Evaluating The Transcriptional Regulation of Cdh1 by Grhl3 in Different Cellular Models

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ABSTRACT: The calcium-dependent adhesion protein E-cadherin encoded by the Cdh1 gene is a member of a large family conferring proper establishment of adherens junction. The expression of Cdh1 is critical and is observed in epithelial cells. E-cadherin expression is also essential for establishing the pluripotent state in embryonic stem cells. Cdh1 transcriptional regulation has been the focus of research for many years; early reports identified repressors of Cdh1 since its downregulation is essential for the initiation of the epithelial to mesenchymal transition. Restoring the expression of Cdh1 is thought to be simply due to the disengagement of Cdh1 silencers from its promoter. Recent studies supported the presence of dedicated activators of Cdh1 expression, including members of the Grhl and Ets family of transcription factors. Here we evaluated the regulatory potential of Grhl3 on the Cdh1 promoter in different cellular models to understand the extent of the transcriptional relationship with Cdh1. We utilized several approaches, such as the correlation of expression by loss and gain of function, ChIP, and luciferase reporter assays. As a result, we found that Grhl3 is a potent regulator of Cdh1 in cells of epithelial origin. Moreover, Grhl3 was sufficient to re-establish Cdh1 expression in the murine hepatoma cells Hepa1-6. Improved understanding of the regulation of Cdh1 is essential for the perception of how the epithelial to mesenchymal and the mesenchymal to epithelial transitions are regulated, as they play a crucial role in metastasis, which will pave the way for better management of the metastatic disease.

Keywords: E-cadherin, transcriptional regulation, Grhl3

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INTRODUCTION

E-cadherin (E-cad), encoded by the gene Cdh1, is a member of the cadherin superfamily. E-cad is a Ca\(^{2+}\)-dependent cell-cell adhesion glycoprotein, which shares about 70% similarity with N-cadherin (N-cad) (Bedzhov et al., 2013). Cadherins anchor the cytoskeleton through interactions with p120-catenin, alpha-catenin, and beta catenins to form adherens junctions (Stemmler, 2008). E-cad and N-cad are expressed in a mutually exclusive manner; E-cad is restricted to epithelial cells, while N-cad is expressed in mesenchymal cells. The cadherin switch is a vital step during the epithelial to mesenchymal transition (EMT) (Stemmler, 2008). EMT is a critical developmental process hijacked by tumor cells during metastasis (Brabletz et al., 2018; Kalluri and Weinberg, 2009; Terashima et al., 2018). During EMT, E-cad is downregulated after the activation of a repertoire of silencers dubbed the EMT inducers, including Snail and Zeb1, among others (Stemmler, 2008; Thiery et al., 2009). While E-cad repression during EMT is well studied (Fazilaty et al., 2019; Lavin and Tiwari, 2020), its reactivation during mesenchymal to epithelial transition (MET) is not fully understood, and only recently has it been under investigation (Alotaibi et al., 2015; Sengez et al., 2019; Werth et al., 2010). Understanding how Cdh1 is regulated is of great importance to better comprehend the biological processes it is involved in, understand metastasis, and develop novel therapeutics for the management of the disease.

Few regulators of Cdh1 have been reported (Alotaibi et al., 2015; Li et al., 2010; Sengez et al., 2019; Werth et al., 2010). One of the earlier reports revealed Klf4 as a key regulator of Cdh1 (Li et al., 2010). During the initiation of induced pluripotent stem cells (iPSC), the activation of Cdh1 expression via direct binding of exogenous Klf4 to specific sites at the promoter (Li et al., 2010) triggers MET, which is then followed by the activation of the endogenous transcription factors of the core pluripotency network (Li et al., 2010). More recently, members of the Grhl family of transcription factors have been reported to activate Cdh1 expression, thus contributing to MET initiation (Alotaibi et al., 2015; Werth et al., 2010). Grainyhead-like 2 (Grhl2) was found to activate Cdh1 expression by binding to an intronic enhancer during the formation of uretic bud (Werth et al., 2010). We have also reported that Grhl3 plays a crucial role in the initiation of MET. After activating Hnf4α expression, both Grhl3 and Hnf4α bind to separate enhancers in intron 2 and physically interact to activate Cdh1 expression (Alotaibi et al., 2015). Despite the presence of several publications focusing on how Cdh1 expression is downregulated during EMT, the positive regulation of Cdh1 expression is limited to a few reports. Studies regarding E-cad function or regulation are usually limited to few cellular models such as embryonic stem cells for its role in pluripotency (Bedzhov et al., 2013; Niwa, 2007; Redmer et al., 2011; Voutsadakis, 2015), or EMT/MET models where its expression is modulated by extracellular signals such as TGFβ (Alotaibi et al., 2015). This limits the potential of discovering novel aspects of its regulation. To overcome this limitation, we carried out several experiments to assess the regulatory potential of Grhl3 in different cellular models. We included cell lines of embryonic, epithelial, and cancer origins. The variety of cells would allow us to better understand Cdh1 expression. We found that the E-cad negative cell line Hepa1-6 is an excellent cellular model for analyzing Cdh1 transcriptional regulation, which could be used to identify novel regulators of Cdh1 that are not limited to pluripotency or EMT/MET.

