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RESEARCH ARTICLE

Effect of ReNeg-AID peptide overexpression in MDA-MB-231 triple-negative breast cancer cells: the noncanonical Notch pathway

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ABSTRACT

The Notch pathway has two general regulatory mechanisms: its canonical pathway and the non-canonical pathway. The non-canonical Notch pathway is known that acts independently of its transcriptional factor to activate its target genes which do not belong to the *HER, HES* and *HERP* gene family, and has been linked to oncogenic processes and immune cell activation. Here we report the behavior of triple-negative breast cancer cells that maintain high activity of the non-canonical Notch pathway at the presence of ReNeg-AID a peptide which seems to be provoking both a change in the phenotype of the triple-negative breast cancer cells from epithelial cell to non-functional mesenchymal cell, as well as a cell's attempt to regain canonical Notch pathway activity. Finally, overexpression of the ReNeg-AID peptide in the triple-negative breast cancer cell line promoted a negative regulation of its transcriptional factor, intensifying the non-canonical Notch pathway activity and causing an oscillation or combination between the activities of the canonical and non-canonical mechanisms of Notch pathway due to the activation of Notch-1 and 3 receptors and the repression of Notch-2 and 4. Added to this must be the interactions that exist between Notch pathway with other signaling pathways that may also be in a state of deregulation in the cancer microenvironment.

Introduction

The non-canonical Notch pathway (NP$^{\chi}$) was described by Aster and Pear in 1990. They discovered a chromosomal translocation of the intracellular domain of the Notch-1 gene receptor in T-cell acute lymphoblastic leukemia causing constitutive canonical Notch pathway (NP) activation, which causes T cells to be unable to differentiate to T helper (Th) cells and activate into Th1, Th2 and Th17 cells forms, as T cell activation requires the mechanism of NP$^{\chi}$ to activate specific interleukins for each of the T helper cells activation forms. From this discovery of a new mechanism of the Notch pathway, its study was extended to more cell types such as lung, ovaries, blood system, bone, and epithelium; and this coincides with organs and systems where either the NP$^{\chi}$ or NP are deregulated or have changed their activation rhythms caused by a carcinogenic event[1,2].

Two mechanisms have been postulated that can activate NP$^{\chi}$ and they are:

- *Ligand-dependent or ligand-independent activation*. The Notch receptor physically interacts in the membrane with certain proteins of other signaling pathways such as Hedgehog (Hh), Wingless (Wnt), Nuclear factor kappa B (NF-kappa B) or Catenin beta-1 (beta-catenin), or, once the Notch receptor is proteolytically processed by the gamma secretase (gamma-secretase) complex and released as Notch intracellular domain (NICD); in the course from the membrane to the nucleus NICD interacts with the same proteins mentioned above, this makes NP$^{\chi}$ also dependent and independent of gamma-secretases[3-5].
- *The Notch pathway transcriptional factor (CSL)-independent activation*. This is based on the capacity of the Notch receptor to bind with certain transcriptional factors and increase their half-life in the cytoplasm. These transcriptional factors such as hypoxia-inducible factor 1 alpha subunit (HIF-1A), transcriptional repressor YY1 (YY1) or beta-catenin; this interaction promotes the activation of the target genes of these transcriptional factors, which causes the feedback between signaling pathways to have a stricter but effective level of homeostatic regulation[6,7].

The role of NP is strictly necessary in the embryonic stage. NP$^{\chi}$ seems to be exclusive in fully differentiated adult organism processes and acts in regulatory processes of the same NP and other pathways such as NF-kappa B, PI3K/Akt/mTOR pathway, Hh and Wnt, it's also involved in regulatory processes of apoptosis through the breast cancer type 1 susceptibility protein (BCRA1) and with physical interactions with mitochondria, related to the regulation of cancer cell metabolism under the interaction with beta-catenin[8-14].

