptosis. This could promote carcinogenesis and the appearance and maintenance of cancerous precursor cells [26].

The NS does not have second messengers or enzymes that enhance its activity. The strength of the NS signal, in practical terms, is directly related to the nuclear accumulation of active NICD. As a result, NS is highly dose-dependent of NICD at the nucleus, which in turn is determined by the cellular context [27]. For this reason, the biological consequences of the NS are always different depending on the cell type [28]. Also, it is important to mention that NS can self-regulate their ligands and receptor genes transcription according to the cell environment [29].

In a cancer context, the cells present alterations in the normal activity of different signal transduction pathways as occurs with the NS. In this regard, the NS was recently considered to be the key signaling transduction pathway to understanding the entire process in the establishment of some types of cancer, as well as their incidence and metastasis. For this reason, in the last several years, strategies had been developed to inhibit the activation of NS to avoid ligand and receptor binding, but no strategies have been developed to avoid the constitutive NICD activity at the nuclear level.

1.3. Notch and Breast Cancer

NS plays a definitive role in the biology of breast cancer development, as it amplifies and suppresses elementary communication signals with several signaling pathways involved in the oncogenesis processes, such as WNT, ERK, β-catenin, and Her2/VEGFR, among others, thereby controlling important aspects of the cell cycle and differentiation [30].

In this context, genes directly regulated by NS are involved in the cell cycle, apoptosis, cell development [31, 32], and metabolism [33]. We know that about 20% of tumors in the mammary gland are initiated by aberrations in the *Notch4* gene expression and more than 50% of the cases by the *Notch1* aberrant gene expression [34,35]. These aberrations are related to a constitutive performance of NS in cancer cells and studies have shown that aberrant *Notch1* gene activity during a translocation from chromosome 9 to 7 causes a fusion between two DNA sequences: the gene fragment that codes for the N-terminal domain of the TCR-β (Transforming Factor Beta), which is responsible for controlling certain aspects in the cell cycle and metabolism, with the fragment of the gene that encodes the intracellular domain of Notch 1 (NICD1). This translocation causes a constitutive expression of the intracellular domain of NICD1, overpassing the negative regulation of NS and deviating normal differentiation in cell growth [36], provoking the appearance of cancerous progenitor cells and promoting mobility between tissues, causing metastasis and angiogenesis, and thereby developing the phenomenon known as Epithelial to mesenchymal transition (EMT) [37].

The constitutive activation of both *Notch1* and *Notch4* genes prevents the ductal ramifications of the mammary glands from adequately responding to growth and differentiation signals such as hematopoietic growth factor (HGF) and transforming factor beta (TGF-β) and so they are induced to differentiate from an invasive phenotype [38]. In this context, deregulation or aberrations in the functioning of the NS can stop the normal differentiation of the mammary gland cells, eliminating the mechanism by which the cell maintains its homeostasis as a differentiated cell, giving rise to cancer cells and keeping them in a state of accelerated proliferation that will eventually become a carcinoma [39].

Previously, it was thought that the structure of the mammary gland was composed of two cell types— myoepithelial and epithelial cells—but recent research suggests that there is a third cell type between these two cell lines that share characteristics of both cell phenotypes. It is in this type of cell where the effects of NS overexpression have been studied more closely [40, 41].

In order to identify the nature of breast carcinomas, several special markers have been identified for each type of cell lineage, including the following, which are found in most mammary epithelium: E-cadherin, desmosome cadherin, integrins α2β1, α3β1, α6β4, cytokeratin CK14 [42], cadherins associated with myoepithelium and epithelium DSg3, DSc3, DSc2, and DSg2, the P9Ka protein and the CALLA and MUC1 proteins [43,44]. Although there are unique markers for each cell type, those mentioned above are strongly related to aggressive and invasive behaviors in breast cancer cell lines when they are overexpressed due to the abnormal function of signal transduction pathways, among them, the NS [45].

The combination of different phenotypes in a cell type may be related to the interactions of the signaling pathways responsible for carrying out both the correct differentiation process and cell fate decision. The NS, along with other signaling pathways, could be orchestrating this combination of phenotypes, therefore a slight change in the expression level of each of its components can generate uncontrolled and aggressive behavior in the cell, giving it the power to evade cellular controls such as apoptosis and the cell cycle [46].

It is well known that different consequences of NS activity can be found in different breast adenocarcinomas. For example, the *Notch1* gene overexpression is frequently found in breast cancers that overexpress the H-ras protein, considered a breast cancer marker. Also, studies have shown that the overexpression of the Notch-3 receptor in some breast cancer cell lines, such as MCF-7, promotes apoptosis regulation. On the other hand, the overexpression of the ligand Jagged2 regulates the activation of both intrinsic and extrinsic caspase mechanism regulation in breast cancer cell lines. Consequently, NS exerts different effects depending on the combination of ligand-receptor interactions [47].

There are many genes that are regulated by the NS in the cellular context of breast cancer. Among the most studied are *Hes* and *Hey* gene families, members of the super family of genes that encode for proteins of the bHLH type [48,49], which function as transcriptional factors. However, the core proteins of the pathway also come into play with the transcriptional factor known as CSL, Delta and Jagged ligands, and Notch receptors, as well as their direct modulators such as Lunatic Fringe, ADAM10, Mastermind, and Deltex proteins. The absence or overexpression of these has been related to tumor characteristics in mammary epithelial cells [50, 51].

The NS can establish cross-talk communication with other signal transduction pathways. For example, in mammary epithelial cells, NS is able to activate GSK-3β and increase the resistance to apoptosis by the interaction of the WNT/β-catenin and Hh pathways (Fig. 2) [52]. Another example of cross-talk communication involves the WNT pathway, which helps to regulate events such as cell cycle and cell differentiation by way of lineage decision. When this communication is compromised, breast cancer occurs and a group of cancer cells undergoes a gradual transformation into cancer progenitor cells [53].

Two mechanisms of NS activation in breast cancer have been described. The activation of the NS canonical pathway strictly involves a ligand-receptor binding to activate the cellular response and trigger, with the help of NS modulating proteins and the transcriptional factor CSL, and an expression response or repression of their target genes. The activation of the non-canonical NS pathway involves other mechanisms and triggers a different response in the pattern of the target genes expression or repression. It is important to know what type of NS activation occurs in the cell since the influence of each activation type can determine the cell fate [54-56].

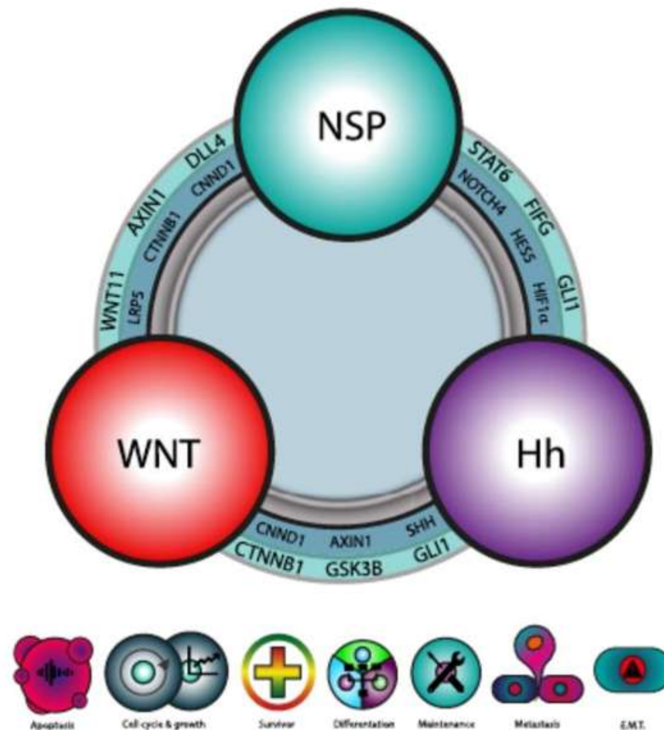

Fig. (2). Cross-talk communication between the Notch (NS), Wingless (WNT) and Hedgehog (Hh) signaling pathways and the genes that promote this communication between each of them as well as the cellular processes promote.

1.3.1. Notch Target Genes in Breast Cancer

At present, there are studies that consider the NS to be a possible and interesting target for the treatment and development of new therapies against breast cancer. NS was found to be related to breast cancer in mouse models [57], where overexpression of a fragment of the Notch-4 receptor was observed. This phenomenon shows many similarities to the one described in T-cell cancer (T-ALL), where overexpression of a fragment of the Notch-1 receptor was identified. This is one of the causes of spontaneous tumor formation that can lead to mammary carcinogenesis [31, 32]. On the other hand, research has demonstrated that the expression of the Notch-2 receptor is related to a favorable prognosis in breast cancer [58].

It is now known that each Notch receptor activates different target genes depending on the affinity for each ligand in the pathway, and had its own form of expression. However, there are several genes that are always expressed through any one of the Notch receptors and these can be genes from the *Hes*, *Hey* families such as *Hes1* and *HeyL*, *c-Myc, CyclinD1*, *CyclinD3*, *BCR1-2*, and *BCL2*. Each of these influences a broad range of biological events including the cell cycle, cell differentiation, apoptosis and survival, and maintenance of cancerous cells progenitors [59-61].

1.3.2. HES Gen Family

The *Hes* genes family in mammals belongs to the super genes helix-loop-helix (bHLH) type family and is very important in diverse processes. The most important process is the maintenance of the progenitor cells in an undifferentiated state and the regulation of the lineage decision in the progenitor cells of the mammary gland. Initially, these genes were identified by their participation in processes during embryogenesis, where they are responsible for regulating cell proliferation and differentiation. The Hes family of proteins also acts as a molecular oscillator and controls the timing of some biological events, such as somite segmentation. The Hes family proteins have pleiotropic effects ranging from cell development to cross-talk communication with several gene networks from other signal transduction pathways and can affect anything from metazoan embryogenesis to progenitor cell maintenance in adult organisms [62].

In an adult organism, the *Hes1* gene encodes a nuclear protein that actively and passively represses the transcription of several genes, such as *Ptf1* and *Ngn3*. These, in turn, are transcriptional factors of the bHLH type that activate the expression of genes during cell differentiation [63]. Active repression mediated by the Hes transcription factors works with the help of co-repressors such as Groucho (Gro) [21]

and has been shown to cause changes in chromatin since Gro recruits histone deacetylases to inhibit gene transcription. On the other hand, Hes proteins can form heterodimers with Hey proteins, another bHLH factor. These heterodimers have a higher efficiency in repressing gene transcription when NS is activated [64].

Reports relate how chromosomal translocations that occur in cancer cells cause an inappropriate functioning of the *Hes1* gene and that this phenomenon is associated with the appearance of malignant lymphomas, including B cell leukemia, which has a known translocation (t (1; 19) with the *PBX1* gene, (t (19; 19) with the *TFPT* gene and a translocation (t (12; 19) with the *ZNF384* gene, and probably with the translocation that occurs with the *Notch4* gene (t (9; 7) in breast cancer [65]. Studies have shown that low levels of the transcriptional factor Hes1 promote the progression of the cell cycle through the negative regulation of p21 and p27 [66]. Also, research shows that high levels of Hes1 protein expression have been related to cell cycle inhibition, probably because Hes1 also represses the expression of some cell cycle regulators such as E2F-1, which promotes the transition of the cell cycle from the G1 phase to the S phase [67]. Therefore, when there is a high expression of Hes1 the cell cannot reach a final cell fate and remains in an undifferentiated state, or different from the normal lineage, causing it to gain attributes that are not within normal parameters. As a result, it can evade biological controls of great importance such as the same cell cycle or differentiation [68].

Variations in the transcription levels of the *Hes1* gene are attributed to its nature as a molecular oscillator, since it acts as an internal biological clock that controls the level of expression of each of its dependent genes, making it a gene with pleiotropic effects in certain stages or cellular conditions, and functioning as a switch in other cellular systems [69]. This oscillation of *Hes1* gene expression has been reported in different tissues such as fibroblasts, myoblasts, neuroblasts, and epithelia, producing stimulation in the induction of NS [70].

The heterodimer Hes1/Hes7 transcriptional factor negatively regulates the expression of the *Lunatic fringe* gene (*Lfng*), which, in turn, is a regulator of the NS pathway by way of the glycosylation of the receptors. This feedback network is very common in the signal transduction pathways and especially in the NS where it has its own self-regulatory controls, both positive and negative. It is not surprising that in the breast cancer context, alterations in the oscillating expression of Hes1 and Hes7 transcription factors can trigger effects that lead the cell towards a state of differentiation favorable to the development of cancer cells and the occurrence of metastasis [13].

Evidence shows that Hes1 is negatively regulated by 17β-estradiol in breast cancer cells that express the alpha receptor for estrogen (ERα). This negative regulation is essential to the normal differentiation of the mammary glands [71], where Hes1 participates in proliferation inhibition stimulated by 17β-estradiol in breast cancer cells. Research also indicates that the interaction between factor Hes1 and 17β-estradiol generates alterations in the cell cycle by joining ERα and inhibiting the expression of Cyclin D1 in breast cancer cell lines [72].

In breast cancer, the Hes1 factor, together with ERa and 17β-estradiol, induces a network of gene expression that depends on the differentiation state and the position that the cell occupies in space. This interaction seems to be intimately related to transformative effects in its phenotype due to the alteration of the expression levels of genes dependent on this interaction. This results in an imbalance in cell cycle controls and differentiation, causing a phenomenon known as EMT, due to the overexpression of several metalloproteases, especially the MMP7 metalloprotease, which can degrade the extracellular matrix and make the anchoring base of the epithelial cells disappear [73].

1.3.3. Cyclin D1

The gene that encodes for cyclin D1 is considered a potent oncogene that can modulate the activity of multiple hormone families, such as steroids, so they can be recognized by nuclear receptors in an inhibitory or stimulating way and thus regulate proliferation in a positive way [74]. However, overexpression of cyclin D1 increases the activity of ERα by recruiting coactivators for type 1 steroid receptors that respond to different stimuli such as cell cycle proliferation or progression [75,76].

Overexpression of cyclin D1 occurs mainly in malignant cells that function positively with the receptors of these hormones and there may be an unfavorable prognosis if their expression occurs in an autocrine manner in the cell [74,77]. According to research, the progesterone beta receptor (PR-β) induces the positive regulation of cyclin D1 mRNA and increases the stability of the cyclin D1-dependent MAPK protein [78] in malignant breast cancer cells, increasing the lifespan of cyclin D1 in the cell [79].

Cyclin D1 protein is related to the transcriptional regulation of numerous genes, which in turn, are related to the progression of cancer [80, 81]. Cyclin D1 is normally found regulating the activity of cyclin-dependent kinases (CDK) 4 and 6. It is also able to form complexes with CDK2, which regulates the cell cycle progression at the G1/S phase transition [82]. The cyclin D1 / CDK2 complex has been detected in roughly 70% of breast cancers. This cyclin D1 / CDK2 complex controls the transformation into a malignant phenotype of the mammary gland cells [83,84]. The direct interaction between the PRs and Erα complexes with cyclin D1 and CDK provides a mechanism to help understand the phenotype of proliferative cells in cancer tumors of the mammary glands [85].

The effects of cyclin D1 overexpression are present in most malignant cells, especially in the formation of carcinomas and in the EMT transition in different parts of the body such as the colon and the mammary glands. The overexpression of the cyclin D1 protein causes important metabolic changes in the cytosol of the cells; for example, it changes the expression rate of other proteins such as E-cadherins, Axin, the APC complex, and GSK-3β [86, 87].

Changes in the cytosolic concentration rates affect crosstalk communications between signaling pathways such as Notch, WNT and Hh. This allows the cells to avoid metabolic controls in the normal cell functional state [88, 89]. The interaction between the β-catenin signal and the NS produces synergistic effects in the progenitor cells that have

suffered some damage in their DNA causing overexpression of cyclin D1. The overexpression of cyclin D1, together with cyclin D3, takes over control of cell proliferation in the cancer progenitor cells since the β-catenin/TCF complex, which is important for the self-control of the cell cycle, is negatively regulated for the progression between phases G1 and S [90, 91].

Directly proportional feedback between the expression levels of the Notch-1 receptor and cyclin D1 in several types of cancer has been demonstrated [92]. The overexpression of both proteins decreases the levels of other proteins, especially PTEN and p53 and, in turn, increases the expression levels of the *c-myc* gene. The loss of PTEN or p53 due to an overexpression of the NS dependent on cyclin D1 promotes the survival of mammary gland cells. It also reduces the activity of apoptosis by decreasing the expression of PTEN and p53 proteins [93].

