

Nidogen 1 regulates proliferation and migration/invasion in murine claudin-low mammary tumor cells

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Abstract. Nidogen 1 (NID1) is a glycoprotein found in basement membranes involved in cross-linking collagen IV and laminin. The role of NID in breast cancer has only been evaluated in a small number of studies and the findings of these studies have been inconsistent. Our previous work revealed that highly tumorigenic murine mammary tumor cells express high levels of *Nid1* while weakly tumorigenic mammary tumor cells express low levels of *Nid1*. To investigate *Nid1*, two stable knockdown lines were created, and *Nid1* knockdown was confirmed at both the mRNA and protein level. *Nid1* knockdown significantly reduced cell proliferation and migration/invasion and these reductions in proliferation and migration/invasion could be rescued by conditioned media containing NID1 protein. The reduced migration/invasion observed in the *Nid1* knockdown cells was not associated with significant alterations in the epithelial gene *Cdh1* or the mesenchymal genes *Snai1*, *Snai2*, *Twist1*, *Twist2*, *Zeb1* and *Zeb2*. Therefore, suppression of *Nid1* expression reduces proliferation and migration/invasion in claudin-low murine mammary tumor cells.

Introduction

The basement membrane is a highly specialized extracellular matrix (ECM) that is located around all cell monolayers and multi-layered epithelia within the body (1-4). Its functions include structural support, regulation of cell signalling behaviors and providing a protective barrier. The main components of basement membranes include collagen IV, laminin, perlecan and nidogen. Of these components, it is known that collagen IV and laminin can self-assemble into superstructures individually, which plays a vital role in the stability of BMs. Perlecan and nidogen (NID) influence the

structural integrity through connecting the collagen IV and laminin networks, increasing stability (1-4). NID, also known as entactin, mimics the function of perlecan by connecting the superstructures of collagen IV and laminin (4). Collagen IV contains a triple helical domain that fuses to the G2 domain of NID, while the γ -3 chain of laminin binds to the G3 domain of NID, effectively linking the two components (1,2,4).

Only a small number of studies have investigated the impact of NID1 in cancer with only 3 studies evaluating breast cancer (5-7). A study by Alečković *et al* (5) performed a secretome analysis of lung metastatic sublines of the human breast cancer cell line MDA-MB-231 and identified NID1 as potential regulator of metastasis. The importance of NID1 in lung cancer metastasis was further examined in MDA-MB-231 overexpressing *NID1*. Overexpression of *NID1* increased lung metastases 10-fold and reduced survival in an experimental metastasis assay as well as promoting invasion and migration in a transwell assay (5). These investigators also showed that *NID1* expression in breast cancer patients was associated with lung metastasis and poor patient prognosis (5). However, a recent study by Ferraro *et al* (7) showed the opposite; secretion of NID1 from endothelial cells inhibited the migration of the human breast cancer cell line SK-BR-3. Therefore, the impact of NID1 on breast cancer migration is inconclusive. The third study evaluating the function of NID1 in breast cancer found that a disintegrin and metalloproteinase with thrombospondin repeats 1 (ADAMTS1) can cleave NID1 inhibiting its function. ADAMTS1 levels were shown to be reduced in breast cancer samples and this was associated with reduced NID1 proteolysis and increased deposition of NID1 into tumor blood vessels (6).

A previous study from our lab looking at miR-200 levels found that re-expression of the miR-200b/200a/429 cluster impaired tumor growth and metastasis (8). RNA-sequencing revealed that *Nid1* was the most significantly reduced gene in miR-200 transfected cells compared to control cells (8). Given that a higher *Nid1* expression was associated with increased tumorigenicity and metastatic capacity this study evaluated the impact of *Nid1* on mammary tumor cell proliferation and migration/invasion.

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Materials and methods

Cell lines. RJ423 cells have been previously described (8-11). To reduce *Nid1* expression, RJ423 cells were transfected with four

Table I. Characteristics of murine mammary tumor cells.