The mechanisms of NP$^{\chi}$ are confounded by its ambiguous nature of interacting with many proteins from other signaling pathways. This intricate nature has provided new ways of regulation for NP itself and for the cellular events involved such as differentiation, apoptosis, cell cycle and metabolism. The importance of understanding NP$^{\chi}$ could give us a new perspective to understand a new mechanism of gene regulation, not exclusive to NP but in the complex network of interactions that occur in the background between many signaling pathways to maintain and regulate vital processes, if this delicate mechanism is altered, as with any regulatory process will cause complications in the functioning and homeostasis of the organism.

Methods.

For cell culture: Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Cat. No. 12100046) for MDA-MB-231 cells, DMEM-F12 Ham (Gibco, Cat. No. 12500096) for MCF12-F cells, fetal bovine serum (FBS, Gibco, Cat. No. 10437028), penicillin, streptomycin, and trypsin-ethylenediaminetetraacetic acid (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™.

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ReNeg-AID peptide. The ReNeg-AID peptide (patent 415236) was previously described at Saucedo-Correa *et al.* (2021)[15].

Cell culture. The non-cancer cell lines of human epithelial breast MCF-12F (ATCC CRL-3599) and tumorous triple-negative MDA-MB-231 (ATCC CRM-HTB-26) cells were purchased from ATCC (Manassas, VA, USA).

Transfection & electroporating. Cells were incubated at 5% of CO_2 at 37°C until reaching 80% confluence. The culture was then tripzinated and 1×10^6 cells were harvested and centrifuged until the pill was formed. Then, a 100 µl nucleofection solution (Cell Line Nucleofector® Kit V, protocol T/C-28a2, AMAXA®) was prepared with 2 ng of the plasmid pReNeg-AID and 400 µl of OptiMEM medium (GIBCO-USA, Cat. No. 31985062). The cells were resuspended in nucleofection solution and then were placed in a 4 mm electroporating cuvette in an electroporating system (BTX® Harvard Apparatus ECM 630 exponential decay wave electroporating system, item 45-2051). Electroporating conditions: 140 V, 70 ms with one pulse; then the cuvette with the cell solution was incubated for five minutes at room temperature between 18°C and 25°C, and the cells were cultured in six-well plates with 1.5 ml of supplemented DMEM in a humidified 37°C/5% CO_2 incubator for 48 h.

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 and MDA-MB-321, subsequently treated with DNase I, and purified using the RNeasy Mini Kit (Qiagen, Cat. No. 74034). 25 µg of high-quality total RNA was then reverse transcribed using the First Strand Synthesis Kit (Qiagen Cat. No. 330401) and subsequently loaded onto the Human Notch RT2 profiler array (PAHS-059Z). 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. Both the control cells and ReNeg-AID cells were harvested 48 hours post-transfection. A total of 1×10^6 cells in PBS were dyed with 400 µl of IP, followed by adding 50 µl of RNAsa and incubated for 30 minutes to 1 hour. Data were 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 following 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 cytometry analysis were analyzed by student's t-test, and values with $P < 0.05$ were considered statistically significant. All experiments were performed at least three times, with $n = 4$ 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 these housekeeping genes, ACTB and GAPDH. All experiments were performed at least three times, with $n = 4$ for each of them.

Results.

Notch pathway inhibitor-related genes and cell differentiation. The NP inhibitor-related genes analyzed were *amino-terminal enhancer of split* (*AES*), *catenin beta 1* (*CTNNB1*), *deltex E3 ubiquitin ligase 1* (*DTX1*), *GLI family zinc finger 1* (*GLI1*), *nuclear receptor corepressor 2* (*NCOR2*), *O-fucosyltransferase 1* (*POFUT1*) and *smoothened, frizzled class receptor* (*SMO*). Figure 1A shows the effect of ReNeg-AID peptide overexpression in MDA-MB-231 control versus ReNeg-AID cells 48 hours post-transfection (hpt). The *AES*, *DTX1* and *GLI1* genes presented a positive up-regulation with 8-, 3- and 6-fold change with a p-value of 0.002337, 0.00004 and 0.0008 respectively. The *NCOR2* gene presented a negative regulation with 3-fold change with a p-value of 0.0005. *CTNNB1*, *POFUT1* and *SMO* genes did not show significant changes.