MATERIALS AND METHODS

Cell Lines

E14.1; mouse embryonic stem cells, NMuMG; normal murine mammary gland cell line, CSG; Murine submandibular gland carcinoma cell line, Hepa1-6; hepatoma cell line, P19 and F9; mouse embryonic carcinoma cell line, and CMT; mouse polyploid carcinoma cell line were obtained from ATCC. In general, cells were cultured at 37°C in an incubator supplied with 10% CO\(_2\). The culture...
medium was DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS), 10 U ml\(^{-1}\) Penicillin/Streptomycin, 0.1 mM non-essential amino acids, and 2 mM L-glutamine (Gibco). For NMuMG cells medium, 10 µg ml\(^{-1}\) insulin (Sigma) was added. \textbf{E14.1} ES cells were cultured on mitotically inactivated embryonic fibroblasts (MEFs) in complete ES cell medium as explained in (Bedzhov et al., 2013). Culture media were replenished every couple of days. Cell passaging was performed at 80-90% confluence.

**mRNA Expression Level Analysis**

For gene expression analysis, RNA isolation and cDNA synthesis were performed using the methods and reagents as described previously (Alotaibi et al., 2015). qPCR analysis and quantification were performed using the \(\Delta\Delta Ct\) method. For knock-down (loss-of-function) experiments, cultured cells were transfected with 50 nM siRNAs against \textit{Grhl3} (siGrhl3, Ambion) or a non-targeting control scrambled siRNA (siCntrl, Ambion) using the Lipofectamine RNAi Max (Invitrogen) and collected 72 h later. For over-expression (gain-of-function) experiments, cells were transfected with a \textit{Grhl3} expressing plasmid or an empty vector (Mock) using the X-tremeGENE 9 (Roche). RNA used for cDNA synthesis was isolated 48 hours after transfection.

**Luciferase Reporter Assays**

The \textit{Cdh1} promoter (1.5kb region) and the \textit{Cdhl} enhancer at 7.8 kb were cloned in the pGL4.10 and pGL4.23 plasmids, respectively. Details about the reporter plasmids were described previously (Alotaibi et al., 2015). In brief, transfection was done in 48-well plates, and the cells were transfected using the X-tremeGENE 9 (Roche) and 100 ng of each reporter plasmid containing 5 ng pRL-TK (Promega) as an internal control. Luciferase activity was measured in the Centro LB 960 luminometer (Berthold Technologies) using the Dual-Glo Luciferase Assay kit (Promega). Fold induction is relative to the empty vector calculated by normalizing Firefly luciferase values to the Renilla control.

**Chromatin Immunoprecipitation (ChIP)**

For ChIP analysis, cells were cultured in 6-well plates. Following cross-linking with formaldehyde and then lysis, chromatin containing cell lysates were sonicated using the S2 ultrasonicator (Covaris) at the following conditions: frequency sweeping mode and 15 cycles of 60 s at maximum intensity and 15 % duty cycle performed at 4°C. Immunoprecipitation was carried out at 4°C for 16 h using the ChIP-grade antibodies against Grhl3 (S-19, Santa Cruz) and a rabbit control IgG (sc-2027, Santa Cruz). Quantification was performed with qPCR as described previously (Alotaibi et al., 2015).

**Statistical Analysis**

Luciferase reporter assays and qPCR experiments were performed in triplicates. Statistical evaluation of the results was performed using the Student’s t-test; a 95 % confidence interval was applied. All experiments were performed at least three times. Error bars depict “standard error of the mean”.

**RESULTS AND DISCUSSION**

**Correlation of Expression Between \textit{Grhl3} and \textit{Cdh1}**

Initially, we assessed the expression levels of \textit{Cdhl} and \textit{Grhl3} in the cell lines used in this study. Three cell lines are of embryonic origin (E14.1, P19, and F9); these cells express \textit{Cdhl}, among other pluripotency and epithelial genes. Also, somatic cells such as NMuMG, CSG, and CMT express \textit{Cdhl} as well. On the other hand, Hepa1-6 cells lack \textit{Cdhl} expression (Figure 1A). As for \textit{Grhl3}, we detected the transcript in most cells but at varying levels. \textit{Grhl3} expression was highest in E14.1, P19, and F9
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(Figure 1B). The overall evaluation of mRNA levels of Cdh1 and Grhl3 revealed that Cdh1 expression is considerably higher than that of Grhl3.