β-catenin and the WNT pathway, with the help of NS, increase the expression levels of both cyclin D1 and cyclin D3, which together with the transcriptional factor Hes1 creates feedback between these proteins promoting cell proliferation in a cancer context. Studies show that inhibiting Notch-1 signaling causes an arrest in cell proliferation and apoptosis induction through the positive regulation of the MUC2 protein when cyclin D1 and cyclin D3 are not over-expressed [94].

RNA expression levels of *Hes1*, *c-myc*, and *cyclin D1* target genes of Notch-1 and Notch-4 receptors and have been detected at high concentrations when the NS is constitutively active. This produces an increase in the amount of growth and progression signals of the cell cycle in adjacent cells and tissues [95-97]. Cyclin D1 is required in cells where Notch-1 is overexpressed to provide an invasive and malignant phenotype for the mammary gland cells. However, when the genetic background of cyclin D1 is absent, the cancer progenitor cells are not able to expand. This suggests a close relationship between NS and cyclin D1 in the carcinogenic state [98].

According to reports, the four Notch receptors act differently with cyclin D1 depending on each cellular type or cellular context. For example, the Notch-3 receptor and the cyclin D1 are required in a normal manner for the terminal differentiation of the progenitor cells that give rise to the luminal and epithelial lineages of mammary gland cells [99]. Nevertheless, the expression of the Notch-1 receptor in conjunction with the Notch-3 receptor is increased in human breast cancer tumors because of its dependence on cyclin D1 expression levels. This dependence has been associated with an unfavorable prognosis of the disease [100, 101]. A recent discovery shows that Mastermind-1, the Notch pathway coactivator, can positively regulate the cyclin D1 expression independently of the activation of the NS, which converts the Mastermind-1 protein into a potent effector of carcinogenic cells. Mastermind-1 is able to associate with β-catenin both *in vitro* and *in vivo*, allowing the activation of the machinery involved in cell proliferation [102].

This change in the expression pattern of these genes gives the cell a multipotent phenotype with a potential for out of control renewal, turning it into a cancer stem cell, since the overexpression of the Notch-1 receptor has also been detected in high concentrations outside the nucleus in cancer cells of the mammary gland, increasing the effects of deregulation of this cell type. In other words, the Notch pathway does not cause cancer, but rather maintains the carcinogenic phenotype of the cells [103].

1.4. Pathways Involved in Breast Cancer and Crosstalk with Notch Signaling Pathway

Despite the fact that NS is the main effector in cell development and progenitor cell maintenance, this signaling pathway alone is not capable of generating a cancer cell. NS requires other signal transduction pathways also involved in cell development and differentiation, and so it must act together with the Hedgehog (Hh) and Wingless (WNT) pathways, which, in turn, communicate with other pathways essential in the functioning of a cell [104]. NS has been widely studied in triple negative cancer cells (which do not express any type of hormonal receptor, growth or receptors that can be detected by the immune system) since these can show overexpression of Notch-1 and Notch-4 receptors. However, the overexpression of each of these receptors will depend on the subcellular location of the origin of each cell and whether they present positive receptors for certain hormones such as progesterone or estrogen and HER2, in cases with an unfavorable prognosis [100]. The NS can have a direct or indirect effect on many genes. Those most studied are the *VEGF3*, *Hes*, *Hey*, *NF-kB2*, *cMYC*, *CCDN1*, *p21* and *HER2*, and the estrogen receptor ER, which are all genes involved in the growth or that function as regulators of the cell cycle, apoptosis, and angiogenesis [105].

1.4.1. Hedgehog Signaling Pathway

The Hedgehog pathway is highly conserved and functions as a key in the signaling cascade involved in the correct development of the embryo. This pathway has been implicated in the initiation of tumor progression, angiogenesis, and metastasis. It is known that the Hh pathway is responsible for regulating the renewal of progenitor cells in the nervous system and skin cells [106].

Activation of the Hh pathway together with NS generates metastasis and a transition from epithelium to mesenchyme by deregulating the apoptosis and angiogenesis controls since it enhances the overexpression of both SNAIL protein and angiopoietin 1 and 2. The SMO protein also has an overexpression in breast cancer cells that, in combination with the overexpression of the Notch-1 receptor, activate the direct expression of the MYC protein, which promotes proliferation by raising cyclin levels D1 and FOXM1. The FOXM1 protein is involved in the appearance of triple-negative malignant cells when the overexpression of NS is present [107–110].

1.4.2. WNT/ β-catenin

The WNT / β-catenin pathway plays an important role in the development of the embryo and may allow the formation of tumors when they are over-expressed. There is evidence indicating that this signaling pathway is positively regulated when malignant cells of different types of cancer appear in conjunction with the over-expression effect of NS in mammary gland cells [111, 112].

Cross-talk communication between NS, Hh, and WNT / β-catenin is essential when cancer cells appear in most cell types [113]. The communication of these three pathways is reflected in the effects of several important proteins during growth, apoptosis, and differentiation, such as PARP1, BCRA1, and BCRA2, which act in the DNA damage repair process [114,115]. Several growth receptors such as EGFR, HER2, and C-erbB1 are involved in the progression and maintenance of malignant cells in breast cancer. If any of these pathways do not work properly, the result is communication problems with the other pathways involved since it is a complex network of information flow [116-118].

1.5. Strategies Against Breast Cancer in the Context of the Notch Pathway

As mentioned above, cross-talk communication between the signaling pathway networks in the cancer microenvironment is disturbed by the accumulation of mutations in the genome of cells that do not allow normal controls of cell function. For this reason, we have begun to pay attention to the molecular mechanisms of certain signal transduction pathways; mainly those that regulate the cell cycle, differentiation, and apoptosis.

New strategies are being combined recently with existing therapies against cancer due to the particularities that this condition involves. The NS is an interesting target that has currently garnered a lot of interest because of its capacity to regulate cross-talk communication between the main signal transduction pathways involved in the appearance, survival, and maintenance of the disease. The NS oversees the regulation of the renewal of normal and carcinogenic progenitor cells, but its effects may vary according to the cell microenvironment and the participation of cross-talk communication with other transduction pathways, as well as with WNT and Hh pathways, which synergistically promote the rapid proliferation of cells. The main strategies used to counteract the overexpression of NS focus on the use of monoclonal antibodies (mab) against the γ-secretases complexes; however, the use of these inhibitors of γ-secretases (GSIs) has been shown to have significant side effects in the digestive system, mainly in the differentiation process of colon cells. This causes severe to intense diarrhea because the inhibition of γ - secretases is a key point in the systematic repression of the NS, which, in turn, causes a non-accurate cell differentiation process in the newborn cells in the crypts of the colon epithelium tissue. These cells only differentiate to cells of the globular type and, therefore, the nutrients cannot be absorbed by this cell type in the normal digestion process [119].

GSIs not only act on the four receptors of the Notch-1 – 4 receptors, they also affect Delta-like and Jagged's ligands, APP proteins, and markers of cancer progenitor cells such as CD44, ErbB4, E-cadherins, N-cadherins, and sindecaine-3, which are among the most studied; however, it should be noted that their efficacy focuses on the Notch receptors, especially in the inhibition of signaling initiated by the Notch-1 receptor [120]. There are several drugs with this function of GSIs that are in the pharmacological phases I and II; for example, MK-0752, RO-4929097, and PF-03084014 [121,122]. The combination of these GSIs with chemotherapy promotes the reduced expression of certain markers such as CD44 + / CD24-, potentiating the effect of alternative therapies, but only with cancers that are positive to hormone receptors, such as progesterone and estrogens. Otherwise, these drugs still present adverse reactions when applied for prolonged periods.

The drug PF-03084014 acts against mutated Notch-1 found in 50% of breast cancers, leukemia, and colorectal cancer [36], with effects on the activation of apoptosis, and it is a strong inhibitor of γ-secretases. This drug, in combination with hormonal chemotherapies, is able to slow down the growth of CSCs. However, its cytotoxic effects are broad and medium risk when it is administered over the long term. This characteristic of GSIs presenting serious adverse reactions has limited their prolonged use even if their effectiveness is of great impact [123].

A second group of molecules that have been developed against cancer in the context of NS and its application in breast cancer are the monoclonal antibodies against key proteins that participate in the activation of the pathway. These strategies are directed against the receptors and ligands of the NS and promise to have a friendlier impact compared to the GSIs and their side effects. When using monoclonal antibodies, certain receptors are inhibited simultaneously, unlike the GSs, which do not all inhibit at the same time. This potentially decreases the side effects of the systematic inhibition of the NS in patients. For example, one of the most promising monoclonal antibodies is demcizumab, which selectively inhibits signaling mediated by Notch-1 and Notch-4 with the ligand Delta-4 and has a direct effect on cell arrest in cancer progenitor cells in the colon and mammary epithelium. Nevertheless, the use of these types of therapies also generates adverse reactions, but not as noticeable as those generated by the GSIs [124].

Finally, there are new types of molecules available known as anchoring peptides. These act directly on the proteins of the signaling pathway to prevent the formation of protein complexes and, as a result, the activation of the pathway. The main anchor peptides have an effect on the γ-secretases, in particular on specific subunits of this protein complex known as PSEN or PSENEN, which selectively prevent the detachment of the cell membrane of the intracellular part of Notch receptors, and as a result, they are able to avoid the NICD - Mastermind complex and prevent the transcription of the NS target genes. Reports show that these anchoring peptides act by inhibiting the formation of cancer progenitor cells through their signaling effect mediated by Notch-1 in solid tumors such as breast adenocarcinoma [125].

2. DISCUSSION

NS normally acts as a regulator of other signaling pathways in different cellular processes, exerting a selective and complex control so that these cellular processes can fulfill their objectives. The simplicity and elegance of the NS operation allow for a broader scope of molecular mechanisms whose effects can be studied at a systemic level—whether at the single-cell level or at the multicellular level—of an organism such as the human being. The study of NS for over a decade has provided us with new clues so that we can elucidate on the mechanisms of their finely orchestrated molecular functioning and how this signaling path interacts with

other signaling pathways in differentiation, survival, cell cycle, and apoptosis. It is not surprising that if an error occurs in one of its main proteins, such as its receptors (Notch-1 - Notch-4) or ligands (Delta 1, 2 and 4, Jagged-1, 2), the complex structure of crossed-talk communication networks in the cell have effects as dramatic and malignant as cancer itself.

Studies show that NS alone is not capable of causing the appearance of a cancer cell; however, its role is known to generate populations of progenitor cancer cells and to maintain their lineage once the NS has altered its normal functioning [126]. This is explained by the fact that the NS is a professional in the process of differentiation and cross-talk communication with other signaling pathways, especially with the WNT and Hh pathways.

The main responsibility of the WNT pathway is to maintain the survival and growth controls, with direct effects on the cell cycle. The WNT pathway is one of the initiators of Notch signaling in the early stages of embryo development, promoting the expression of Delta-like ligands [86]. Cross-talk communication between the WNT pathway and the NS converges at different points; however, this relationship is focused on cell growth and survival controls as well as cell cycle progression, especially in the progression from the G1 phase to the S phase. This close relationship between WNT and NS regulates normal accelerated growth in a cell and gives it a sense of survival, where, if necessary, the cell will program its apoptosis. However, when the regulatory capacity of the NS does not work, the cell loses its way of surveying its environment and suffers over-stimulation with signs of growth. The relationship between the Hh and NS pathway, unlike WNT, seems to be narrower and with greater regulatory effects in a majority of processes such as cell migration, cell cycle, differentiation and the response to angiogenesis growth stimuli [27].

CONCLUSION

The complex communication network between the WNT, Hh, and NS has resulted in therapies in the cancer field not being entirely effective. However, most of the therapies that are currently being developed include NS as quite a promising target because of its regulatory effect with other signaling pathways, since Notch acts as a judge when deciding how to regulate the effects of these other signaling pathways. The molecules that currently exist for the regulation of the NS focus on the repression of these at the membrane level in their majority. For example, the GSI's and most of the mab that work against ligand and receptors of the NS are just a few molecules that act at the nuclear level, inhibiting NS signaling, taking into account that therapies that act at the membrane level, such as GSIs, have collateral effects that can range from moderate to severe. If new strategies aimed at blocking NS at a nuclear level are focused, we can achieve the finest control in the regularization of each of the receptors that are being over-expressed or repressed in the cancer microenvironment. However, in-depth research into this question is still lacking.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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IV. Hipótesis

La sobrexpresión del péptido ReNeg-AID derivado de la proteína Hairless de *Drosophila melanogaster* regula de manera negativa la activación constitutiva y aberrante de la vía de señalización Notch en células de cáncer de mama humano.

V. Objetivos

General:

Evaluar la sobrexpresión del péptido ReNeg-AID en la línea celular MCF-12F, MCF-7 y MDA-MB-231 bajo el perfil genético de la vía de señalización Notch.

Específicos:

- Evaluar la proliferación de la línea celular MCF-12F, MCF-7 y MDA-MB-231 transfectadas con el péptido ReNeg-AID.
- Obtener el perfil de expresión de los genes dependientes de VN en la línea celular MCF-12F, MCF-7 y MDA-MB-231; control y transfectadas con el péptido ReNeg-AID.
- Evaluar el efecto de la sobreexpresión del péptido ReNeg-AID en ciclo celular en la línea celular MCF-7.

VI. Resultados

Capítulo 2: ReNeg-AID y la inducción de la regulación negativa de vN.

A peptide derived from *D. melanogaster* Hairless protein promotes the negative regulation of Notch Aberrant Constitutive Signaling on human breast cancer cells.

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Running title: ReNeg-AID peptide derived from Hairless protein of *D. melanogaster* can promote the negatively regulate Notch Signaling Pathway on mammary cancer cells inhibiting the G1/S cell cycle phase.

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Abstract: The Notch Signaling Pathway (NSP), by a Notch intracellular domain (NICD) constitutive overexpression, has been related to many cancer types. In breast cancer,

the constitutively activated NSP plays a principal role in aberrant cell cycle progression, poor cellular differentiation and apoptosis inhibition. It is well known the high conservation of Notch proteins through the metazoarians. Hairless is a negative regulator of NSP in *D. melanogaster*, but a homologous Hairless protein in mammals is unclear. The design an expression plasmid, pReNegAID, which encodes a peptide based on the CSL binding domain of the Hairless protein of *D. melanogaster* will be used for analyzing the ReNegAID peptide participation at the negative regulation of NSP in the mammary gland cancer context. Both, the pReNeg-AID plasmid and the mock plasmid were transfected into breast cancer cells in order to analyze cell proliferation (MTT assay) and gene expression pattern related to the NSP common genes between Hedgehog and Wingless pathways and genes related to apoptosis, cell differentiation and cell cycle. Moreover, control expression of Luciferase reported plasmid was performed and data showed that ReNeg-AID peptide induces a switch in the gene expression pattern related to the NSP and induce G1/S cell cycle arrest by the negative regulation of the Notch-1 receptor expression and it suggests cross talking between Hedgehog pathway (Hh) and NSP on mammary cancer cells to avoid the molecular machinery of initial EMT.

Keywords: Breast cancer, MCF-7, Negative regulation, cancer therapy, Notch-1, Notch Signaling Pathway.

Introduction

The Notch Signaling Pathway (NSP) (Supplement figure 1) is an ancestral cell communication circuit highly conserved in all metazoan. Their transcription activation complex evolution through time has been minimal, however, the transcription repression complex presents more variability and specific tissue behavior at different organisms where it has been described [1, 2]. In the last years the NSP activation mechanism has been elucidated and in recent years the Notch mechanism of negative regulation, mainly on different cells populations and different cellular context, as well as its implications in different diseases such as cancer [3-5].

In the cancer context, it has been known that NSP has a principal role in both promoting the cancer cells appearing and maintaining the disease state [6, 7]. The role of the NSP in cancer has been firstly described on acute lymphoblastic leukemia. Today, the NSP is considered like a primordial target at the strategy against cancer disease, especially to breast cancer [6, 8, 9].

The NSP exerts control over different events essentials for the proper function of the cell, such as differentiation, apoptosis, signaling pathways cross talking with both Hh and Wnt pathways, it is an essential regulator of the cell cycle G1/S phases transitions and it has been shown if the Notch signaling pathway is not regulated properly, aberrations arise in its information flow, leading the cell to a cancer state [10, 11].