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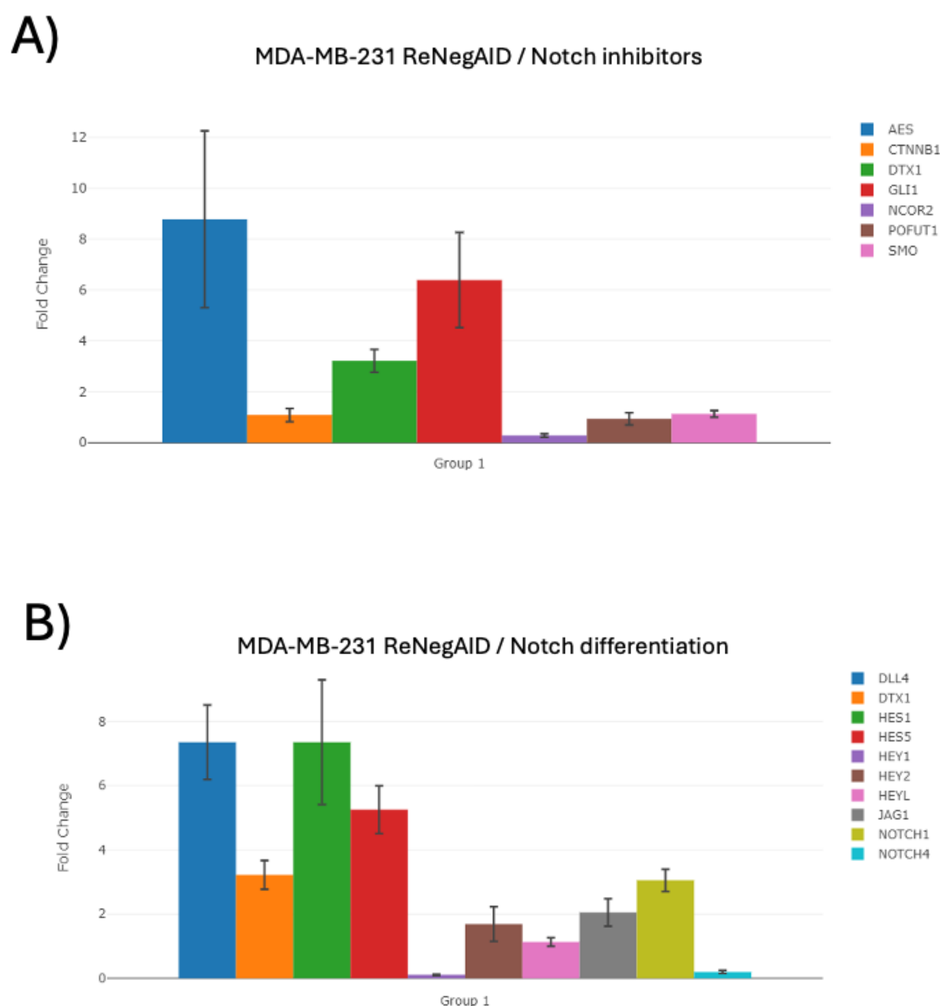

Figure 1. Times of change of NP-dependent genes in MDA-MB-231$_{ReNeg-AID}$ versus MDA-MB-231$_{control}$ cells. **A)** NP inhibitory genes. **B)** NP-related differentiation genes. *ACTB* and *GAPDH* genes were used to normalize mRNA quantifications. The normalized threshold in times of change is ± 2-fold. Genes with statistically significant results are marked with a [*]; (P < 0.05), with n=4.