Cell lines	miR-200 expression	<i>Nid1</i> expression	Tumorigenicity	Metastatic
RJ345	High	Low	Low	No
RJ423	Low	High	High	Yes
RJ423-200ba429	High	Low	Low	No
RJ423EV	Low	High	High	Yes

miR-200, microRNA-200; *Nid1*, nidogen 1.

unique shRNAs targeting *Nid1* (cat. no. TL501474; Origene Technologies Inc.) or a control shRNA (cat. no. TR30021; Origene Technologies Inc.). Stable clones were selected using 5 µg/ml puromycin (InvivoGen). Two knockdown lines (RJ423sh*Nid1A* and RJ423sh*Nid1D*) were identified as having the greatest *Nid1* mRNA knockdown relative to a control line (RJ423con). All cells were cultured as described in (8). RJ423sh*Nid1A*, RJ423sh*Nid1D* and RJ423con cells were maintained in 5 µg/ml puromycin.

RNA isolation and RT-qPCR. RNA isolation and RT-qPCR were performed as previously described (8). Primers for *Nid1* (qMmuCID0018281), *Cdh1* (qMmuCED0044197), *Vim* (qMmuCID0005527), *Snai1* (qMmuCID0024342), *Snai2* (qMmuCED0046072), *Twist1* (qMmuCED0004065), *Twist2* (qMmuCID0009652), *Zeb1* (qMmuCID0009095), *Zeb2* (qMmuCID0014662) and *Hprt* (qMmuCID0005679) were purchased from Bio-Rad Laboratories (Canada) Ltd. Gene expression was quantified and normalized to *Hprt* using the normalized expression ($\Delta\Delta Cq$) (12) mode in CFX-Manager 3.1 software [Bio-Rad Laboratories (Canada) Ltd.].

Collection of conditioned media. Cells were seeded in 10 mm culture dishes at a density of 2×10^6 cells per dish overnight in fully supplemented media. The next day, the fully supplemented media was removed, the cells were washed twice with phosphate-buffered saline (PBS) and 10 ml of serum-free media containing sodium pyruvate, glutamine and antibiotic-antimycotic was added to each dish. Twenty-four hours later, conditioned media was collected and either used immediately or stored at -20°C .

Western blotting. Western blotting was performed as previously described (13). NID1 protein was detected using a primary antibody against NID1 (cat. no. NBP1-97701; Novus Biologicals) at a 1:200 dilution while β -actin was detected using a 1:5,000 dilution of a primary antibody against β -actin (cat. no. 8457; Cell Signaling Technologies, Inc.). The appropriate HRP-linked secondary antibody (Cell Signaling Technology, Inc.) was used at a 1:2,000 dilution.

Cell proliferation. Immunofluorescence for phospho-histone H3 (PH3) was used to determine cell proliferation as previously described (8). For the conditioned media experiments, cells were allowed to attach to the coverslips overnight in fully supplemented media. The next day, the fully supplemented media was removed, the cells washed twice with PBS,

and then the cells were cultured in conditioned media for 24 h prior to assessing proliferation.

Cell apoptosis. Apoptosis was assessed using the Annexin V-FITC Apoptosis Detection kit (eBioscience) according to the manufacturer's protocol. A BD Accuri C6 flow cytometer (BD Biosciences) was used to measure fluorescence at a flow rate less than 400 events/second and analyzed using the Accuri C6 software (BD Biosciences).

Transwell invasion chamber assay. Transwell assays were performed as previously described (8) with minor alterations. Since Matrigel contains NID1, the Cultrex® 24 Well Collagen IV Cell Invasion Assay kit (cat. no. 3458-024-K) was used for the transwell inserts. The amount of cell migration was analyzed using ImageJ software (National Institutes of Health). To determine whether NID1 served as a chemoattractant, cells were cultured in serum-free media in the upper chamber with conditioned media from the various cell lines in the lower chamber. To determine whether NID1 directly interacted with cells to alter migration, cells were cultured in conditioned media in the upper chamber and fully supplemented media was placed in the lower chamber.

Statistical analysis. Statistical analysis was conducted using one-way ANOVA followed by Tukey's test using GraphPad Prism 8 (GraphPad Software, Inc.). Statistical significance ($P < 0.05$) is indicated by asterisks.