It is known that in breast cancer the NSP presents a constitutive activation mainly through Notch1 and Notch2 receptors and rarely through Notch4 receptor, promoting a strike on the mammary gland cells differentiation, an epithelial to mesenchymal transition (EMT) through deregulating the cross talking between Hh and NSP, promoting high levels of cell migration and more aggressive metastasis. Also, this constitutive activation of the NSP promotes the stop of the cell cycle checkpoints performed by cyclin D1 and cyclin E1, as well as changes at the transcriptional rates of Fos and FosL genes promoting the apoptosis evasion [11-15].

The strategies focused against breast cancer related to NSP distorted activity are based mainly in the γ-secretase inhibitors (GSI's) to block the NICD release from the

cellular membrane avoiding its transport into the nucleus. Also, antibodies directed against ligands and receptors interactions are used to prevent the NSP cascade. Antibodies against co-activators Mastermind are also used, to prevent the transcriptional activation complex inhibiting the CSL/Mastermind interaction [16, 17]. However, these strategies still have collateral damage to the patients since the total inhibition of the NSP on healthy cells can have contradictory and lethal effects in the long-term [18-20].

Due to the high conservation of the NSP through the metazoan kingdom, inter species experiments have been conducted to corroborate the correct interaction between proteins from D. melanogaster NSP and proteins from the Mus musculus and human NSP, mainly between proteins involved on the transcriptional negative regulation. It has been demonstrated that D. melanogaster Hairless protein, responsible for negative regulation of the NSP at the fruit fly early embryo development is capable to bound, with high affinity, to mammal CSL transcription factor and downregulating the NSP activity[21-23].

The aim of this study was to demonstrate that the ReNeg-AID (Regulation Negative – AID) peptide (Supplement figure 2), derived from the CSL binding domain of the D. melanogaster Hairless protein (Figure 1-A), is capable to modify the transcriptional pattern of genes related to the NSP which are involved in both proliferation inhibition and cell cycle arrest on mammary cancer cells. Interestingly, the transcriptional pattern gene on non-cancer mammary cells (MCF-12F) has not showed changes, but if it caused changes in MCF7 and MDA-MB-231 cells. In summary, the result supported the hypothesis that pReNeg-AID could be employed as an adjuvant together with other anti-cancer therapies on mammary cancer or other cancer related to aberrant expression of Notch-1 cancer which is opening the opportunity to propose a new strategy against breast cancer where the Notch pathway is involved.

Material & Methods

For cell culture: Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Cat. No. 12100046) to MCF-7 and MDA-MB-231 cells, DMEM-F12 Ham (Gibco, Cat. No. 12500096) to MCF12-F cells, fetal bovine serum (FBS, Gibco, Cat. No. 10437028), penicillin, streptomycin, and trypsin-EDTA (supplied by GIBCO-USA). For DMEM-F12 Ham complete growth medium 20 ng/ml of epidermal growth factor (Gibco, Cat. No. PHG0315), 0.01 mg/ml of human insulin and 500 ng/ml of hydrocortisone were added. The plasmid used for cell transfection pFN21K HaloTag® CMV Flexi® (Vector was supplied by Promega™ G2831 protocol guide).

ReNeg-AID peptide

The ReNeg-AID peptide comes from the Hairless (H) protein of *Drosophila melanogaster*, specifically from the binding domain of the Hairless protein to the transcriptional factor Su(H) [CSL]. The aminoacid sequence of pReNeg-AID is determined from position 1987aa-2782aa sequence for the Hairless protein. The ReNeg-AID peptide was cloned into the vector pFN21K HaloTag CMV (pRegNeg-AID) following the manufacturer's specifications (PROMEGA ™), (Figure 1A).

Cell culture

The non-cancer cell line of human epithelial breast MCF-12F (ATCC Cat# CRL-10783, RRID:CVCL_3745) (CRL-10783), tumorous estrogen receptor-positive MCF-7 (CLS Cat# 300273/p2720_MCF-7, RRID:CVCL_0031) (HTB-22) and tumorous triple negative MDA-MB-231 (ATCC® HTB-26™) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (basal medium) at 5% CO_2 and 37°C for 48 h before transfections. The cell lines were

obtained by the ATCC provider at the beginning of the experiments; therefore, these cell lines are new.

Transfection & electroporation

The cell culture was incubated at 5% of CO2 and 37°C until reaching 80% of confluent, the culture was then tripzinated and an 1X106 cells were harvesting, centrifuged until the pill was formed. Then, a 100 µl nucleofection solution (Cell Line Nucleofector® Kit V, protocol number T/C-28a2, supplied by AMAXA®) was prepared with 2 ng of the plasmid pReNeg-AID and 400 µl of OptiMEM medium (supplied by GIBCO-USA, Cat. No. 31985062). The nucleofection solution was added to the cells and were placed on a 4 mm electroporation cuvette in an electroporator system (supplied by BTX® cuvettes & electroporation systems-Harvard Apparatus ECM 630 exponential decay wave electroporation system, item 45-2051) and the following conditions were applied: 140 V, 70 ms with one pulse; then the cuvette with the cell solution was incubated by five minutes at room temperature between 18°C to 25°C and the cell was cultured in a six-well plates with 1.5 ml of supplemented culture media in a humidified 37°C/5% CO2 incubator by 48h.

MTT assay

Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Before 48 hours for transfection cells were cultured at 3×10^3 cells/well in DMEM basal medium and incubated at 37°C in 96-well plates with 5% CO_2. Then, 20 µL of MTT (5 mg/mL in PBS) was added to each well, and cells were incubated for 4 hours at 37°C. The supernatants were removed, 200 µL of dimethyl sulfoxide (DMSO) was added, and the absorbance (595 nm) was determined by a microplate reader (Bio-Rad, Hercules, CA, USA). For each cell line were 3n of the trials.

Luciferase assay

A previous day to transfection, 450,000 cells were seeded on a petri dish, later, the cell was co-transfected with the pGL4xCSL and the pReNeg-AID vectors, following the protocol to Xfect™ (Clontech). The luciferase activity was measured after 24 and 48 hours post transfection following the Dual-Luciferase® Reporter Assay System (Promega) protocol.

Quantitative real time PCR (RT-qPCR)

RT^2 Profiler PCR (Qiagen, Cat. PAHS-059Z, No. 330231) Notch related gene arrays: Total cell RNA was isolated from MCF12-F, MCF-7 and MDA-MB-321, subsequently treated with DNase I and purified using RNeasy Mini Kit (Qiagen, Cat. No. 74034) according to manufacturer's instructions. 25 µg of high-quality total RNA was then reversed transcribed using the First Strand Synthesis Kit (Qiagen Cat. No. 330401) and subsequently loaded on Human Notch RT2 profiler array (PAHS-059Z) according to manufacturer's instructions. The real time PCR was performed by using SYBR Green as a marker for DNA amplification on a thermocycler StepOnePlus™ System (Applied BioSystems, Thermo Fisher Scientific).

Flow cytometry

For flow cytometry the control cells and ReNeg-AID cells were harvesting before the 48 hours post-transfection, a total of $1X10^6$ cells on PBS was dyed with 400 µl of IP and add 50 µl of RNAsa and were incubated for 30 minutes to 1 hour. Data was collected on the Attune™ NXT Flow Cytometer (Thermo Fischer Scientific) using the BL2-H (lin)/Histogram channel to obtain the cell cycler phases graphics with the next parameters: FCS-260 V, SSC-280 V and BL2-360 V. Analysis was performed with FlowJo software V. 10 (Tree Star, Inc).

Statistical analysis

MTT proliferation, luciferase assay and Cytometric analysis was analyzed by Student's t-test, values with $P < 0.05$ were considered statistically significant. All experiments were made at least three times with 4n for each of them.

For gene expression Qiagen's online web analysis tool was utilized to produce comparative scatter plots and fold change was calculated by determining the ratio of mRNA levels to control values using the ΔCt method (2−ΔΔCt). All data were normalized to an average of two of this housekeeping genes, ACTB and GAPDH. All experiments were made at least three times with 4n for each of them.

Results

Luciferase assay and western

The reporter vector pGL4xCSL was designed for control the gene transcription of the luciferase enzyme under the control of one promoter that contains regulatory binding elements from the CSL transcription factor. The co-transfection results show that MCF-7 cells with the pGL4xCSL and pReNeg-AID vectors decreased the enzymatic activity of luciferase until 41% ($p \leq 0.01$).

The molar relation between the transfection vector pGL4xCSL and pReNeg-AID was 1:1 and 1:3 respectively. The luciferase activity was measured after 24- and 48-hours post-transfection. However, there was no significant difference between the readings of luciferase enzyme activity taken at 24 and 48 hours after transfection. The decrease in the luciferase enzyme activity was not drastic, although H is the largest NSP antagonist in Drosophila melanogaster, possibly because the polypeptide coded by the pReNeg-AID vector lacks the co-binding domains for Groucho and CtBP co-repressors. However, the pReNeg-AID vector was able to decrease the luciferase activity (Figure 1B). The Anti-HalgoTag monoclonal antibody (Promega) was used to detect the ReNeg-AID peptide expression (Figure 1C).

MTT proliferation assay

Figure 2A shows the proliferation behavior of MCF-12F cells after transfection with mock vector pFN21K (black line) and pReNegAID vector (blue line), at 12, 24, 36- and 48-hours post-transfection (hpt). The statistical analysis indicated that there are no significant differences between the pReNeg-AID transfected and mock transfected MCF12-F cells. This result means that the ReNeg-AID peptide activity has no effect on non-cancerous mammary gland cells. Figure 2B shows the behavior of MCF-7 cells proliferation after transfection with both mock vector pFN21K (black line) or pReNegAID vector (green line) at 12, 24, 36 or 48 hpt. The statistical analysis indicated significant differences (*) at 36 hpt ($P \leq 0.005$) and 48 hpt ($P \leq 0.005$), causing a decrease in cell proliferation when the ReNeg-AID peptide is over expressed in this cancer cells. In Figure 2C, the MDA-MB-231 cell line presented an irregular proliferation, due to its

genetic background with respect to the NSP that is activated in its non-canonical way. For this reason, the peptide used is not able to control the constitutive activation of the Notch pathway in triple negative cancer cells.

Fold-change gene expression related to inhibition of the NSP and cell differentiation process mediated by the NSP in mammary gland cells

For gene related to inhibition of the NSP the comparative analysis of the transcriptional rate fold-change of *AES, CTNNB1, DTX1, GLI1, NCOR2, POFUT1* and *SMO* genes, MCF-7 control cells vs MCF-7 ReNeg-AID cells 48 hpt (Figure 3A); shown that the over expression of the ReNeg-AID peptide causes down regulation of the expression pattern for *CTNNB1* ($P \leq 0.00004$, -15.22 times), *NCOR2* ($P \leq 0.00004$, -37.14 times), *POFUT1* ($P \leq 0.000002$, -24.54 times) and *SMO* ($P \leq 0.000104$, its expression is dejected) genes. Also, is caused an up-regulation for the expression pattern of AES ($P \leq 0.000105$, 7.05 times), and there were no changes on *DTX1* and *GLI1* genes.

For gene related to differentiation a comparative analysis of the transcriptional rate fold-change shown that *DLL4, DTX1, HES1, HES5, HEY1, HEY2, HEYL, JAG1, NOTCH1* and *NOTCH4* genes, MCF-7 control cells vs MCF-7 ReNeg-AID cells 48 hpt (Figure 3B); shown that the over expression of the ReNeg-AID peptide causes a down-regulations on the expression pattern for *HES1* ($P \leq 0.000553$, -3.08 times), *HEY1* ($P \leq 0.002341$, -3.86 times), *HEY2* ($P \leq 0.00002$, -58.61 times), *JAG1* ($P \leq 0.0012$, -5.53 times) and *NOTCH1* ($P \leq 0.00005$, -30.41 times) genes. Also, is caused an up-regulation on the expression pattern for *DLL4* ($P \leq 0.000004$, 2.26 times). *DTX1, HES5, HEYL* and *NOTCH4* genes there were no changes.

Transcriptional rate fold-change of genes related to the cross talk between Hh and NSP pathways in mammary gland cells

A comparative analysis (Figure 4) of the transcriptional rate fold-change of *GLI1, GSK3B, HES5, NOTCH4, SHH, SMO* and *SUFU* genes, MCF-7 control cells vs MCF-7 ReNeg-AID cells, 48 hpt; shown that the over expression of the ReNeg-AID peptide

causes a down-regulation of the transcriptional expression pattern for *GSK3B* (P ≤ 0.000069, -17.01 times), *SHH* (P ≤ 0.000017, -21.82 times), *SMO* and *SUFU* (P ≤ 0.000286, -7.47 times). Also, is caused an up-regulation of the transcriptional expression pattern for *GLI1, HES5* and *NOTCH4* genes there were no changes.

Transcriptional rate fold change of genes involved at the cross talk between Wnt and NSP pathways in mammary gland cells

A comparative analysis (Figure 5) of the transcriptional rate fold-change of *AXIN1, CTNNB1, FZD2, FZD3, FZD4, FZD7, GSK3B, LRP5, WISP1* and *WNT11* genes, MCF-7 control cells vs MCF-7 ReNeg-AID cells at 48 hpt. The over expression of the ReNeg-AID peptide causes a down-regulation over the gene expression pattern for *AXIN1*(P ≤ 0.000041, the expression is dejected), *CTNNB1* (P ≤ 0.00004, -15.02 times), *FZD2* (P ≤ 0.00247, -2.02 times), *FZD3* (P ≤ 0.00006, -10.61 times), *FZD4* (P ≤ 0.00026, -4.81 times), *GSK3B* and *LRP5* (P ≤ 0.000073 -10.33 times). *FZD7* showed no statistically significant change. *WISP1* and *WNT11* genes there were no changes.

Transcriptional rate fold change of genes related to apoptosis regulation under the control of the NSP in mammary gland cells

A comparative analysis (Figure 6) of the transcriptional rate fold-change of *AXIN1, CFLAR, CTNNB1, FOS, FOSL1, IL2RA, NEURL1, NR4A2* and *PTCRA* genes, MCF-7 control cells vs MCF-7 ReNeg-AID cells 48 hpt. The over expression of the ReNeg-AID peptide causes a down-regulation at the expression pattern for *AXIN1* and *CFLAR* (P ≤ 0.00004, -1348.80 times), *CTNNB1* and *FOS* (P ≤ 0.000176, -3.74 times), *FOSL1* (P ≤ 0.00004, -3.83 times), *IL2RA* (P ≤ 0.000193 its expression is dejected), *NEURL1* and *PTCRA* (showed no significant change).

Transcriptional rate fold change of genes related to cell cycle under NSP control

A comparative analysis (Figure 7A) of the transcriptional rate fold change of *AXIN1, CCND1, CCNE1, CDK1A, JAG2 and NOTCH2* genes, MCF-7 control cells vs MCF-7 ReNeg-AID cells 48 hpt. The ReNeg-AID peptide over expression causes a down-regulation at the expression pattern for *AXIN1, CCND1* ($P \leq 0.0005$, -392.06 times), *CCNE1* ($P \leq 0.00005$, its expression is dejected), *CDK1A* ($P \leq 0.00006$, -50.59 times), *JAG2* ($P \leq 0.000184$, -12.77 times), *NOTCH1* ($P \leq 0.00005$, -30.41 times), *NOTCH2* ($P \leq 0.000007$, -34.62 times) genes.

Flow cytometry and cell cycle

Figure 7B shows the cell cycle histograms of MCF-7 control cells compared with MCF-7 ReNeg-AID cells, 3n experiments with 3 repeats were performed for each condition. G1 phase peak on MCF-7 control throw an average percentage of 39.9 ± 2.6 vs an average of 34.6 ± 2.5 for MCF-7 ReNeg-AID without statistical significance. S phase valley on MCF-7 control throw an average percentage of 26.4 ± 3.6 vs an average of 37.7 ± 0.98 for MCF-7 ReNeg-AID with statistical significance ($P \leq 0.0382$). The G2/M phases peak on MCF-7 control throw an average percentage of 18.1 ± 3.6 vs an average of 6.91 ± 1.4 for MCF-7 ReNeg-AID with statistical significance ($P \leq 0.0183$).