The differentiation-related genes analyzed were: *delta like canonical Notch ligand 4* (*DLL4*), *DTX1*, *hes family bHLH transcription factor 1* (*HES1*), *hes family bHLH transcription factor 5* (*HES5*), *hes related family bHLH transcription factor with YRPW motif 1* (*HEY1*), *hes related family bHLH transcription factor with YRPW motif 2* (*HEY2*), *hes related family bHLH transcription factor with YRPW motif like* (*HEYL*), *jagged canonical Notch ligand 1* (*JAG1*), *notch receptor 1* (*NOTCH1*) and *notch receptor 4* (*NOTCH4*). Figure 1B shows the effect of ReNeg-AID peptide overexpression in MDA-MB-231 control versus ReNeg-AID cells 48 hpt. The *DLL4* and *JAG1* gene presented a 7.35 and 2.54-fold positive up-regulation with a p-value of 0.000022 and 0.0021 respectively; the *NOTCH1* gene presented a 3.05-fold positive up-regulation with a p-value of 0.00001, while the *NOTCH4* gene presented a -5.09-fold negative down-regulation with a p-value of 0.00005. As well as a positive expression of the *HES1* and *HES5* genes presented a 7.35- and 5.25-fold change up-regulation with a p-value of 0.00001 and 0. 00003 respectively.

The Notch and Hedgehog pathways communication. The NP genes related to the Hh pathway analyzed were: *GLI1*, *glycogen synthase kinase 3 beta* (*GSK3B*), *HES5*, *NOTCH4*, *sonic hedgehog signaling molecule* (*SHH*), *SMO* and *SUFU negative regulator of hedgehog signaling* (*SUFU*). Figure 2A shows the effect of ReNeg-AID peptide overexpression in MDA-MB-231 control versus ReNeg-AID cells 48 hpt. It was observed that the *GLI1* and *HES5* genes showed a positive

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regulation in their times of change, together with the negative regulation of the *NOTCH4*. Specially the *SUFU* gene presented a positive regulation of 14. 58-fold change up-regulation and a p value of 0.000617.