Results

***Nid1* shRNA reduces NID1 protein levels in conditioned media.** Our previous work revealed that re-expressing the miR-200b/200a/429 cluster in murine mammary tumor cells (RJ423-200ba429) suppressed tumor growth and metastasis *in vivo* (8). RNA sequencing of the RJ423-200ba429 cells revealed that *Nid1* was the most significant, downregulated gene (*Nid1* expression was decreased 3,821-fold, P -value 3.5×10^{-268} , GSE113162). Consistent with this finding, *Nid1* expression was reduced 6,654-fold (P -value 3.5×10^{-240}) in the poorly tumorigenic, non-metastatic murine mammary tumor cell line RJ345 compared to the highly tumorigenic, highly metastatic murine mammary tumor cell line RJ423. Since both RJ345 cells and RJ423-200ba429 cells are poorly tumorigenic, non-metastatic (8) and express almost no *Nid1* (Table I) this study examined whether reducing *Nid1* in RJ423 cells could negatively impact proliferation and/or migration.

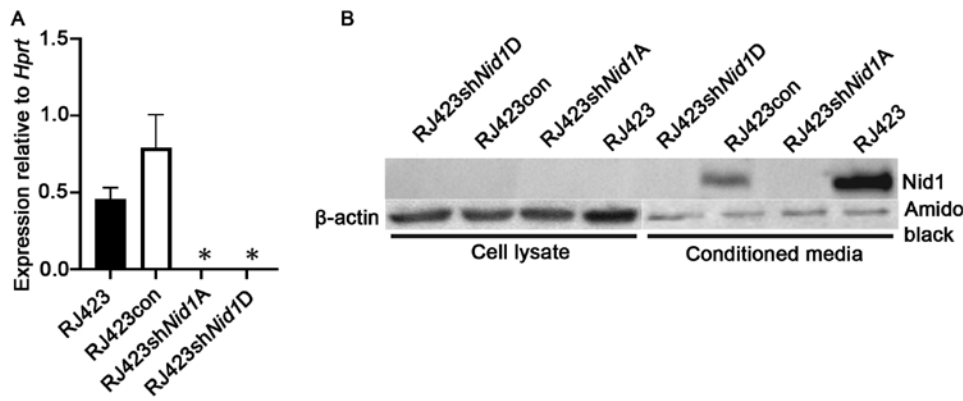


Figure 1. *Nid1* levels in cells stably expressing *Nid1* shRNA. (A) Expression levels of *Nid1* mRNA relative to *Hprt* in parental RJ423, RJ423con, RJ423*Nid1A* and RJ423sh*Nid1D* cells. The bars represent the mean value and the error bars represent the SEM. *Indicates significant difference ($P < 0.05$) from RJ423con cells ($n=6$). (B) Western blot analysis of NID1 protein from cell lysates and conditioned media from RJ423, RJ423con, RJ423sh*Nid1A* and RJ423sh*Nid1D* cells. β -actin was used as a loading control for the cell lysates while amido black was used to ensure similar loading for the conditioned media samples. *Nid1*, nidogen 1; *Hprt*, hypoxanthine phosphoribosyltransferase; con, control; sh, short hairpin RNA.