Discussion

Cellular proliferation. It has been demonstrated that the ReNeg-AID peptide has not got any effect on non-cancerous mammary gland cells at any of the tested time, cell proliferation remains unaltered if we compare transfected (blue line) and not transfected (black line) MCF-12F cells (Fig. 2A). On the other hand, the ReNeg-AID peptide presents an effect over the MCF-7 cells proliferation (Fig. 2B, green line). Cells proliferation began to diminish at 36 hpt, and at 48 hpt is clear that the cell proliferation is severely compromised, compared against MCF-7 cell transfected with the mock plasmid (Fig. 2B, black line). All these together are suggesting that the ReNeg-AID peptide in capable to stop cancer cells proliferation and, moreover, it seems that this peptide in somehow is capable to affect cell proliferation, and did not generate changes in gene expression in the MCF-12F cell line (Supplement figure 3).

Gene related with the NSP inhibition: Expression of the ReNeg-AID peptide in MCF-7 cells promoted that the AES gene, which participates in NSP inhibition, will show an increase in its transcription rate, which codifies for the Groucho protein. This protein is one of the mainly pleiotropic co-repressors of the NSP at nuclear level and has been related to the correct expression of the NSP target genes. The binding activation complex structured by the CSL transcriptional factor, Mastermind (MAML) and NICD proteins, is inhibited by the Groucho protein which recruits histone deacetylases has to inhibit the NSP target genes [5, 21, 24]. no changes in DTX1 gene expression were reported, the gene codifies to a protein named Deltex; this protein has been reported to be able to be physically interacting with the Notch-1 receptor avoiding its translocation to the nucleus, and, as a consequence, the NSP target genes will not be expressed (Figure 3A) [6, 25, 26]. The GLI1 gene, which codes for GLI1 protein, in the context of breast cells can negatively regulate NSP by direct physical interaction or cross-talk with the HIF1α factor [27-29], this prevents NICD1 from entering the nucleus and promotes vacuolar proteolysis when the SMO gene, which encodes the Smoothness protein, is negatively regulated and by not presenting changes these proteins normally function in the context of NSP [15, 30, 31].

Also, the ReNeg-AID peptide expression in MCF-7 cells promotes that three genes experiment a reduction in their transcriptional rate. The first one, *NCOR2* gene, which code for the NCoR2 protein, shows a negative regulation; and it has been known that this is a specific tissue repressor for NSP in mammary gland cells. Although its negative regulation, the *AES* positive regulation is capable to compensate the regulatory protein complexes mechanisms involved in the inhibition of NSP (Figure 3A) [32-34]. The second gene, *CTNNB1*, that codes for the β-catenin protein, shows a negative regulation in its transcriptional rate, which could be indicating that the physic interaction reported between Notch-1 and β-catenin was happening when the ReNeg-AID peptide was expressed; this interaction Notch-1/β-catenin promotes the half-live for Notch-1 and Notch-2 in the mammary gland (Figure 3A) [35, 36]. The third is the *POFUT1* gene, which encodes the OFUT1 protein, which is responsible for a correct ligand-receptor interaction in the NSP transduction mechanism; its negative regulation promotes control at the membrane level to the NSP in a negative way when an interaction between the Jagged-1 ligand and the Notch-1 receptor is established [37-39].

Gene related to NSP and cell differentiation: A negative regulation was observed for the *JAG1* and *NOTCH1* genes, which encodes Jagged-1 and Notch-1 proteins respectively. Overexpression of ReNeg-AID promoted the decrease of ligand / receptor interaction, so that the NSP target genes such as *HES1*, *HEY1* and *HEY2* have a negative regulation. This phenomena would be suggesting that the mammary gland cancer cells comes to a dedifferentiated state in order to recover the normal control of cell cycle and apoptosis [2, 40]. On the other hand, a positive regulation of *DLL4* gene has been observed. *DLL4* code for Delta-4 ligand proteins, this positive regulation at mammary gland cancer cells seems to be promoting the establishment of Delta-4/Notch-4 (ligand/receptor) interaction, in order to try to take control in the absence of Jagged-1/Notch-1 interaction, although it has been shown that Notch-4/Delta-4 is not able to recover the whole cell process mediated by Jagged-1/Notch-1 interaction [26, 41].

When the ReNeg-AID peptide was over expressed at breast cancer cells, *Hes5* did not present changes, promoting the direct cross talking between vessel cells and epithelial

cells through Notch-4/Delta-4 interaction which, in consequence, promotes a normal *de novo* angiogenesis process [42, 43] but the new vessels will not be functional because the Notch-1/Jagged-1 interaction is not occurring and it is necessary to complete the whole vessel differentiation process. This change in the expression pattern of *Notch4* and *Notch1* genes interrupts the differentiation of the Tip/Stalk cells, where the expression of Notch-1 gene is required to get a fully functional vessel cells (Fig. 3B) [42, 44-46].

Cross talking between NSP and Hh pathways: It had been observed that ReNeg-AID peptide overexpression promotes a negative regulation of *GSK3B*, *SHH*, *SMO* and *SUFU* genes (Fig. 4), which encodes for GSK-3β, Sonic Hedgehog, Smoothened and SUFU proteins, respectively. The negative regulation of Smoothened and Sonic Hedgehog proteins has been related to the progenitor cancer cells population reduction [31, 47, 48]. This phenomena, together with the negative regulation of SUFU and GSK-3β proteins, both of them negative regulators of the Hh pathway, prevents the GLI1 and GLI2 proteins degradation, which is essential for the correct differentiation process of progenitor mammary gland cells [49-51]. As a consequence, the GLI1 protein cytoplasm accumulation increases the amount of HIF1α protein in cytoplasm which avoid the NICD1 translocation to the nucleus [15, 52]. All this together has been related with a diminished cell migration and EMT decrease on mammary cancer cells led by Hh pathway and would suggest a cross talking with the γ-secretase activity by the Notch-1 overexpression [53, 54].

Cross talking between NSP and WNT pathways: It had been observed a diminished transcriptional rate of those genes encoding AXIN and β-catenin proteins (Fig. 5). It has been known that the interaction between these proteins results in a transcriptional complex that triggers the expression of genes dependent on the WNT pathway activity, which is involved in cell cycle and cell growth. When the ReNeg-AID peptide was overexpressed, the complex AXIN/β-catenin is not formed. In normal cells, when the β-catenin protein is released from the AXIN/β-catenin complex, it interacts with the NICD present at cell cytoplasm, avoiding its degradation. In these cancer cells the β-catenin in cytoplasm is also diminished by the ReNeg-AID peptide overexpression, which

shrinkage its interaction with NICD causing its degradation which, instead, cause a negative transcriptional regulation of the NSP target genes [35, 55, 56].

It had been observed that the ReNeg-AID peptide overexpression promotes a negative regulation for *FZD2, FZD3, FZD4*, and *LRP5* genes. Therefore, a WNT signaling pathway inactivation occurs because the receptors Frizzled 2 (FZD2), Frizzled 3 (FZD3) and Frizzled 4 (FZD4) are diminished in their membrane concentration. In contrast, the *WNT11* and *WISP1* genes did not show changes, which has not any receptor to interact with. This effect is potentiated by the lower LRP-5 protein concentration, which has the important roll to stabilize ligand/receptor interaction in the WNT pathway context. All this together could be meaning that the WNT pathway is not activated by the deficiency of receptors. Nevertheless, at the same time the ReNeg-AID peptide overexpression on MCF-7 cell promotes a deficiency of the *CTNNB1* gene transcription, avoiding the presence of β-catenin in cell cytoplasm, which instead prevents its translocation into the nucleus, promoting the expression of WNT pathway target genes. The decrease of β-catenin in the cytoplasm promotes a cytoplasmic NICD half-live decrement, because NICD are caught by proteasomes [35, 36, 44, 57].

NSP and apoptosis: A negative regulation of *CFLAR*, *FOS*, *FOSL*, *NR4A2* and *IL2RA* genes was observed (Fig. 6) by the ReNeg-AID peptide overexpression, in MCF-7 cells. Those genes encode for FADD-L1, C-Fos, Fra1, Nurr1 and CD25 proteins, respectively. The ReNeg-AID peptide overexpression is suggesting that FADD-L1/C-Fos/Fra1 interaction is able to be positively regulating the activation mechanism of FAS/FADD apoptotic receptors by the extrinsic way, together with the Nurr1 and CD25 regulation on MCF-7 cells [54, 58]. However, it is known that the NSP is able to regulate the intrinsic apoptotic pathway by the expression of *NEURL1* and *PTCRA* genes. It is also known that these proteins are involved in the differentiation processes and in the negative regulation of the NSP, promoting the regulation of the apoptotic processes mediated by PUMA and Bcl-2 proteins. Nevertheless, it is clear that more experimental data is necessary to elucidate the participation of these proteins in the apoptotic processes regulation in MCF-7 cells carried out by NSP [14, 59].

Cell cycle and NSP: In the Figure 7A has observed a negative regulation of *AXIN1*, *CNND1*, *CNNE1*, *CDK1A*, *JAG2*, *NOTCH1* and *NOTCH2* genes, which are involved in cell cycle. These genes are encoding for Axin1, cyclin D1, cyclin E1, CDK1, Jagged-2, Notch-1 and Notch-2 proteins. It has been reported that in MCF-7 cells the Jagged-2/Notch-2 interaction promotes the cell cycle initiation mediated by the activity of Cyclin D1 [11]. In breast cancer the Notch-1 constitutive overexpression promotes the Notch-2 receptor overexpression and, in consequence, the expression of the NSP target genes. One of these genes are *CNND1* (Cyclin D1) which is responsible of the cell cycle G1/S phase transition. The suggested Cyclin D1 overexpression could be promoting a G1/S checkpoint malfunction and, in consequence, a loop cell cycle that will eventually lead the mammary gland cells to an uncontrolled cell proliferation. The ReNeg-AID peptide overexpression in MCF-7 cells negatively regulates the overexpression of both, Notch-2 and Jagged-2 proteins; and therefore promotes the negative regulation of Cyclin D1 and Cyclin E1 [60, 61]. This negative regulation of those proteins arrests the cell cycle at G1/S phase on MCF-7 cells (Fig. 7B), where the cell population is mainly arrested in the S phase, causing as a consequence a diminished proliferation. Finally, it had been observed that a down regulation of *CDK1A* gene transcription, caused by the ReNeg-AID peptide overexpression, in MCF-7 cells should be causing an instability of the cell cycle associated with an early apoptotic activation process. This could meaning that the combined down regulation of both *CDK1A* and *CNND1* genes, promotes the cell cycle arrest of mammary gland cancer cells [12, 62, 63].

Overexpression of ReNeg-AID peptide on MCF-7 cells regulates negatively the constitutive expression of Notch-1 receptor at different levels. On cellular membrane level regulated negatively the Jagged1/Notch-1 pathway by the negative regulation of POFUT1 and the normal expression of DTX1. At cytoplasmic level, negatively regulates the half-lives of the Notch receptor by the negative regulation of β-catenin and Axin1 and by the normal expression of the GLI1 protein. At nuclear level, the activation complex protein between CSL/MAML/NICD is negatively regulated by the positive expression of Groucho protein and by the very nature of the ReNeg-AID peptide. The most significant effect of the ReNeg-AID overexpression occurs in the cell cycle, the

MCF-7 cells were arrested in the G1/S phase by the negative regulation of Cyclin D1 and Cyclin E1, and since the effect of ReNeg-AID peptide expression on normal cells (MCF-12F) does not cause significant effects on the pattern genes related to the Notch pathway. The effect of the ReNeg-AID peptide overexpression opens the doors for future research based on the negative regulation at nuclear level in cancer cells that present a constitutive activation of the Notch signaling pathway and that can be used as an alternative adjuvant strategy against breast cancer. It remains to analyze the possible routes of administration and/or action, by which the peptide ReNeg-AID can have an effect in vivo. The results of the MDA-MB-231 cell line were performed as with the MCF-7 and MCF-12F cell line, however, the data is not shown as conclusive results due to the nature of its expression of the Notch pathway since it presents a non-canonical signaling of the Notch pathway and merits more detailed studies to solve that question.

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Availability of data and materials

All data from this study are included within this article.

Ethics approval and consent to participate

No applicable

Consent for publication

Not applicable.

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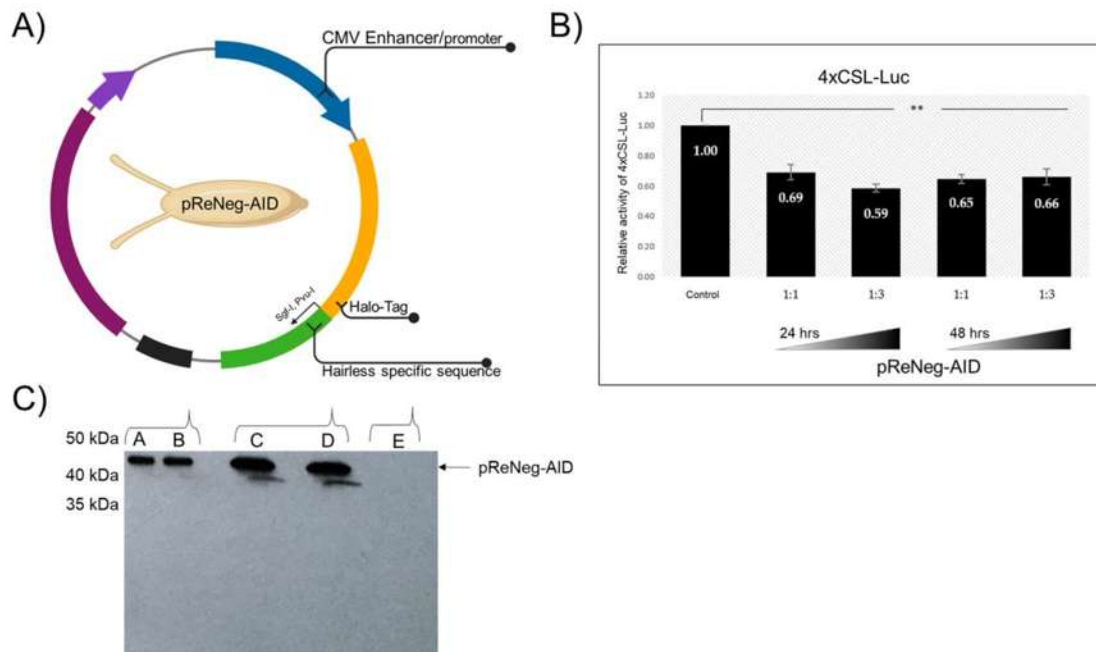


Figure 1. A) Plasmid map of ReNeg-AID. The pFN21K HaloTag® CMV Flexi® Vector (Promega) was used to design the pReNeg-AID. From *Drosophila melanogaster* larvae, the Hairless sequence that joins the CSL transcriptional factor to form a protein repression complex of NSP was obtained and tagged with the HaloTag flag. **B) The vector pReNeg-AID decreases Luciferase activity.** MCF-7 cells were transfected in a molar ratio 1:1 and 1:3, respect to pGL4xCSL and pReNeg-AID vectors at 24 and 48 hours. Relative activity of luciferase was calculated as the luciferase activity of firefly against luciferase activity of Renilla. Each bar represents ± EST of average for triplicated experiments. Values for P were determined for t test from Student (**p ≤ 0.01 vs control. n=4). **C) pReNeg-AID Western.** The ReNeg-AID peptide is bound to a HaloTag flag (Promega) that uses monoclonal antibodies to be detected, the peptide bound to the tag has a relative weight of 45 kDa; lane A and B shows the expression in MCF-7 cells of the mock vector without the fragment of the specific sequence of the Hairless protein; C and D show the expression in MCF-7 cells of the vector containing the fragment of the specific sequence of the Hairless protein bound to HaloTag and lane E shows the negative control in MCF-7 cells.