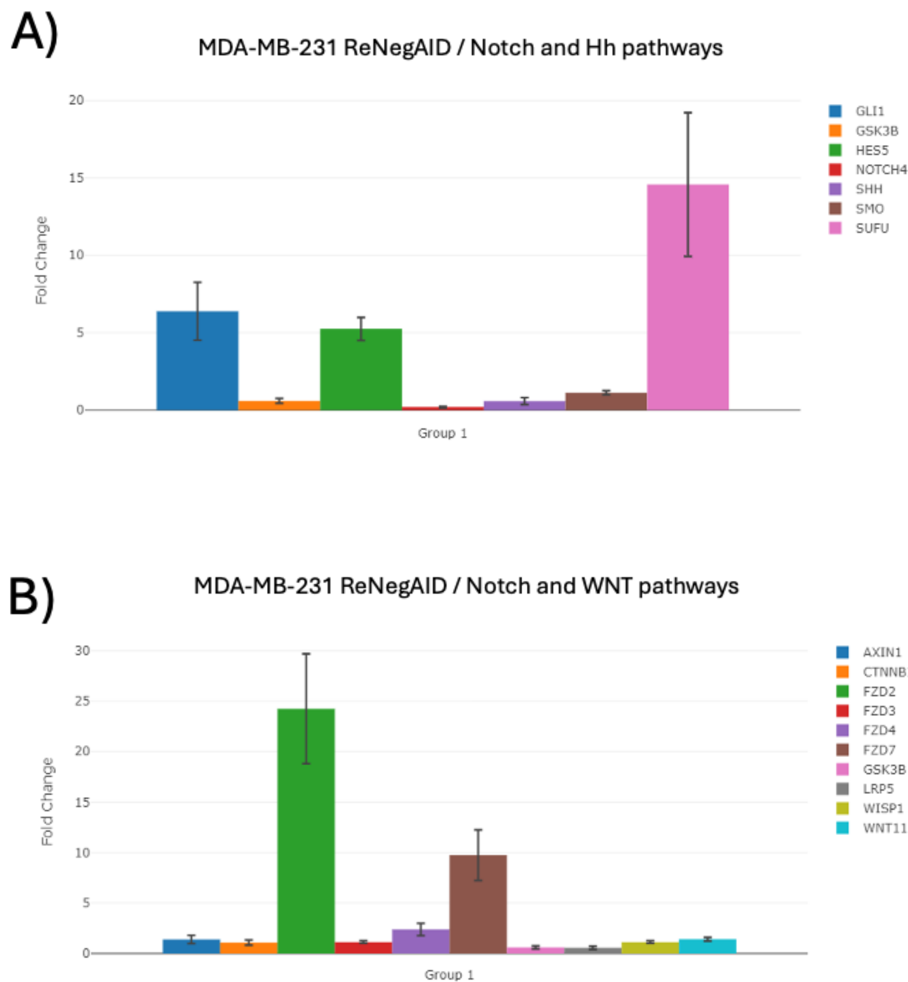

Figure 2. Change times of NP-dependent genes related to the Hh and Wnt pathway in MDA-MB-231$_{ReNeg\text{-}AID}$ versus MDA-MB-231$_{control}$ cells. **A)** Hh pathway-related genes. **B)** Wnt pathway-related genes. *ACTB* and *GAPDH* genes were used to normalize mRNA quantifications. The normalized threshold in times of change is ± 2-fold. Genes with statistically significant results are marked with a [*]; ($P < 0.05$), with n = 4.

The Notch and Wingless pathways communication. The NP genes related to the Wnt pathway analyzed were: *axin 1* (*AXIN1*), *CTNNB1*, *frizzled class receptor 2* (*FZD2*), *frizzled class receptor 3* (*FZD3*), *frizzled class receptor 4* (*FZD4*), *frizzled class receptor 7* (*FZD7*), *GSK3B*, *LDL receptor related protein 5* (*LRP5*), *WNT1 inducible signaling pathway protein 1* (*WISP1*) and *Wnt family member 11* (*WNT11*). Figure 2B shows the effect of ReNeg-AID peptide overexpression in MDA-MB-231 control versus ReNeg-AID cells 48 hpt. The genes with significant changes were *FZD2*, *FZD4* and *FZD7* which presented a positive regulation with 24.24-, 2.39- and 9.74-fold change with a p value of 0.0001, 0.0002 and 0.0003 respectively. *AXIN1*, *CTNNB1*, *FZD4*, *GSK3B*, *LRP5*, *WISP1* and *WNT11* genes showed no significant changes.

Apoptosis-related genes. The apoptosis-related NP genes analyzed were: *AXIN1*, *CASP8 and FADD like apoptosis regulator* (*CFLAR*), *CTNNB1*, *Fos proto-oncogene, AP-1 transcription factor subunit* (*FOS*), *FOS like 1, AP-1 transcription factor subunit* (*FOSL1*), *interleukin 2 receptor subunit alpha* (*IL2RA*), *neuralized E3 ubiquitin protein ligase 1* (*NEURL1*), *nuclear receptor subfamily 4*

group A member 2 (*NR4A2*) and *pre T cell antigen receptor alpha* (*PTCRA*). Figure 3A shows the effect of ReNeg-AID peptide overexpression in MDA-MB-231 control versus ReNeg-AID cells 48 hpt. The only genes that showed significant changes were *FOS* and *NEURL1* both with a positive regulation in their change times with 9.19- and 6.40-fold change and a p-value of 0.00019 and 0.0002 respectively.