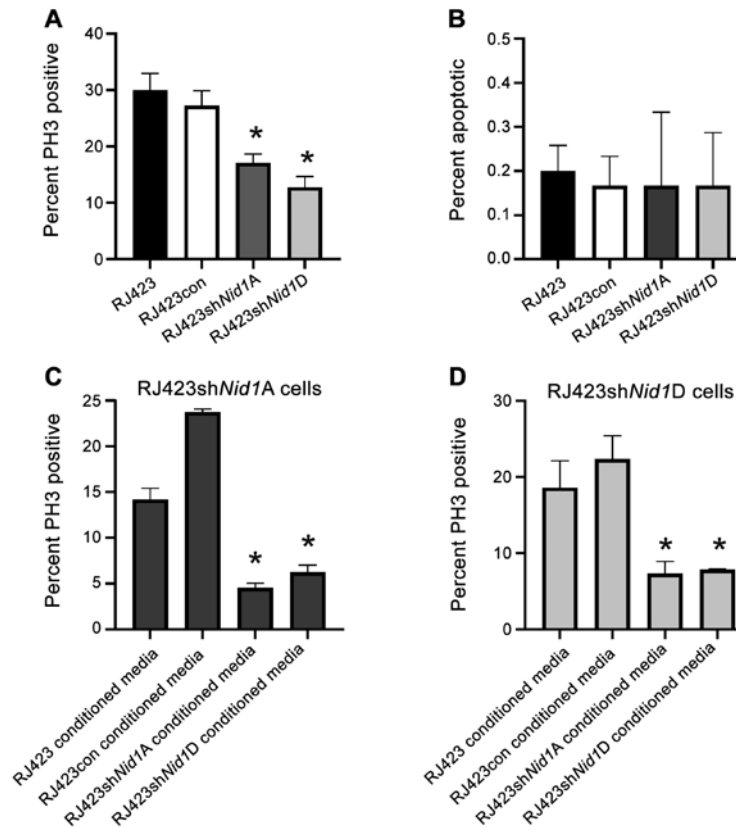


Figure 2. Impact of NID1 conditioned media on cell proliferation and apoptosis. (A) Percentage of PH3-positive cells in parental RJ423, RJ423con, RJ423sh*Nid1A* and RJ423sh*Nid1D* cells. (B) Percentage of apoptotic cells in RJ423, RJ423con, RJ423*Nid1A* and RJ423sh*Nid1D* cells. Percentage of PH3-positive cells from (C) RJ423sh*Nid1A* and (D) RJ423sh*Nid1D* cells treated with conditioned media from RJ423, RJ423con, RJ423sh*Nid1A* or RJ423sh*Nid1D* cells for 24 h. * $P < 0.05$ vs. RJ423con cells ($n=3$). *Nid1*, nidogen 1; con, control; sh, short hairpin RNA; PH3, phospho-histone H3.

To reduce the expression of *Nid1*, RJ423 cells were stably transfected with 4 unique shRNA constructs (A-D) targeting mouse *Nid1* or a control shRNA construct. The two most effective *Nid1* shRNA constructs, sh*Nid1A* and sh*Nid1D*, reduced *Nid1* mRNA levels over 99% compared to RJ423 cells stably expressing the control shRNA construct (RJ423con; Fig. 1A).

To confirm NID1 was suppressed at the protein level western blotting was performed on cell lysates from RJ423, RJ423con, RJ423sh*Nid1A* and RJ423sh*Nid1D* cells. The

NID1 antibody failed to detect NID1 protein in any of the cell lysates (Fig. 1B). Since NID1 is a secreted protein conditioned media was collected from all four cell lines. As shown in Fig. 1B, NID1 was detected in the conditioned media from RJ423 cells and RJ423con cells but not from either RJ423sh*Nid1A* or RJ423sh*Nid1D* cells.

Nid1 knockdown inhibits proliferation but not apoptosis. The impact of *Nid1* suppression on cell proliferation was assessed