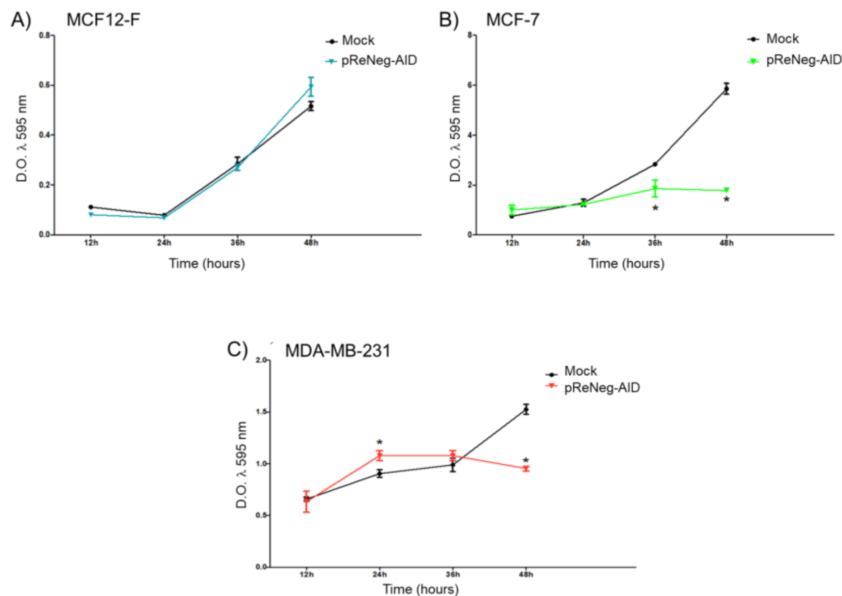


Figure 2. Effect of ReNegAID overexpression on proliferation of MCF12-F, MCF-7 and MDA-MB-231 cells. *A*) shows proliferation of MCF12-F cells after transfection with pFN21K (Mock) or pReNegAID for 12, 24, 36 or 48 h. *B)* Shows proliferation of MCF-7 cells after transfection with pFN21K (Mock) or pReNegAID for 12, 24, 36 or 48 h. C) Shows proliferation of MDA-MB-231 cells after transfection with pFN21K (Mock) or pReNegAID for 12, 24, 36 or 48 h. Both determined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Significant differences in *, control cells vs transfected cells ($P < 0.05$) between its respective control (one-way ANOVA and Tukey´s test). (n=4 individual samples).

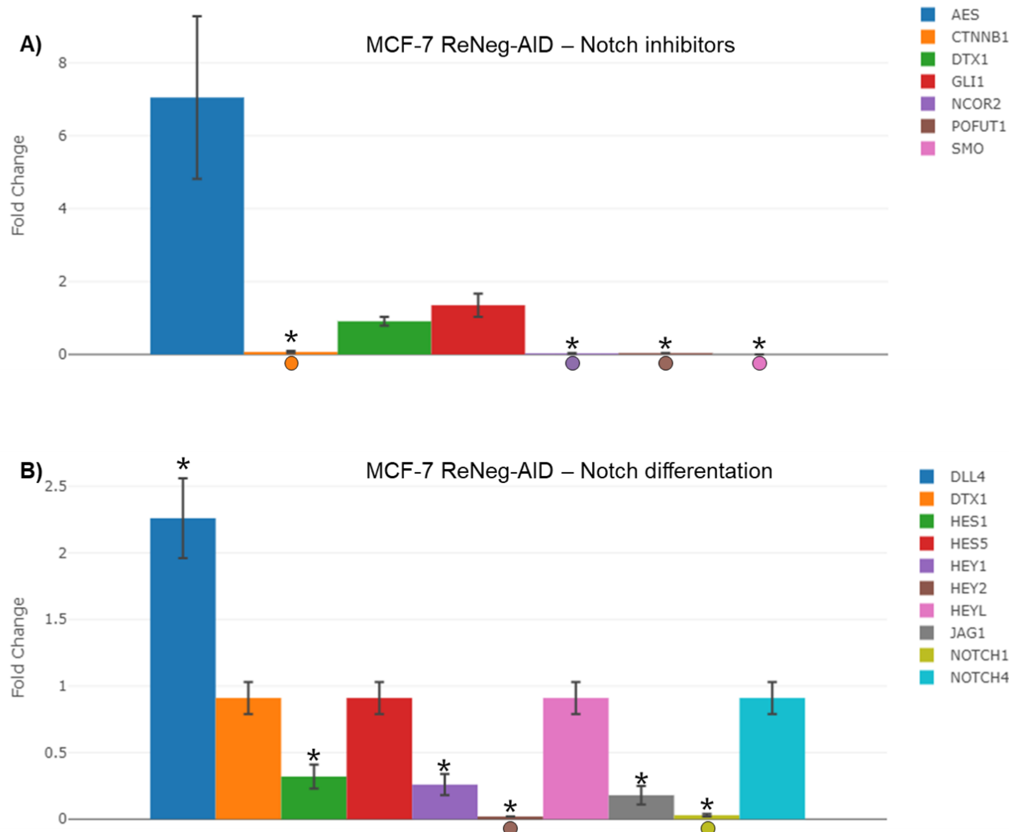


Figure 3. Profile of gene expression related to differentiation and Inhibition of Notch Pathway after transfection with pRegNegAID on MCF-7 cells in contrast with Mock transfected cells. *A)* Fold change of the expression of genes related to the inhibition of NSP. mRNA for *AES*, *CTNNB1*, *DTX1*, *GLI1*, *NCOR2*, *POFUT1* and *SMO* was quantified by qPCR method. *B)* Fold change of the expression of genes related to cellular differentiation. mRNA for *DLL4, DTX1, HES1, HES5, HEY1, HEY2, HEYL, JAG1, NOTHC1* and *NOTCH4* were quantified by qPCR. In all cases mRNA was quantified using a real-time PCR method (RT^2 Profile^{TM} PCR Array Human Notch Signaling Pathway, Qiagen). *ACTB* and *GAPDH* served as internal control and was used to normalize for differences in input RNA, the fold change threshold was cut-off in 2. Significant differences in *, MCF-7 transfected cells (*P* < 0.05) between its respective control (one-way ANOVA and Tukey´s test). (n=4 individual samples).

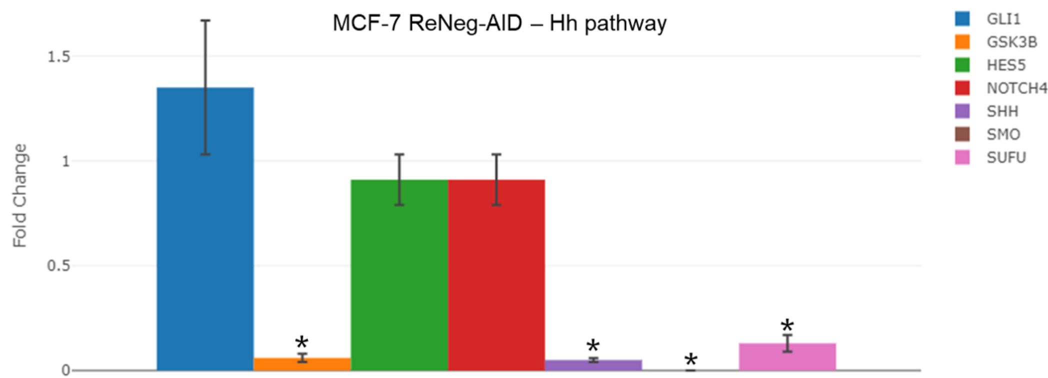


Figure 4. Profile of gene expression of genes interconnected between Notch and Hh pathways, after transfection with pRegNegAID on MCF-7 cells in contrast with Mock transfected cells. *A)* Fold change of the expression of genes interconnected between Notch and Hh signaling pathway. mRNA for *GLI1, GSK3B, HES5, NOTCH4, SSH, SMO* and *SUFU* was quantified by qPCR method. mRNA was quantified using a real-time PCR method (RT^2 Profile^{TM} PCR Array Human Notch Signaling Pathway, Qiagen). *ACTB* and *GAPDH* served as internal control and was used to normalize for differences in input RNA, the fold change threshold was cut-off in 2. Significant differences in *, MCF-7 transfected cells ($P < 0.05$) between its respective control (one-way ANOVA and Tukey´s test). (n=4 individual samples).

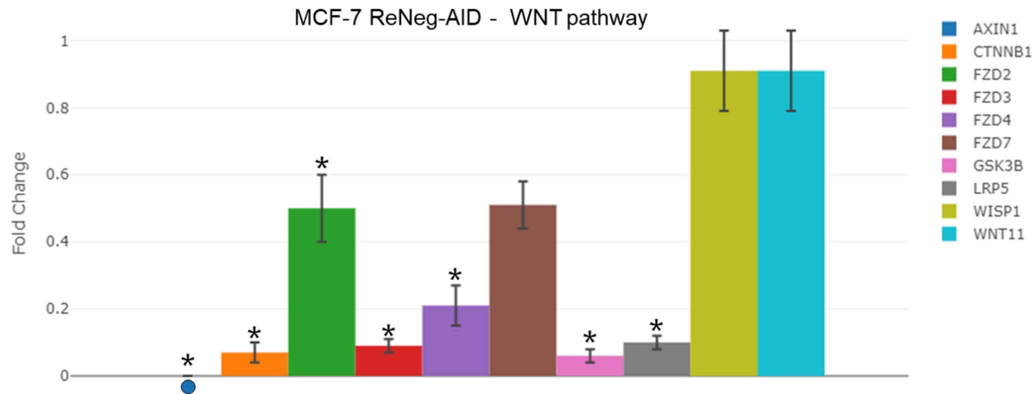


Figure 5. Profile of gene expression of genes interconnected between Notch and Wnt pathways, after transfection with pRegNegAID on MCF-7 cells in contrast with Mock transfected cells. *A)* Fold change of the expression of genes interconnected between Notch and Hh signaling pathway. mRNA for *AXIN1, CTNNB1, FZD2, FZD3, FZD4, FZD7, GSK3B, LRP5, WISP1* and *WNT11* was quantified by qPCR method. mRNA was quantified using a real-time PCR method (RT^2 Profile^{TM} PCR Array Human Notch Signaling Pathway, Qiagen). *ACTB* and *GAPDH* served as internal control and was used to normalize for differences in input RNA, the fold change threshold was cut-off in 2. Significant differences in *, MCF-7 transfected cells ($P < 0.05$) between its respective control (one-way ANOVA and Tukey´s test). (n=4 individual samples).

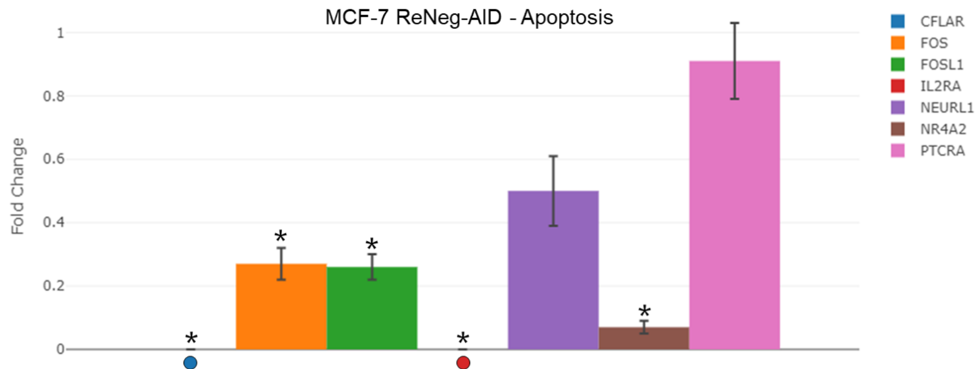


Figure 6. **Profile of gene expression of genes related to regulation of the apoptosis by the control of Notch Signaling Pathway**. *A)* Fold change of the expression of genes related to the regulation of the apoptosis. mRNA for *AXIN1, CFLAR, CTNNB1, FOS, FOSL1, IL2RA, NEURL1, NR4A2* and *PTCRA* was quantified by qPCR method. mRNA was quantified using a real-time PCR method (RT^2 Profile^{TM} PCR Array Human Notch Signaling Pathway, Qiagen). *ACTB* and *GAPDH* served as internal control and was used to normalize for differences in input RNA, the fold change threshold was cut-off in 2. Significant differences in *, MCF-7 transfected cells ($P < 0.05$) between its respective control (one-way ANOVA and Tukey´s test). (n=4 individual samples).

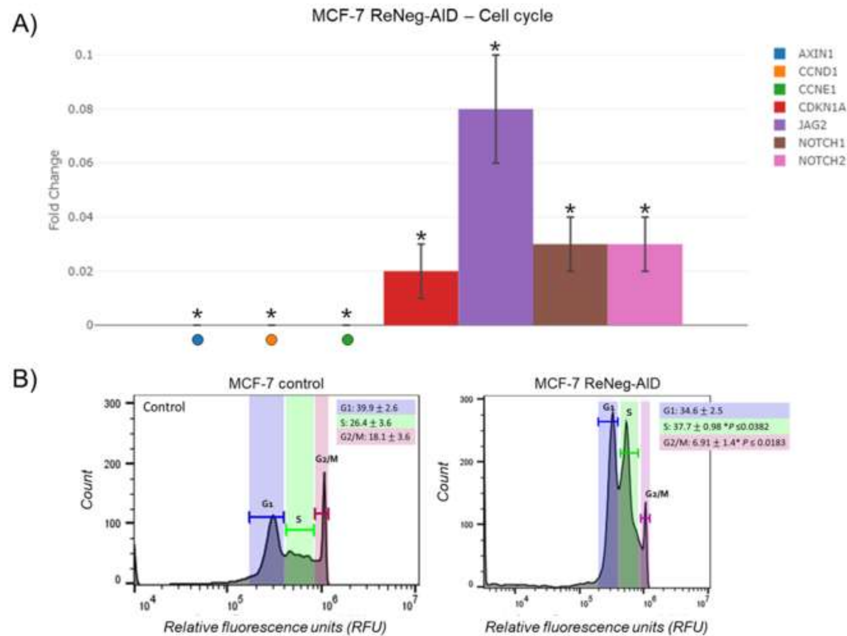
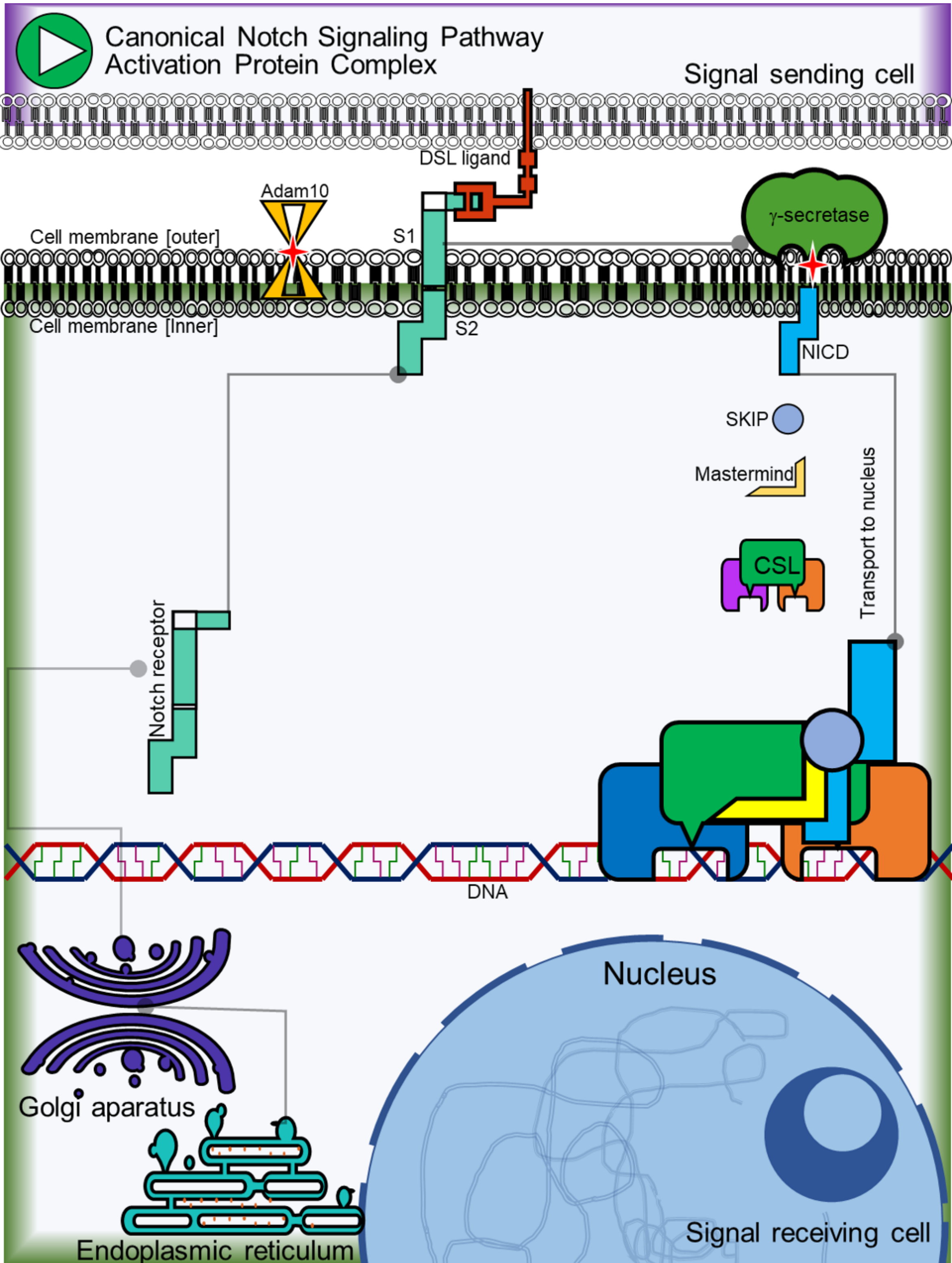
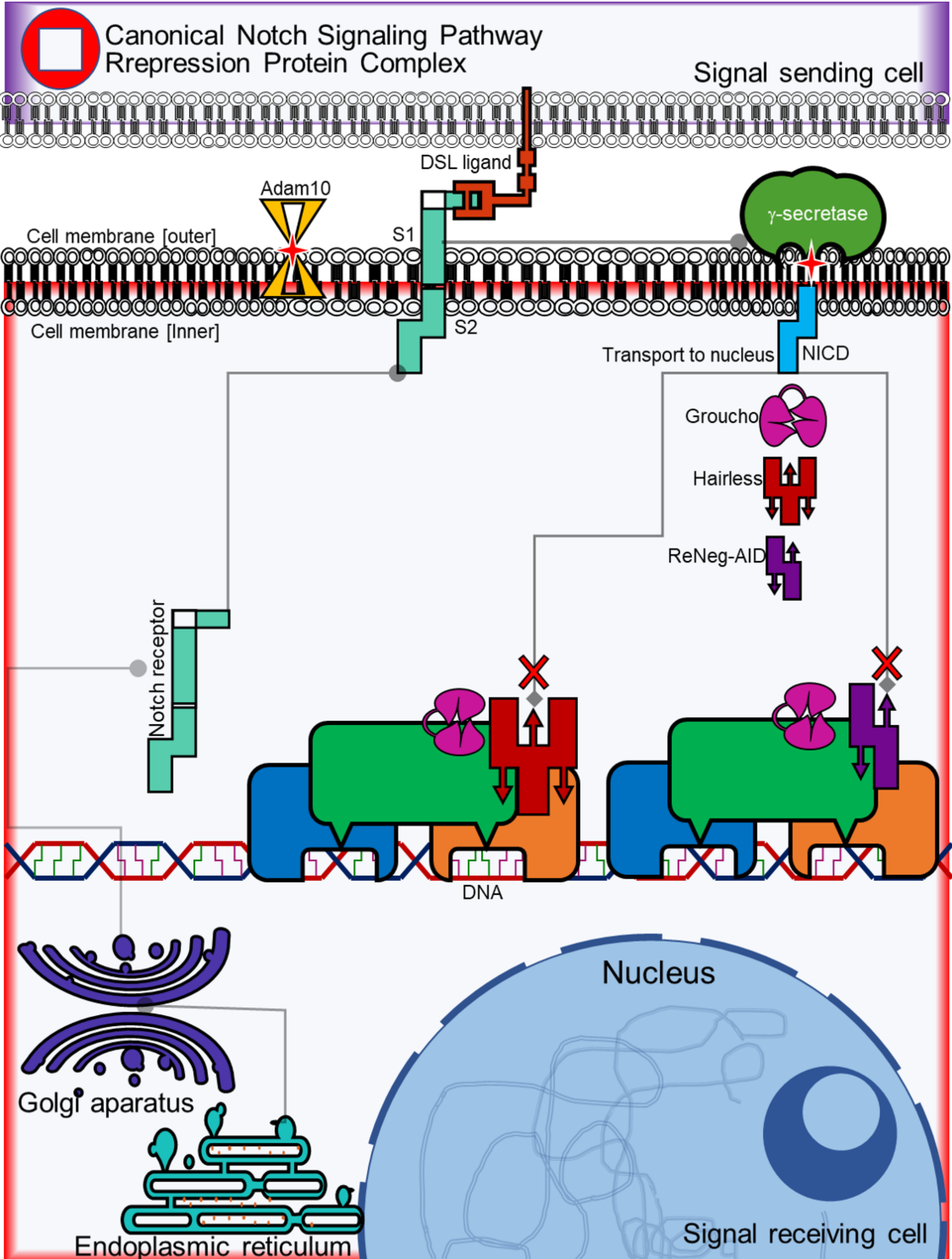