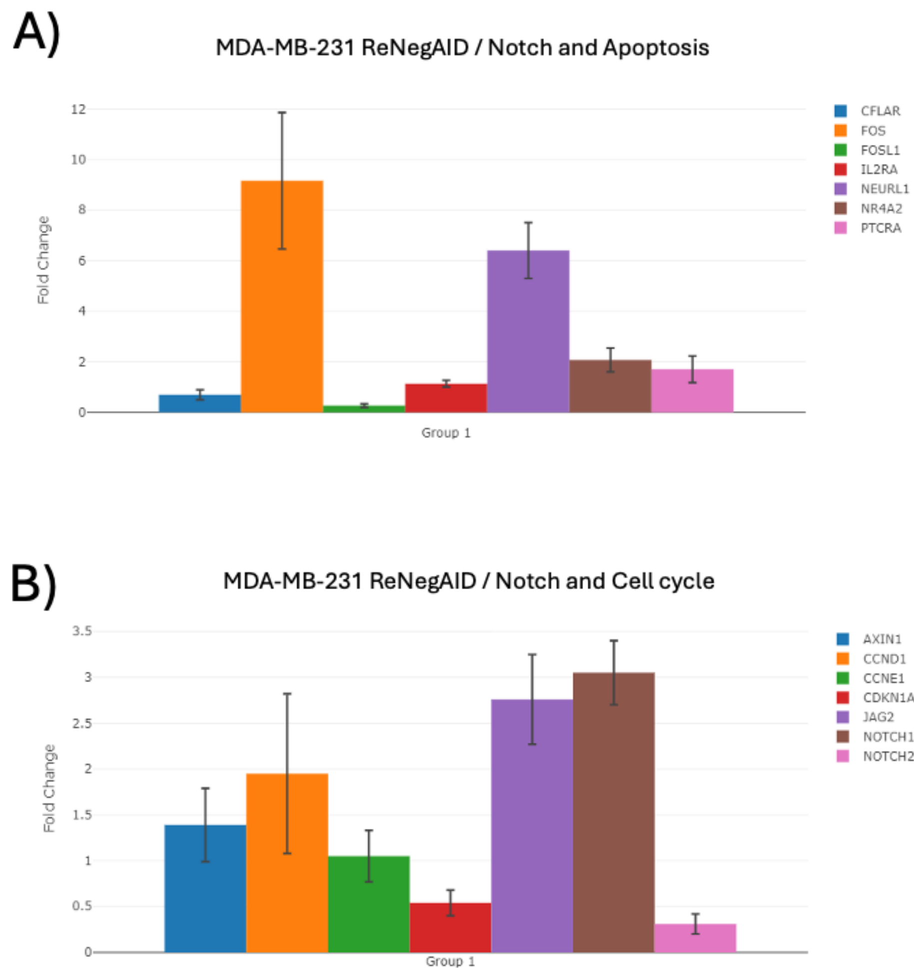

Figure 3. Change times of NP-dependent genes related apoptosis and cell cycle in MDA-MB-231$_{ReNeg\text{-}AID}$ versus MDA-MB-231$_{control}$ cells. **A)** Apoptosis-related genes. **B)** Cell cycle-related genes. *ACTB* and *GAPDH* genes were used to normalize mRNA quantifications. The normalized threshold in times of change is ± 2-fold. Genes with statistically significant results are marked with a [*]; ($P < 0.05$), with n=4.

Cell cycle-related genes. The cell cycle-related NP genes analyzed were: *AXIN1*, *cyclin D1* (*CCND1*), *cyclin E1* (*CCNE1*), *cyclin-dependent kinase 1 S homeolog* (*CDK1A*), *jagged canonical Notch ligand 2* (*JAG2*) and *notch receptor 2* (*NOTCH2*). Figure 3B shows the effect of ReNeg-AID peptide overexpression in MDA-MB-231 control versus ReNeg-AID cells 48 hpt. The only genes that showed significant changes were *JAG2* and *NOTCH1* with a positive regulation in their change times of 2.76- and 3.05-fold change with a p-value of 0.0001 and 0.00001 respectively, while the *NOTCH2* gene underwent a negative regulation in its change times of -3.25-fold change with a p-value of 0.0016.

Discussion.

Notch pathway inhibitor-related genes and cell differentiation. The positive regulation of *DTX1*, *AES* and the negative regulation of *NCOR2* indicates that the canonical NP is being negatively regulated at the level of protein complex formation by the action of *AES* and at the cytoplasmic level by the action of *DTX1*[16,17] (Fig. 1A), however, the

GLI1 gene could be promoting the expression of the Hypoxia-inducible factor 1-alpha (HIF-1a) factor of the Hh pathway and thus contributing to the positive regulation of NP$^{\chi}$; although the effect of the interaction of the Notch receptor with HIF-1a assists the expression of genes towards the oxidative stress response such as *BCL2 binding component 3* (*PUMA*), which encodes to a protein that cooperates with direct activator proteins to induce mitochondrial outer membrane permeabilization and apoptosis, or *BCRA*; it has also been reported that the HIF-1a factor in turn regulates the intensity of the expression of NF-kappa B dependent genes involved in the recognition response of the immune system such as IL-6 and 17 as well as the metabolism of cancer cells. The fact that *CTNNB1*, *POFUT1* and *SMO* genes did not show a change in expression after transfection with pReNeg-AID could indicate that the aberrant state of the Wnt pathway has not undergone changes in this cell type; the case of the *POFUT1* gene suggests a correlation between the ligand-dependent pathway that NP$^{\chi}$ can exert[4,9,17].