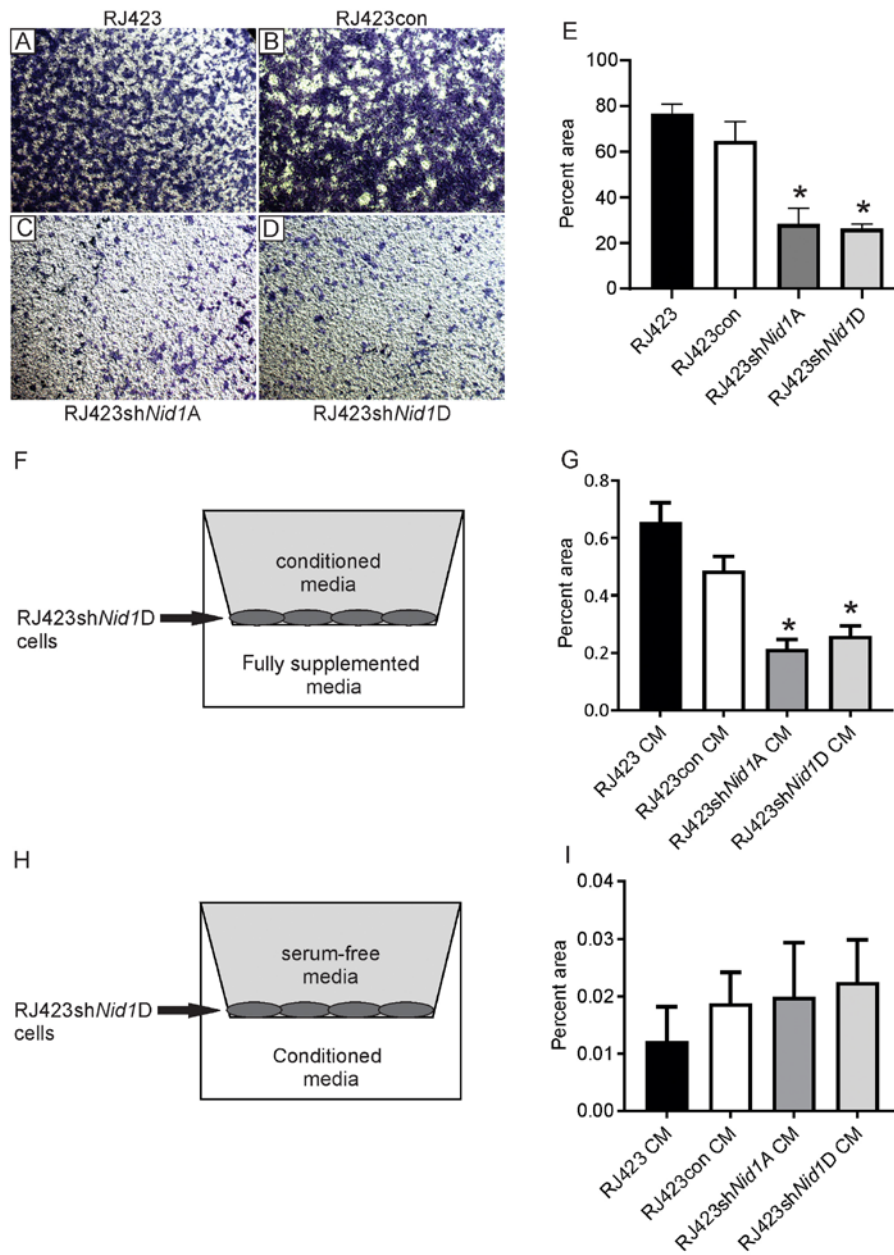


Figure 3. Impact of NID1 conditioned media on cell migration. Representative images of toluidine blue stained cells on the bottom of collagen-coated transwell inserts for (A) parental RJ423, (B) RJ423con, (C) RJ423shNid1A and (D) RJ423shNid1D cells (magnification, x4). (E) Quantification of the percent area occupied by parental RJ423, RJ423con, RJ423shNid1A and RJ423shNid1D cells. Migration and invasion of RJ423shNid1D cells when conditioned media from RJ423, RJ423con, RJ423shNid1A or RJ423shNid1D cells were placed (F and G) in the upper chamber of the well with the cells or (H and I) in the lower chamber. (F and H) Schematic indicating the location of the conditioned media and cells. (G and I) Quantitative data from these experiments. * $P < 0.05$ vs. RJ423con cells ($n=3$). Nid1, nidogen 1; con, control; sh, short hairpin RNA; CM, conditioned media.

using PH3 immunofluorescence. Both *Nid1* shRNA constructs significantly reduced proliferation compared to the control cell line, RJ423con (Fig. 2A). The impact of *Nid1* suppression on apoptosis was evaluated using Annexin V flow cytometry. RJ423, RJ423con, RJ423shNid1A and RJ423shNid1D cells all had very low levels of apoptosis (<1%) and this level of basal apoptosis when cultured in fully supplemented media (Fig. 2B). Since there was no significant difference in apoptosis this cell response was not further evaluated.