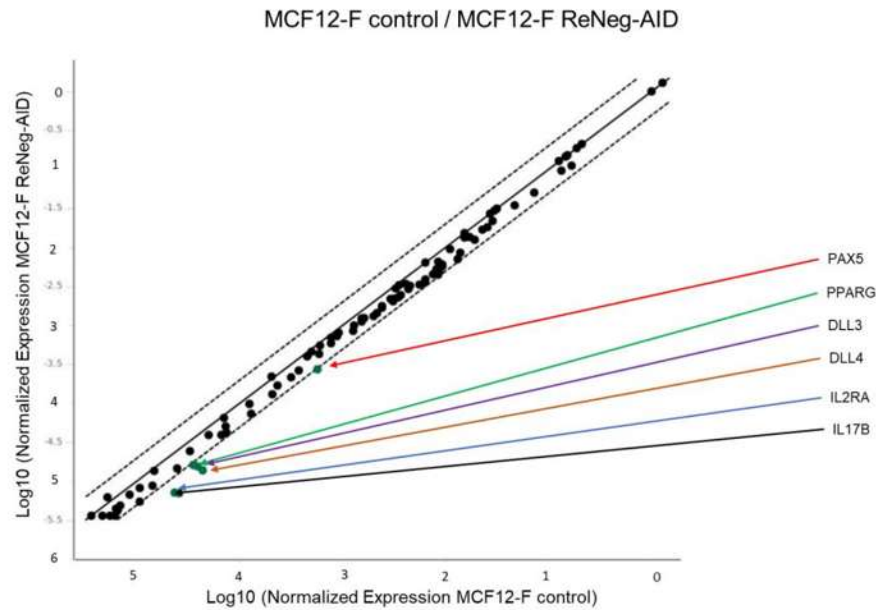
Figure 7. Profile of gene expression of genes related to cell cycle control and analysis of cell cycle arrest. *A)* Fold change of the expression of genes related to the cell cycle control. mRNA for *AXIN1, CCND1, CCNE1, CDKN1A, JAG2* and *NOTCH2* was quantified by qPCR method. mRNA was quantified using a real-time PCR method (RT² Profile™ PCR Array Human Notch Signaling Pathway, Qiagen). *ACTB* and *GAPDH* served as internal control and was used to normalize for differences in input RNA, the fold change threshold was cut-off in 2. Significant differences in *, MCF-7 transfected cells ($P < 0.05$) between its respective control (one-way ANOVA and Tukey´s test). (n=4 individual samples). *B*) Cell cycle analysis. After 48 h cell transfection Mock and pRegNegAID respectively, the cells were pooled, stained with Propidium Iodide (PI), and analyzed by flow cytometry as described in the Materials and Methods section. Each histogram shows a flow cytometric plot of 10,000 cells per sample and is representative of three independent experiments. The percentage of cells (mean ±S.D.) in G1, S, and G2/M phases is listed. Significant differences in *, MCF-7 control vs MCF-7 transfected cells ($P < 0.05$) between its respective control (one-way ANOVA and Tukey´s test).


Canonical Notch Signaling Pathway
Activation Protein Complex
Signal sending cell
DSL ligand
Adam10
S1
γ-secretase
Cell membrane [outer]
Cell membrane [Inner]
S2
NICD
SKIP
Mastermind
Transport to nucleus
CSL
Notch receptor
DNA
Nucleus
Golgi aparatus
Endoplasmic reticulum
Signal receiving cell

Supplement figure 1. NSP canonical activation protein complex. The Notch receptor in mammals (Notch1-4) undergoes post-transcriptional modifications in the endoplasmic reticulum and in the Golgi apparatus where it is finally granted the specificity of binding by its ligands of the DSL family (Delta, Serrate and Lag-1). Once the Notch receptor is found on cell membrane it is recognized by the ADAM10 / TACE metallopreotease that makes a proteolytic cut by separating the extracellular domain of the Notch protein once it is bound to its ligand (S1). The Notch intracellular domain (NICD) is then released from the cell membrane by the action of g-secretase (S2). Once NICD is released from the cell membrane it is directed to the nucleus where it forms an activation complex by recruiting the Mastermind and SKIP co-activators to bind to the CSL transcriptional factor and initiate the expression of genes dependent on the Notch pathway like Hes and Hey family genes, CCND1, CCND3, Notch receptor and Notch ligands.


Canonical Notch Signaling Pathway
Rrepression Protein Complex
Signal sending cell
DSL ligand
Adam10
Cell membrane [outer]
S1
γ-secretase
Cell membrane [Inner]
S2
Transport to nucleus
NICD
Groucho
Hairless
ReNeg-AID
Notch receptor
DNA
Nucleus
Golgi aparatus
Endoplasmic reticulum
Signal receiving cell

Supplement figure 2. ***NSP repression protein complex***. The Hairless protein competes for the union of the CSL transcriptional factor against NICD with a similar binding affinity to form a repression complex with the help of co-repressor Groucho. The ReNeg-AID peptide having part of the CSL binding domain of the Hairless protein competes in the same way against NICD to form a repression complex and change the expression pattern of genes dependent on the Notch pathway.



Supplement figure 3. Scatter Plot profile of the gene expression related to Notch Signaling Pathway on MCF-12F cells. mRNA was quantified using a real-time PCR method (RT^2 Profile^{TM} PCR Array Human Notch Signaling Pathway, Qiagen). *GAPDH* served as internal control and was used to normalize for differences in input RNA. No significant differences were detected in MCF-12F control vs MCF-12F transfected cells between its respective control (one-way ANOVA and Tukey´s test). (n=4 individual samples). Only *PAX5, PPARG, DLL3, DLL4, IL2RA* and *IL17B* gene were regulated negatively without significant differences.

Efecto de la sobrexpresión del péptido ReNeg-AID en células de cáncer de mama triple negativo MDA-MB-231: La vía no canónica de Notch.

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Resumen: La vN posee dos mecanismos generales de regulación, su vía canónica; donde estrictamente ocurren los tres cortes proteolíticos para liberar el dominio intracelular de Notch (DIN) de la membrana celular y asi DIN pueda formar los complejos de activación o represión con su factor transcripcional CSL. La vía no canónica es conocida como la vía independiente de CSL para la activación de sus genes diana, sin embargo, los genes blancos que regula la vía no canónica no pertenecen a la familia de genes *HER, HES* y *HERP* y esta activación se ha relacionado con procesos oncogénicos asi como en la activación de las células inmunitarias. Debido a la naturaleza del péptido ReNeg-AID, que regula de manera negativa la vía canónica de Notch, las interacciones en células cancerígenas triple negativo con una vía de Notch activa de forma no canónica al ser transfectadas con el plásmido pReNeg-AID presentaron un nuevo orden en el patrón de expresión de genes bajo la señalización de la vía Notch; esto provocó una reconfiguración en el aumento del nivel de expresión de los receptores Notch-1 y Notch-3 y una disminución para los receptores Notch-2 y Notch-4; esto podría indicar que la identidad celular mediada por la vN está provocando un cambio en el fenotipo epitelial de la línea celular MDA-MB-231 a un fenotipo mesenquimal no funcional. El efecto del péptido ReNeg-AID tambien promovió la sobrexpresión de los ligandos Delta-3 y 4, Jagged 1 y 2, probablemente como un intento de recuperar el mecanismo canónico de la vN bajo el fondo genético caótico de este tipo celular. Finalmente, la sobrexpresión del péptido ReNeg-AID promovió una regulación negativa del factor transcripcional CSL, esto origina que la señalización no canónica de la vN sea más intensa y tenga un oscilación o combinación entre el mecanismo canónico y no canónico de la vN debido a la activación de los receptores Notch-1 y 3 y la represión de Notch-2 y 4. Aunado a esto se debe sumar las interacciones que existen entre la vN con las otras vías de señalización que de igual manera puedan estar en un estado de desregularización en el microambiente del cáncer.

Palabras clave: Notch canónico, Notch no canónico, MDA-MB-231, cáncer de mama, regulación negativa de Notch.

Introducción

La vía no canónica de Notch (vN$^{\chi}$) fue descrita inicialmente por Aster y Pear durante la década de los años 90's; ellos descubrieron una translocación cromosómica del dominio intracelular del receptor Notch-1 en leucemia linfoblástica aguda de células T provocando una activación constitutiva de la vN. Esta activación constitutiva de la vN provoca que las células T sean incapaces de diferenciarse y de activarse en su forma Th1, Th2 y Th17, ya que la activación de las células T requieren el mecanismo de la vN$^{\chi}$ para activar a las interleucinas específicas para cada una de las formas de activación en células T. A partir de este descubrimiento de un nuevo mecanismo de la vN se extendió su estudio a más tipos celulares como pulmón, ovarios, sistema sanguíneo, hueso y epitelio; y esto coincide con órganos y sistemas donde la vN$^{\chi}$ o la vN se encuentran desreguladas o han cambiado sus ritmos de activación ocasionados por un evento carcinogénico (1,2).

Se han postulado 2 mecanismos que pueden activar la vN$^{\chi}$ y son:

- ***La activación dependiente o independiente de ligando***. En este escenario el receptor Notch interactúa físicamente en la membrana con ciertas proteínas de otras vías de señalización como Hh, WNT, NF-kB o β-catenina, o bien, una vez es procesado proteolíticamente por el complejo de las γ-secretasas y es liberado como DIN; en el transcurso de la membrana al núcleo, DIN interactúa con las mismas proteínas antes mencionadas, esto hace que la vN$^{\chi}$ sea tambien dependiente e independiente de las γ-secretasas (3–5).
- ***La activación independiente de CSL***. Esta activación está basada en la capacidad del receptor Notch por unirse con ciertos factores transcripcionales y amentar su vida media en citoplasma, como HIF-1a, YY1 o β-catenina; esta interacción promueve que los genes diana de estos factores transcripcionales sean activados, esto provoca que la retroalimentación entre vías de señalización tenga un nivel más estricto pero eficaz de regulación homeostática (6,7).

El papel de la vN es estrictamente necesario en el estado embrionario, pero la vN$^{\chi}$ parece ser exclusiva en procesos de organismo adultos totalmente diferenciados y actúa en procesos regulatorios de la misma vN y de otras vías como NF-κB, PI3K, AKt, mTOR, Hh y WNT, tambien está involucrada en procesos regulatorios de la apoptosis por medio de la proteína BCRA y con interacciones físicas con la Mitocondria, relacionado con la regulación del metabolismo de células cancerígenas bajo la interacción con β-catenina (8–10).

Los mecanismos de activación y regulación de la vN, en su mayoría, están totalmente descritos, sin embargo, los mecanismos de la vN$^{\chi}$ son confusos por su naturaleza ambigua de interactuar con muchas proteínas de otras vías de señalización. Esta naturaleza intrincada ha proporcionado nuevas formas de regulación para la propia vN y para los eventos celulares involucrados como diferenciación, apoptosis, ciclo celular y metabolismo. La importancia de comprender la vN$^{\chi}$ nos podría dar una nueva perspectiva de entender un nuevo mecanismo de regulación genética, que no sea exclusivo de la vN si no en la compleja red de interacciones que se dan en segundo plano entre muchas vías de señalización para mantener y regular los procesos vitales, si este mecanismo tan delicado es alterado, al igual que con cualquier proceso regulatorio provocará complicaciones en el funcionamiento y homeostasis del organismo.

Materiales y Métodos

- *Los materiales y métodos usados en este capítulo son los mismos que se incluyen en el capítulo número 2 “Resultados” excluyendo los análisis de citometría de flujo y ensayos de luciferasa*.

Resultados y Discusión

Veces de cambio en la expresión de genes relacionados con inhibidores de la vN y diferenciación celular.