On the other hand, the discrepancy between the positive expression of Delta4, Jagged1 ligands and Notch-1 receptor but a negative expression of Notch-4 receptor would suggest that NP$^{\chi}$ is being promoted in its ligand-dependent form at a higher frequency (Fig. 1B), however, the positive expression of the *HES1* and *HES5* genes suggests that NP is being favored with ReNeg-AID peptide expression and this suggests a regulation and attempt to regain cellular identity in this type of mammary glandular tissue, however further evidence is needed to fully elucidate the NP$^{\chi}$ machinery and its ultimate implications with the other signaling pathways related to cell differentiation[16-18]. The expression of *HEY1*, *HEY2* and *HEYL* genes did not present significant changes, this coincides with what has been reported for the differential phenotype of this cell type[17,18-21].

The Notch and Hedgehog pathways communication. The genes with a positive regulation (Fig. 2A) in their times of change such as *GLI1* and *HES5*, together with the negative regulation of the *NOTCH4* gene suggests that the regulatory effect that occurs between NP and the Hh pathway through the Notch-4/HIF-1a receptor is being negatively regulated, implying that cell migration or metastatic behavior reported by this interaction is being promoted, however, a positive regulation of the *SUFU* gene means that the NP$^{\chi}$ is compromised, since the *SUFU* gene codes for the protein with the same name and is a negative regulator of Hh pathway activation. This could indicate an approach to understand that, although the NP$^{\chi}$ via HIF-1a factor and GLI1 protein is being activated by MDA-MB-231 cells, the overexpression of the *SUFU* gene promoted by the ReNeg-AID peptide prevents the metastatic phenotype in this cell line from being mechanistically non-functional[22,23].

The Notch and Wingless pathways communication. In this context between the intercommunication occurring with NP and NP$^{\chi}$ suggests that Wnt pathway signaling is being activated by positive expression of its *FZD* ligand gene (Fig. 2B), however, *WISP1* and *WNT11* ligands genes did not undergo significant changes causing the Wnt pathway to be unaffected by NP, but the effects of NP$^{\chi}$ are uncertain in this context and require further analysis in understanding the molecular mechanisms that may be affected[24,25].

Apoptosis-related genes. The positive expression of *FOS* and *NEURL1* genes (Fig. 3A) suggest an up regulation of NP-dependent apoptotic processes by the intrinsic pathway. Nevertheless, the MDA-MB-231 cells cannot terminate their process of apoptosis signaling path. These should be possible because the *FOS* gene is dependent on the joint function with *FOSL1* gene who showed a diminished expression; and the *NEURL1* gene is not functional since this gene and *NR4A* gene, who showed a diminished expression, are known to be both the last mediators in the apoptosis signaling steps under the control of NP[1,26]. All this data together suggests that the attempt to recover this apoptosis signaling path in the NP context fails because some of the proteins involved are not present.

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Cell cycle-related genes. The fact that *CNND1* and *CNNE1* genes showed no significant changes under the influence of the ReNeg-AID peptide can be directly related to a non-functional canonical NP (Fig. 3B), because both genes are direct targets of canonical NP. On the other hand, the ReNeg-AID peptide expression promotes a positive regulation for *JAG2* and *NOTCH1* which in the MDA-MB-231 cell type context are strictly required for differentiation (Fig. 3B), however, the negative regulation of the Notch-2 receptor indicates that the cell cycle is not being adequately regulated by NP, or, alternatively, it is promoting a transcriptional factor CSL-dependent NP^{χ} by presenting a regulation of the Notch-1 receptor but not of the Notch-2 receptor; recalling that the Notch-2 receptor in mammary gland epithelial cells is mainly responsible for activating the cell cycle and carrying out the final progression of differentiation in this cellular context[27-29].