To confirm the decrease in proliferation resulted from *Nid1* suppression, RJ423shNid1A and RJ423shNid1D cells were cultured in the presence of conditioned media from RJ423 cells, RJ423con cells, their own conditioned media and condi-

tioned media from the other knockdown line. It was anticipated that conditioned media from RJ423 or RJ423con cells would increase proliferation in the *Nid1* knockdown cells as both RJ423 and RJ423con cells secrete NID1 into the media. As shown in Fig. 2C and D proliferation of RJ423shNid1A and RJ423shNid1D cells was significantly increased when the cells were grown in conditioned media from RJ423con cells compared to when these cells were grown in conditioned media from either *Nid1* knockdown cell line. Proliferation of RJ423shNid1A cells was also significantly increased when the cells were grown in conditioned media from RJ423 cells compared to when these cells were grown in conditioned media from either *Nid1* knockdown cell line while the proliferation

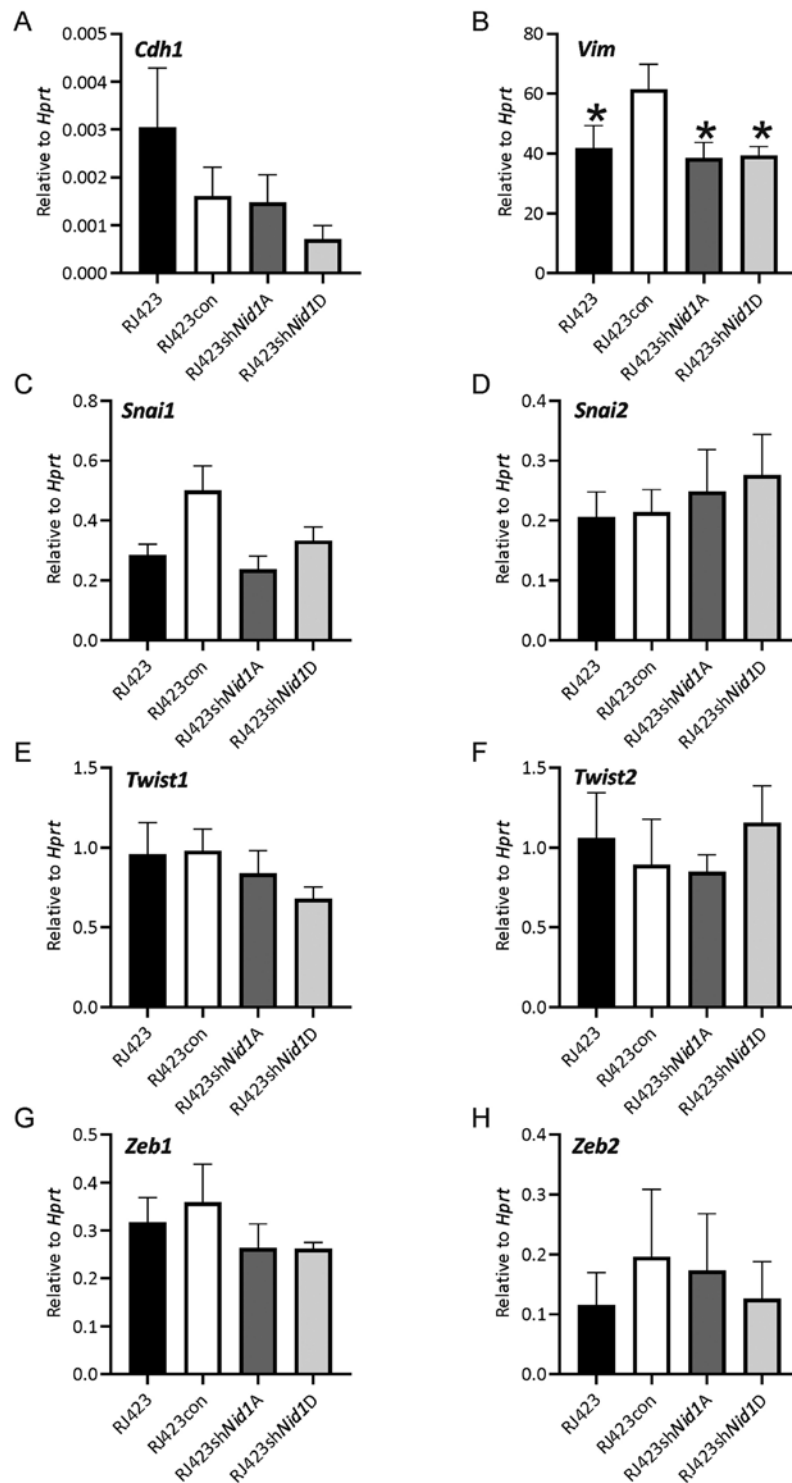


Figure 4. Expression levels of epithelial and mesenchymal genes following *Nid1* knockdown. mRNA expression levels of (A) *Cdh1*, (B) *Vim*, (C) *Snai1*, (D) *Snai2*, (E) *Twist1*, (F) *Twist2*, (G) *Zeb1* or (H) *Zeb2* relative to *Hprt*. * $P < 0.05$ vs. RJ423con cells (n=5). *Nid1*, nidogen 1; *Hprt*, hypoxanthine phosphoribosyltransferase; con, control; sh, short hairpin RNA.

in the RJ423sh*Nid1D* cells was close to being significant when these cells were grown in condition media from RJ423 cells compared to condition media from RJ423sh*Nid1A* ($P=0.058$) or RJ423sh*Nid1D* ($P=0.081$) cells.

Nid1 knockdown impairs cell migration. Next, migration and invasion were assessed using a transwell assay. The transwell inserts were coated with collagen IV rather than Matrigel

as Matrigel contains NID1. Fig. 3A-D shows representative images of the stained cells that invaded and migrated through the collagen IV coated inserts. Quantification of these images (Fig. 3E) revealed that RJ423sh*Nid1A* and RJ423sh*Nid1D* cells were significantly less effective at invading and migrating than either the RJ423 or RJ423con cells.