Los genes relacionados con la inhibición de vN analizados fueron: *AES, CTNNB1, DTX1, GLI1, NCOR2, POFUT1* y *SMO*. La figura 1A muestra el efecto de la sobrexpresión del péptido ReNeg-AID en células MDA-MB-231$_{control}$ contra MDA-MB-231$_{ReNeg\text{-}AID}$ 48 horas post-transfección. Los genes *AES*, *DTX* y *GLI1* presentaron una regulación positiva con **8, 3 y 6** veces de cambio con un valor de *p* de **0.002337**, **0.00004** y **0.0008** respectivamente. El gen *NCOR2* presento una regulación negativa con -3 veces de cambio con un valor de *p* de 0.0005. Los genes *CTNNB1*, *POFUT1* y *SMO* no presentaron cambios significativos en este tipo celular. La regulación positiva de *DTX, AES* y la regulación negativa de *NCOR2* es un indicativo de que la vN canónica está siendo regulada negativamente a nivel de la formación de complejos proteicos por la acción de *AES* y a nivel citoplasmático por la acción de *DTX*, sin embargo, el gen *GLI1* pudiera estar promoviendo la expresión del factor HIF-1α de la vía Hh y asi contribuyendo a la regulación positiva de la vN^{χ}; si bien, el efecto de la interacción del receptor Notch con HIF-1α favorece la expresión de genes hacia la respuesta por estrés oxidativo como *PUMA* o *BCRA*, tambien se ha reportado que el factor HIF-1α regula a su vez la intensidad de la expresión de genes dependientes de NF-κB involucrados en la respuesta de reconocimiento del sistema inmune como la interleucina 6 y 17 asi como el metabolismo de las células cancerosas. El hecho que *CTNNB1*, *POFUT1* y *SMO* no presentaran un cambio su expresión por la transfección del péptido ReNeg-AID podría estar indicando que el estado aberrante de la vía WNT no ha sufrido cambios en este tipo celular; el caso del gen *POFUT1* sugiere correlación entre la vía dependiente de ligando que la vN^{χ} puede ejercer (4,9,11).

Los genes relacionados con la diferenciación analizados fueron: *DLL4, DTX1, HES1, HES5, HEY1, HEY2, HEYL, JAG1, NOTCH1* and *NOTCH4*. La Figura 1B muestra el efecto de la sobrexpresión del péptido ReNeg-AID en células MDA-MB-231$_{control}$ contra MDA-MB-231$_{ReNeg\text{-}AID}$ 48 horas post-transfección. El gen *DLL4* y *JAG1* presentaron una regulación positiva de **7.35** y **2.54** veces de cambio con un valor de *p* de **0.000022** y **0.0021** respectivamente; el gen *NOTCH1* presento una regulación positiva de **3.05** veces de cambio con un valor de *p* de **0.00001**, mientras que el gen *NOTCH4* presento una regulación negativa de **-5.09** veces de cambio con un valor de *p* de **0.00005**. Esta discrepancia entre la expresión positiva de los ligandos Delta4, Jagged1 y del receptor Notch-1 pero una expresión negativa del receptor Notch-4 estaría sugiriendo que la vN$^{\chi}$ está siendo promovida en su forma dependiente de ligando con una frecuencia mayor, sin embargo, la expresión positiva del gen *HES1* y *HES5* (**7.35** y **5.25** veces de cambio con un valor de *p* de **0.00001** y **0.00003** respectivamente) sugiere que la vN canónica está siendo favorecida con la expresión del péptido ReNeg-AID y esto sugiere una regulación e intento de recuperar la identidad celular en este tipo de tejido glandular mamario, no obstante son necesarios más pruebas para elucidar por completo la maquinaria no canónica de la vN y sus repercusiones finales con las demás vías de señalización relacionadas con la diferenciación celular (12–14). La expresión de los genes *HEY1, HEY2* y *HEYL* no presentaron cambios significativos, esto coincide con lo reportado para el fenotipo diferencial de este tipo celular (12,15,16).

Los genes de la vN relacionados con la vía Hh analizados fueron: *GLI1, GSK3B, HES5, NOTCH4, SHH, SMO* y *SUFU*. La Figura 2A muestra el efecto de la sobrexpresión del péptido ReNeg-AID en células MDA-MB-231$_{control}$ contra MDA-MB-231$_{ReNeg\text{-}AID}$ 48 horas post-transfección. Los genes que presentaron cambios significativos con una regulación positiva en sus veces de cambio como *GLI1* y *HES5*, aunado a la regulación negativa del gen *NOTCH4* sugiere que el efecto regulatorio que se da entre la vN y la vía Hh por medio del recetor Notch-4/HIF-1α está siendo negativamente regulada, implicando que la migración celular o el comportamiento metastásico reportado por esta interacción este siendo promovido, sin embargo, se presentó una regulación positiva del gen *SUFU* con **14.58** veces de cambio y un valor de p de **0.000617**; esta

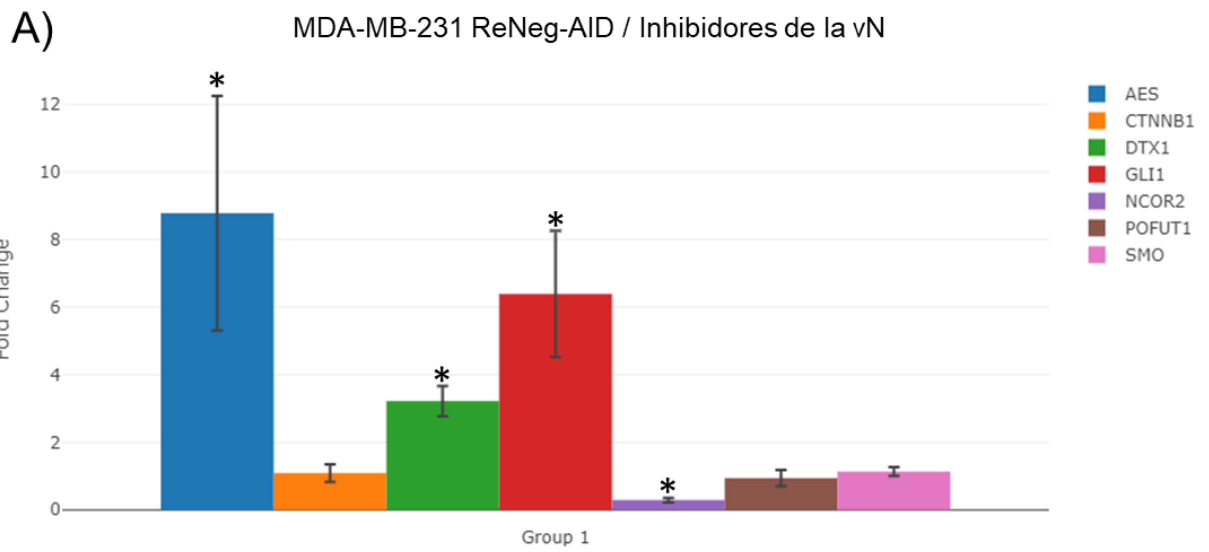
regulación positiva promueve que los efectos de la sobrexpresión de la vía Hh llevada a cabo por la vN^{χ} se vea comprometido, ya que el gen *SUFU* codifica para la proteína con el mismo nombre y es un regulador negativo de la activación de la vía Hh. Esto podría indicar un acercamiento para comprender que, aunque la vN^{χ} por medio del factor HIF-1α y la proteína GLI1 esté siendo activada por las células MDA-MB-231 la sobrexpresión del gen SUFU promovida por el péptido ReNeg-AID evita que el fenotipo metastásico en esta línea celular sea mecánicamente no funcional (17,18).

Los genes de la vN relacionados con la vía WNT analizados fueron: *AXIN1, CTNNB1, FZD2, FZD3, FZD4, FZD7, GSK3B, LRP5, WISP1* y *WNT11*. La Figura 2B muestra el efecto de la sobrexpresión del péptido ReNeg-AID en células MDA-MB-$231_{control}$ contra MDA-MB-$231_{ReNeg\text{-}AID}$ 48 horas post-transfección. Los únicos genes con cambios significativos fueron *FZD2*, *FZD4* y *FZD7* presentaron una regulación positiva con **24.24**, **2.39** y **9.74** veces de cambio con un valor de *p* de **0.0001**, **0.0002** y **0.0003** respectivamente. Los genes *AXIN1, CTNNB1, FZD4, GSK3B, LRP5, WISP1* y *WNT11* no presentaron cambios significativos. En este contexto entre la intercomunicación que se da con la vN y vN^{χ} sugiere que la señalización de la vía WNT está siendo activada por la expresión positiva de sus ligandos FZD, sin embargo, los ligandos WISP1 y WNT11 no han sufrido cambios significativos provocando que la vía WNT no esté siendo afectada por la vN canónica, pero los efectos de la vN^{χ} son inciertos en este contexto y requieren más análisis en comprender los mecanismos moleculares que puedan ser afectados (19,20).

Los genes de la vN relacionados con apoptosis analizados fueron: *AXIN1, CFLAR, CTNNB1, FOS, FOSL1, IL2RA, NEURL1, NR4A2* y *PTCRA*. La Figura 3A muestra el efecto de la sobrexpresión del péptido ReNeg-AID en células MDA-MB-$231_{control}$ contra MDA-MB-$231_{ReNeg\text{-}AID}$ 48 horas post-transfección. Los únicos genes que presentaron cambios significativos fueron *FOS* y *NEURL1* ambos con una regulación positiva en sus veces de cambio con **9.19** y **6.40** y un valor de p de **0.00019** y **0.0002** respectivamente. Aunque ambos genes participan en la regulación de los procesos apoptóticos dependientes de la vN por la vía intrínseca; el gen *FOS* es dependiente de la función conjunta del del *FOSL1*, esto sugiere que el proceso de la apoptosis no

puede concluir su señalización haciendo que la célula MDA-MB-231 intente recuperar este mecanismo, pero falla en el intento. La expresión positiva del gen *NEURL1* refuerza la idea de un mecanismo activado de regulación de la apoptosis, pero no funcional ya que este gen y *NR4A* son conocidos como uno de los últimos mediadores en los pasos de la señalización de la apoptosis bajo el control de la vN (1,21).

Los genes de la vN relacionados con el ciclo celular analizados fueron: *AXIN1, CCND1, CCNE1, CDK1A, JAG2* y *NOTCH2*. La Figura 3B muestra el efecto de la sobrexpresión del péptido ReNeg-AID en células MDA-MB-$231_{control}$ contra MDA-MB-$231_{ReNeg\text{-}AID}$ 48 horas post-transfección. Los únicos genes que presentaron cambios significativos fueron *JAG2* y *NOTCH1* con una regulación positiva en sus veces de cambio de **2.76** y **3.05** con un valor de *p* de **0.0001** y **0.00001** respectivamente, mientras que el gen *NOTCH2* sufrió una regulación negativa en sus veces de cambio de **-3.25** con un valor de p de **0.0016**. En este contexto el hecho que los genes *CNND1* y *CNNE1* sean blancos directos de la vN y que no hayan presentado cambios puede relacionarse directamente con una vN canónica no funcional, no obstante, la expresión del péptido ReNeg-AID promueve una regulación positiva para *JAG2* y *NOTCH1* que en el tipo celular de las MDA-MB-231 son estrictamente necesarios para su diferenciación, sin embargo, la regulación negativa del receptor Notch-2 indica que el ciclo celular no esta siendo regulado adecuadamente por la vN, o bien, se esta promoviendo una vN^{χ} dependiente del factor transcripcional CSL por el hecho de presentar una regulación del receptor Notch-1 pero no del receptor Notch-2; recordando que el receptor Notch-2 en las células epiteliales de glándula mamaria es el principal responsable de activar el ciclo celular y de llevar a cabo la progresión final de la diferenciación en este contexto celular (22–24).


A)
MDA-MB-231 ReNeg-AID / Inhibidores de la vN
Fold Change
Group 1
AES
CTNNB1
DTX1
GLI1
NCOR2
POFUT1
SMO

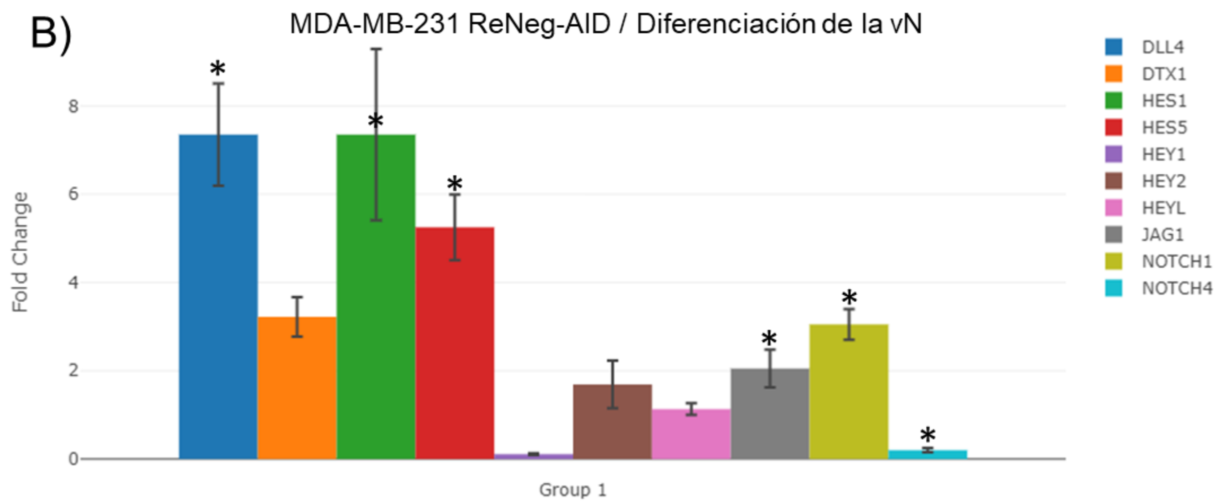
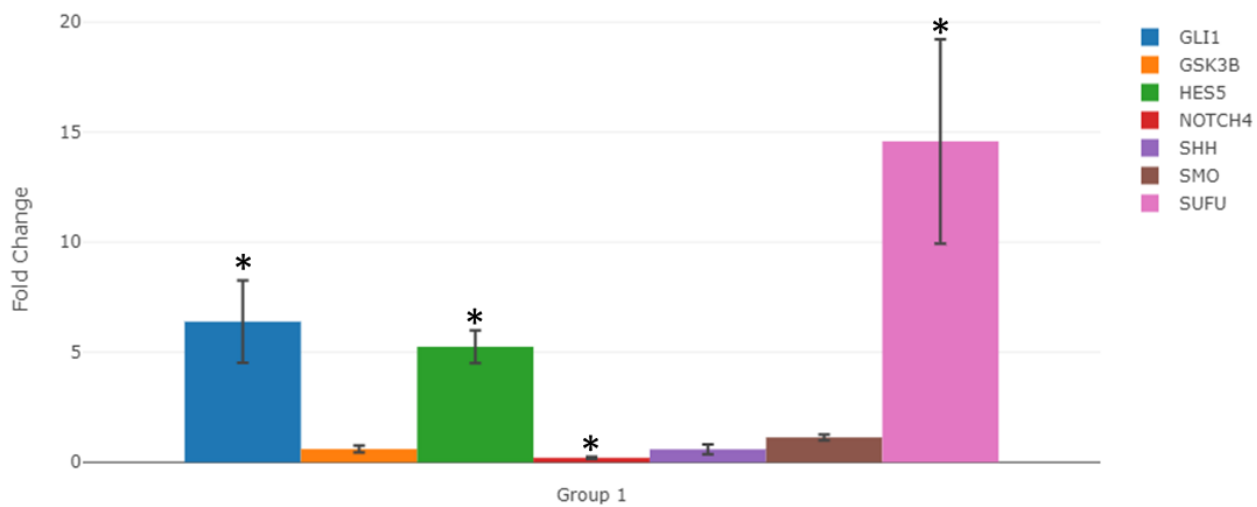

B)
MDA-MB-231 ReNeg-AID / Diferenciación de la vN
Fold Change
Group 1
DLL4
DTX1
HES1
HES5
HEY1
HEY2
HEYL
JAG1
NOTCH1
NOTCH4

Figura 1: Veces de cambio de los genes dependientes de la vN en la diferenciación e inhibidores de la vía en células MDA-MB-231$_{ReNeg\text{-}AID}$ contra MDA-MB-231$_{control}$. A) Veces de cambio de los genes inhibidores de la vN. B) Veces de cambio de los genes de la diferenciación relacionados con la vN. Se cuantifico el ARNm de los genes por RT-PCR (RT^2 Profile^{TM} PCR Array Human Notch Signaling Pathway, Qiagen). Se usaron los genes de ACTB y GAPDH para normalizar las cuantificaciones de ARNm. El umbral normalizado en las veces de cambio es de ± 2 veces. Se marca con un [*] los genes con resultados estadísticamente significativos; ($P < 0.05$), con n=4.