Previosly, Saucedo-Correa *et al.* (2021), had been reported that in a MTT proliferation assay the MDA-MB-231 cell line presented an irregular proliferation, due to its genetic background with respect to the NP that is activated in its non-canonical way, which is more evident when the ReNeg-AID peptide has no effect on non-cancerous MCF-12F mammary gland cells at any of the tested time, and cell proliferation remains unaltered.

Conservation of NP cellular communication mechanism at the molecular level over time and under the inherent effects of evolution, has allowed the emergence of specific clades of metazoans from simple animals such as sponges to complex animals such as mammals. NP is part of the 7 major pathways that give rise to the intricate cellular and systemic morphological patterns of metazoans and at the same time with the interaction of other signaling-pathways promotes cell-cycle regulation, apoptosis, differentiation, and cell migration. These cellular events are the key points for maintaining the state of homeostasis that is characteristic of a healthy state of the individual. If the NP mechanism ceases to function properly it promotes a drastic change in the dynamics of information flow and cell-cell communication. These changes manifest themselves in a loss of cellular identity, meaning that the cell has stopped receiving or has increased the number of commands to other cells; stimulating the cell cycle to lose its self-regulation, apoptosis to fail to activate, cell migration to be activated in cell types that no longer need it, causing the cell to adopt a cancerous and metastatic behavior.

With the above described and integrating the results obtained in this research and based on the principle of conservation of the domains that make up the family of proteins belonging to and related to NP, we were able to design a molecular switch from a domain of the H protein of *D. melanogaster*[15] that is used to bind the transcriptional factor CSL, which negatively regulates the activation of the target genes of NP in human mammary epithelial cancer cells with aberrant constitutive activation. Although mammalian mechanisms of negative regulation of NP exist; the mammalian genome has apparently lost the Hairless protein gene that has been found only in insects and mollusks, however, the mammalian CSL transcriptional factor is still able to recognize the Hairless protein domain[15,16]. This recognition may indicate that the loss of a gene does not necessarily mean that the entire system has lost the ability to use or recognize it, and that the H protein functions as a synapomorphy for insects and mollusks.

The understanding of this mechanism will benefit and facilitate new and future ways to take advantage of this quality in scenarios where constitutive activation of NP is present and is the origin of diseases such as cancer in different tissues. This also implies that the same method can be applied to design strategies in scenarios where NP is constitutively repressed and even to design peptides with the same principle that function in other signaling pathways with malfunctioning mechanisms.

Conclusions.

The design and nature of the ReNeg-AID peptide directly promotes a negative regulation of the

Notch-1 receptor but maintains normal Notch-4 receptor levels in MCF-7 cells[15]; however, in MDA-MB-231 cells it promotes an opposite effect, negatively regulating the Notch-4 receptor and positively regulating the Notch-1 receptor. While the effect of ReNeg-AID peptide under the context of the canonical Notch pathway works in a predicted manner in MCF-7 cells; but the effect in MDA-MB-231 cells is unclear, however, it retains certain patterns suggesting that NP^{χ} is promoted by its two described forms; ligand-dependent and ligand-independent and CSL-independent.

The main effect of the ReNeg-AID peptide in MCF-7 cells was the interruption of the cell cycle in the transition from G1 to S phase[15]; this has a direct impact on the arrest of uncontrolled cell growth characteristic of cancer. In contrast, the main effect in MDA-MB-231 cells was not on the cell cycle but on the cross talk that exists between the NP and the Hh pathway, promoting the positive regulation of the SUFU protein and consequently negatively regulating the expression of Hh pathway target genes that in the context of cancer are directly related to cell metastasis and cancer metabolism.

It is advisable to do more detailed studies, such as step-by-step analysis of the possible interactions that can occur with Notch receptors with undescribed proteins belonging to the Hh and Wnt pathways; since cross-communication between these three pathways govern and determine much of the nature of cancer as well as promote its occurrence and maintain its homeostatic state of cancer itself, and thus understand whether the non-canonical Notch pathway serves as a regulatory, promoter or inhibitory mechanism in cellular processes that regulates canonically way.

Declaration of interest:

None.

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