To confirm that migration and invasion was dependent on NID1, migration of RJ423sh*Nid1D* cells was assessed using

conditioned media from RJ423, RJ423con, RJ423sh*Nid1A* or RJ423sh*Nid1D* cells in the upper chamber and fully supplemented media in the lower chamber (Fig. 3F). The presence of conditioned media from RJ423 and RJ423con cells significantly increased invasion and migration of RJ423sh*Nid1D* cells compared the conditioned media from either RJ423sh*Nid1A* or RJ423sh*Nid1D* cells (Fig. 3G).

To assess whether NID1 served as a chemoattractant, RJ423sh*Nid1D* cells were grown in the upper chamber in serum-free media and conditioned media from RJ423, RJ423con, RJ423sh*Nid1A* or RJ423sh*Nid1D* cells was placed in the lower chamber (Fig. 3H). As shown in Fig. 3I, RJ423sh*Nid1A* cells invaded and migrated very poorly independent of the conditioned media used as a chemoattractant and there were no significant differences observed.

Enhanced migration and invasion are often associated with increased expression of mesenchymal genes and loss of epithelial genes in a process known as epithelial to mesenchymal transition (EMT). It was anticipated that one or more mesenchymal genes would be expressed at significantly reduced levels or an increase in expression of the epithelial gene, *Cdh1*, would be observed in the *Nid1* knockdown lines compared to RJ423con and RJ423 parental cells. The only gene significantly altered was *Vim* in the *Nid1* knockdown lines, however *Vim* expression in the *Nid1* knockdown lines was almost identical to the parental RJ423 cells (Fig. 4).

Discussion

Our previous work looking at highly tumorigenic and metastatic murine mammary tumor cells compared to weakly tumorigenic, non-metastatic tumor cells identified *Nid1* as a potential promoter of tumor development and metastasis (8). RJ423 cells are murine mammary tumor cells with characteristics of human claudin-low breast cancer that express high levels of *Nid1*. RJ423 cells rapidly form tumors when injected into the mammary fat pad of mice and induce lung metastases in mice following tail vein injection (8).