A)
MDA-MB-231 ReNeg-AID / vía Hh
Fold Change
20
15
10
5
0
Group 1
GLI1
GSK3B
HES5
NOTCH4
SHH
SMO
SUFU
*
*
*
*

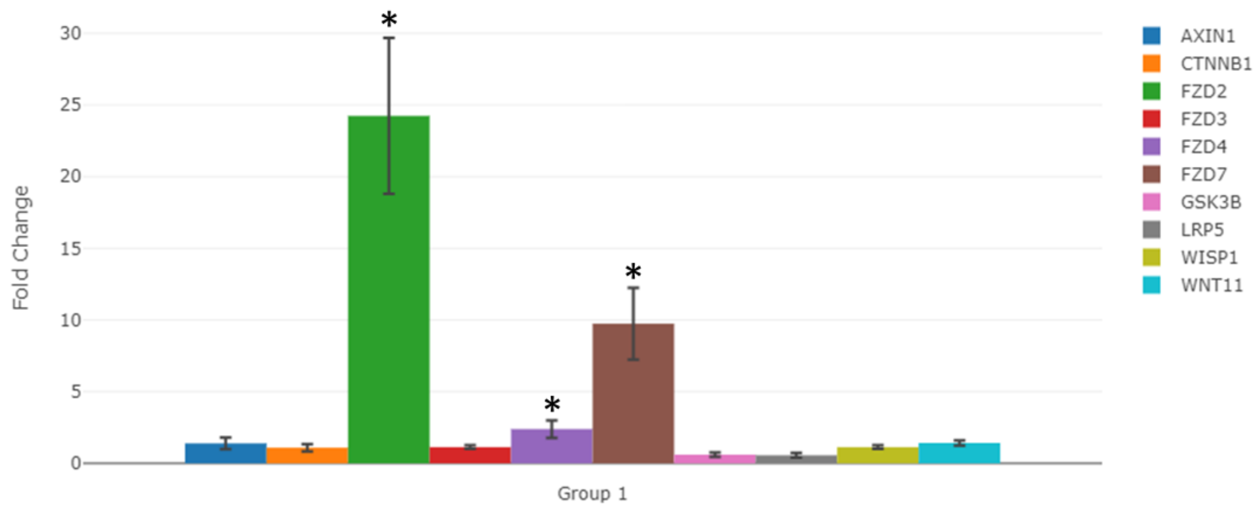
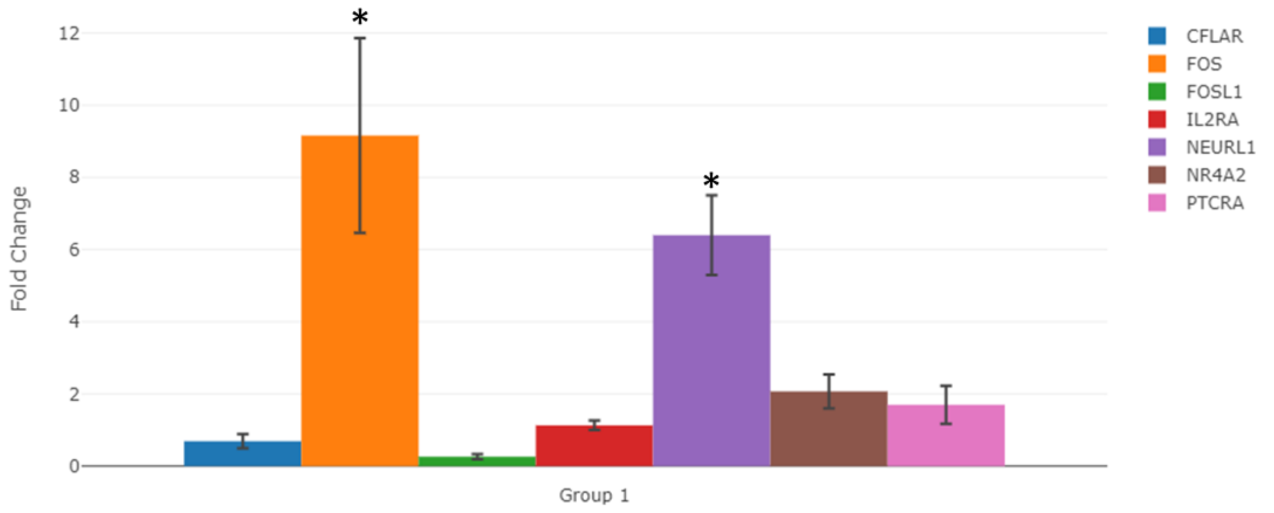

B)
MDA-MB-231 ReNeg-AID / vía WNT
Fold Change
30
25
20
15
10
5
0
Group 1
AXIN1
CTNNB1
FZD2
FZD3
FZD4
FZD7
GSK3B
LRP5
WISP1
WNT11
*
*
*

Figura 2: Veces de cambio de los genes dependientes de la vN relacionados con la vía Hh y WNT en células MDA-MB-231$_{ReNeg\text{-}AID}$ contra MDA-MB-231$_{control}$. A) Veces de cambio de los genes de la vN relacionados con la vía Hh. B) Veces de cambio de los genes de la vN relacionados con la vía WNT. Se cuantifico el ARNm de los genes por RT-PCR (RT^2 Profile^{TM} PCR Array Human Notch Signaling Pathway, Qiagen). Se usaron los genes de ACTB y GAPDH para normalizar las cuantificaciones de ARNm. El umbral normalizado en las veces de cambio es de ± 2 veces. Se marca con un [*] los genes con resultados estadísticamente significativos; ($P < 0.05$), con n=4.


A)
MDA-MB-231 ReNeg-AID / Apoptosis
Fold Change
12
10
8
6
4
2
0
Group 1
*
*
CFLAR
FOS
FOSL1
IL2RA
NEURL1
NR4A2
PTCRA

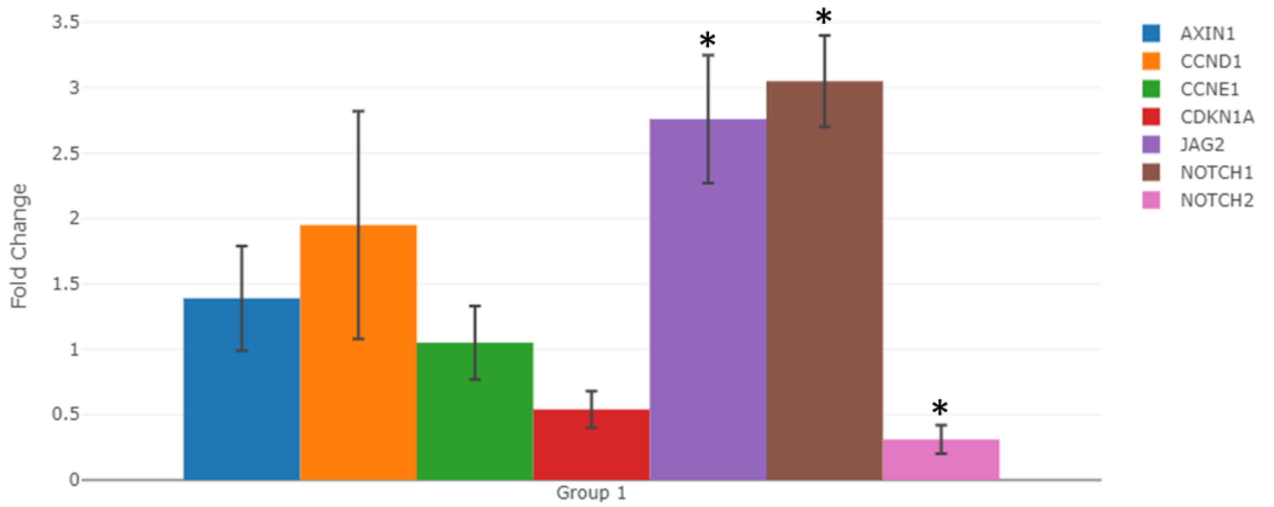

B)
MDA-MB-231 ReNeg-AID / Ciclo celular
Fold Change
3.5
3
2.5
2
1.5
1
0.5
0
Group 1
*
*
*
AXIN1
CCND1
CCNE1
CDKN1A
JAG2
NOTCH1
NOTCH2

Figura 3: Veces de cambio de los genes dependientes de la vN relacionados la apoptosis y el ciclo celular en células MDA-MB-231$_{ReNeg-AID}$ contra MDA-MB-231$_{control}$. A) Veces de cambio de los genes de la vN relacionados con la apoptosis. B) Veces de cambio de los genes de la vN relacionados con el ciclo celular. Se cuantifico el ARNm de los genes por RT-PCR (RT^2 Profile^{TM} PCR Array Human Notch Signaling Pathway, Qiagen). Se usaron los genes de ACTB y GAPDH para normalizar las cuantificaciones de ARNm. El umbral normalizado en las veces de cambio es de ± 2 veces. Se marca con un [*] los genes con resultados estadísticamente significativos; ($P < 0.05$), con n=4.

Conclusiones

El diseño y naturaleza del péptido ReNeg-AID promueve directamente una regulación negativa del receptor Notch-1 pero mantiene los niveles normales del receptor Notch-4 en células MCF-7, sin embargo, en células MDA-MB-231 promueve un efecto contrario, regulando negativamente el receptor Notch-4 y positivamente el receptor Notch-1. Si bien el efecto del péptido ReNeg-AID bajo el contexto de la vía canónica de Notch funciona de manera predicha en células MCF-7; pero, el efecto en células MDA-MB-231 es confuso, sin embargo, conserva ciertos patrones que sugieren que la VN$^{\chi}$ es promovida por sus dos formas descritas; dependiente e independiente del ligando e independiente de CSL.

El efecto principal del péptido ReNeg-AID en células MCF-7 fue la interrupción del ciclo celular en la transición de la fase G1 a S; esto repercute directamente en la detención del crecimiento celular descontrolado característico del cáncer. En contraste, el efecto principal en las células MDA-MB-231 no ocurrió en el ciclo celular pero sí sobre la comunicación cruzada que existe entre la vN y la vía Hh, promoviendo la regulación positiva de la proteína *SUFU* y en consecuencia regulando negativamente la expresión de los genes blanco de vía Hh que en el contexto del cáncer están directamente relacionados con la metástasis celular y el metabolismo del cáncer.

Es recomendable hacer mas estudios minuciosos, como analizar paso a paso las interacciones posibles que pueden darse con los receptores Notch con proteínas no descritas que perteneces a la vía Hh y WNT; ya que la comunicación cruzada entre estas tres vías rigen y determinan gran parte de la naturaleza del cáncer asi como promover su aparición y mantener su estado homeostático del mismo cáncer, y asi comprender si la vía no canónica de Notch funge como un mecanismo regulador, promotor o inhibitorio en los procesos celulares que regula de manera canónica.

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VII. Conclusiones y discusión general

Gracias a los trabajos realizados por autores como Kovall (2009) y Dieter (2011) donde lograron consolidar la hipótesis de que proteínas y dominios proteicos del factor transcripcional CSL, ligandos y receptores de la vía de señalización Notch poseen la capacidad de interactuar con sistemas proteicos entre distintos organismos dentro del grupo de los metazoarios.

Una excepción ocurre con la proteína Hairless (H), que solo ha sido identificada en dos clados de metazoarios, en insectos y moluscos. La proteína H actúa como el único regulador negativo de la VN en estados embrionarios donde hay un gran número de poblaciones de células progenitoras y células madre que darán comienzo a la organogénesis mediante la diferenciación celular. Después de que se demostrara el alto nivel de conservación de las proteínas relacionadas con la VN en los metazoarios junto con su alta afinidad de unión entre ellas se llegó a la conclusión que la proteína H y el receptor Notch comparten el mismo sitio de unión al factor transcripcional CSL.

Bajo esta premisa de afinidad y conservación de dominios de unión entre H y Notch por CSL se diseñó y construyó un péptido derivado de la proteína H de D. melanogaster que contiene el dominio de unión y reconocimiento hacia el factor CSL y usarlo como un regulador negativo de la VN en contextos de cáncer de mama humano, donde se sabe que la VN esta constitutiva y aberrantemente expresada, promoviendo el escenario perfecto para la iniciación de la carcinogénesis en células progenitoras, epitelio y endotelio mamario; promoviendo de esta forma que los receptores Notch 1 – 4 compitan con el péptido ReNeg-AID por el dominio de unión al factor CSL.

El péptido ReNeg-AID no tuvo efectos significativos en la línea celular MCF-12F, lo cual indica que las células no cancerígenas son capaces de autorregular el exceso de un agente que atenúa la activación de la VN. El efecto en la línea celular MCF-7 fue directo sobre la expresión de la ciclina D1 y E1, esto promovió que el avance de la fase G1/S del ciclo celular fuera arrestada en un ambiente de sobreestimulación del ciclo celular como lo es el cáncer de mama. El efecto del péptido en la línea celular MDA-

MB-231 tuvo efectos contrastantes y contradictorios, ya que en este tipo celular predomina la vía no canónica de Notch, sin embargo, se logró determinar que ciertos genes involucrados en la diferenciación celular fueron reactivados, tambien promovió la expresión de genes íntimamente ligados con el funcionamiento canónico de la vía Notch.

VIII. Perspectivas y Recomendaciones

La conservación del mecanismo de comunicación celular de la vN a nivel molecular a través del tiempo y bajo los efectos inherentes de la evolución, han permitido la aparición de clados específicos de metazoarios desde animales simples como las esponjas a animales complejos como los mamíferos. La vN forma parte de las 7 vías principales que dan origen a los intrincados patrones morfológicos celulares y sistémicos de los metazoarios y al mismo tiempo con la interacción de otras vías de señalización promueve la regulación del ciclo celular, la apoptosis, la diferenciación y la migración celular. Estos eventos celulares son los puntos clave para mantener el estado de la homeostasis que es característica de un estado sano del individuo. Por esta razón, si el mecanismo de la vN deja de funcionar adecuadamente promueve un cambio drástico en la dinámica del flujo de información y comunicación entre célula – célula. Estos cambios se manifiestan en una pérdida de la identidad celular, esto quiere decir que la célula ha dejado de recibir o ha aumentado la cantidad de comandos hacia otras células; estimulando que el ciclo celular pierda su autorregulación, que la apoptosis no logre activarse, que la migración celular se active en tipos celulares que ya no la necesitaban, haciendo que la célula adopte un comportamiento cancerígeno y metastásico.

Con lo anterior descrito e integrando los resultados obtenidos en esta investigación y basándonos en el principio de conservación de los dominios que conforman la familia de proteínas pertenecientes y relacionadas con la vN, se logró diseñar un switch molecular proveniente de un dominio de la proteína H de *D. melanogaster* que usa para unirse al factor transcripcional CSL, que regula de manera negativa la activación de los genes blanco de la vN en células cancerígenas de epitelio mamario humano con una activación constitutiva aberrante. Aunque en mamíferos existen mecanismos propios de regulación negativa de la vN; el genoma de los mamíferos aparentemente ha perdido el gen de la proteína Hairless que ha sido encontrada solo en insectos y moluscos, sin embargo, el factor transcripcional CSL de mamíferos aun es capaz de reconocer el dominio de la proteína Hairless. Este reconocimiento nos puede indicar que la perdida de un gen no necesariamente significa que el sistema completo haya

perdido la capacidad de usarlo o reconocerlo, tambien nos estaría indicando que la proteína H funciona como una sinapomorfía para los insectos y moluscos.

El entendimiento de este mecanismo beneficiará y facilitará nuevas y futuras formas de aprovechar esta cualidad en escenarios donde la activación constitutiva de la vN esté presente y sea el origen de padecimientos como el cáncer en diferentes tejidos. Esto implica, además, que el mismo método sea aplicado en diseñar estrategias en escenarios donde la vN este constitutivamente reprimida e incluso diseñar péptidos con el mismo principio que funcionen en otras vías de señalización con un mal funcionamiento en sus mecanismos.

Es necesario mencionar que se requieren de estudios futuros como pruebas *in-vivo* y con modelos animales para poder asegurar que este conocimiento pueda funcionar a un nivel de complejidad mayor, como tejidos y órganos animales. Tambien es de importancia señalar que el uso y manejo de vías ancestrales de señalización celular deben ser manipuladas minuciosamente ya que son el núcleo que da origen a la regulación, activación y represión de genes que a su vez gobiernan los procesos vitales de la célula como el ciclo celular, apoptosis, diferenciación, identidad y migración celular.

IX. Bibliografía complementaria

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