Suppression of *Nid1* expression in RJ423 cells significantly reduced cell proliferation and this decrease in proliferation was rescued by incubating cells in conditioned media containing NID1. While it is possible that the conditioned media from RJ423 and RJ423con cells contain proteins other than NID1, presumably the conditioned media from RJ423con and RJ423sh*Nid1A*/RJ423sh*Nid1D* cells should be highly similar as the only difference between these cells was the stable knockdown of *Nid1*. While treating the cells with recombinant NID1 would confirm the impact of NID1 on proliferation, commercially available, full-length recombinant murine NID1 could not be found. In a study by Alečković *et al* (5), proliferation of MDA-MB-231 cells overexpressing *NID1* cultured *in vitro* was not examined. However, this group did assess tumor size *in vivo* and found that overexpression of *NID1* in MDA-MB-231 did not increase primary tumor growth. No other study investigated the impact of NID1 on breast cancer cell proliferation *in vitro*.

NID1 did not impact basal levels of apoptosis as RJ423con, RJ423sh*Nid1A*, and RJ423sh*Nid1D* cells. As no study has investigated the impact of NID1 on breast cancer cell apop-

tothis this is the first study showing that a reduction of NID1 does not increase mammary tumor cell apoptosis.

Reducing NID1 levels significantly impaired migration and invasion through collagen IV coated membranes and migration/invasion could be rescued when conditioned media containing NID1 was placed in the upper chamber with the RJ423sh*Nid1D* cells but not when the conditioned media containing NID1 was placed in the lower chamber. These findings suggest that NID1 is interacting directly with the cancer cells and not acting as a chemoattractant. It is not clear how NID1 alters cell migration and invasion, but it could be through binding to receptors on the cell and influencing signaling. NID1 has been reported to bind to integrins $\alpha\beta3$ and $\alpha3\beta1$ (14-16) and RNA sequencing by our lab has shown the presence of *Itga3*, *Itgb3*, and *Itgb1* but not *Itgav* in RJ423 cells (GSE113162). Therefore, NID1 is presumably signaling through integrin $\alpha3\beta1$ or another receptor that has yet to be identified. The observation that NID1 promotes migration and invasion is consistent with the findings of Alečković *et al* (5) who showed that MDA-MB-231 cells overexpressing *NID1* increased migration and invasion *in vitro* and lung metastatic colonization *in vivo*. However, Ferraro *et al* (7) found that NID1 secreted by endothelial cells inhibited invasion a migration of the human breast cancer cell line, SK-BR-3. The main difference between the study by Ferraro *et al* (7) and our study and the study by Alečković *et al* (5) was that while the Ferraro *et al* (7) study utilized breast cancer cells with HER2 overexpression while our study (9) and the study by Alečković *et al* (5) used tumor cells with characteristics of claudin-low breast cancer. Thus, NID1 may promote migration and invasion of claudin-low breast cancer cells but not in other breast cancer subtypes.

Enhanced invasion and migration are often accompanied by an increase in mesenchymal gene expression, reduced epithelial gene expression and a more spindled-shape morphology. Moreover, NID1 has been implicated in inducing EMT in colorectal cancer cells (17) and ovarian cancer cells (18) but has not been investigated in breast cancer. No consistent increase in mesenchymal gene expression or decrease in epithelial gene expression was observed in either RJ423sh*Nid1A* or RJ423sh*Nid1D* cells compared to RJ423con cells suggesting that NID1 cannot restore epithelial gene expression patterns in claudin-low mammary tumor cells.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the Gene Expression Omnibus repository, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE113162>

Authors' contributions

RJ performed reverse transcription-quantitative PCR, western blotting for NID1, proliferation assays, apoptosis assays and Transwell migration assays. CJM performed reverse transcription-quantitative PCR on some of the samples for *Nid1* and some of the epithelial and mesenchymal genes. RAM created the cell lines, edited the manuscript and ran the project. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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