To assess the correlation of expression between the two genes in the cell lines used in this study, we first ectopically expressed Grhl3 from a plasmid and compared the expression levels of Cdh1 to those in mock-transfected cells. As a result, Cdh1 mRNA levels increased 2-fold in F9 and CMT cell lines. On the other hand, the Cdh1 negative cell Hepa1-6 response was strongest among the other cell lines tested, with a more than 3-fold increase (Figure 1C).

![Figure 1](image)

**Figure 1.** Correlation of expression between Grhl3 and Cdh1. qPCR analysis of the basal expression levels of Cdh1 (A) and Grhl3 (B) in different cell lines. C) Grhl3 over-expression leads to an increased Cdh1 expression in 3 of the cell lines studied. Results represent 3 independent experiments. *: P <.05, **: P <.01

We extended the correlation of expression studies to loss of function experiments. For this purpose, we utilized siRNAs against Grhl3 and transfected the cell lines with either siGrhl3 or siCtrl siRNAs. After 72 hours of treatment, RNA was isolated, and changes in Grhl3 and Cdh1 mRNA levels were determined by qPCR (Figure 2). The Grhl3 depletion efficiency was assessed by the apparent decrease in Grhl3 levels, while P19 cells were not responsive to the siRNA treatment (Figure 2B). The overall knock-down of Grhl3 was efficient, ranging from 70-90% in different cell lines. mRNA levels of Cdh1 were measured in response to Grhl3 depletion, although the decrease in Cdh1 expression was up to 40% (Figure 2E), the overall decrease among the cell lines was statistically significant, which is in support of the previously measured positive correlation (Sengez et al., 2019). In conclusion, we have established experimental evidence for the correlation of expression between Grhl3 and Cdh1 in different cellular models.
Figure 2. Correlation of expression between \textit{Grhl3} and \textit{Cdhl1}. qPCR analysis of \textit{Grhl3} and \textit{Cdhl1} in response to the siRNA mediated silencing of \textit{Grhl3} in different cell lines. Each panel represents the results of a distinct cell line as indicated on top of each panel. The expression of \textit{Grhl3} indicates the silencing efficiency. Results represent 3 independent experiments. *: $P<.05$, **: $P<.01$

\textbf{Grhl3 Can Activate the Promoter of \textit{Cdhl1}}

After establishing the correlation of expression between \textit{Cdhl1} and \textit{Grhl3}, we investigated the regulatory potential of Grhl3 over the \textit{Cdhl1} promoter and the previously annotated enhancers (Alotaibi et al., 2015). A graphical representation of these enhancers within the \textit{Cdhl1} locus is shown in Figure 1A. We first transfected different cell lines with a luciferase reporter construct containing the \textit{Cdhl1} promoter. \textit{Cdhl1} promoter responded to Grhl3 only in two cell lines, NMuMG and Hepa1-6 (Figure 3B). The observed promoter activation was in agreement with the results presented in figure 1C. We then studied the activation of the Grhl3-activated enhancer (enhancer at position 7.8 in Figure 3A), which we identified previously (Alotaibi et al., 2015); unlike the promoter, this enhancer within the second intron
of Cdh1 was responsive in four cell lines; in addition to NMuMG and Hepa1-6, CSG and P19 showed responsiveness to Grhl3 activation (Figure 3C). To better understand the regulatory potential of Grhl3 on Cdh1 expression, we performed ChIP experiments using antibodies against Grhl3 and measured its enrichment on the intronic enhancer. For this, we only used CSG and CMT cells. We measured a strong enrichment of Grhl3 in CSG cells only on the enhancer at position 7.8kb, and in agreement with the results from the reporter assay, Grhl3 was not enriched on any of the other enhancers (Figure 3D).

Figure 3. Regulation of Cdh1 by Grhl3. Ectopic expression of Grhl3 activates the Cdh1 promoter (A) and the intronic enhancer (B) as measured by luciferase reporter assay. In A and B, the effect of Grhl3 induction was compared to a mock plasmid. C) qPCR results of ChIP experiments indicating the enrichment of Grhl3 at the intronic enhancer in two cell lines, CSG and CMT. Results represent 3 independent experiments. *: P < .05, **: P < .01, ***: P < .001

We have previously reported a strong correlation of the expression between Grhl3 and Cdh1 with a correlation score (Rho) of more than 0.81 with a P-value of 2.22e-16 (Sengez et al., 2019). This high correlation also reflected the expression of these two genes between the epithelial and mesenchymal states. Members of the Grhl family of transcription factors have been described as regulators of Cdh1 expression (Alotaibi et al., 2015; Werth et al., 2010). While Grhl3 was attributed to the regulation during the mesenchymal to epithelial transition, its regulatory potential in other models remained unclear. The
expression of Cdh1 in the studied cell lines clearly reflected an epithelial and a mesenchymal state, yet the expression level varied between different cell lines; this might be due to the tissue origin of the cell line.

On the other hand, the expression pattern of Grhl3 reflected its expression between developmental and post-natal patterns. Grhl3 expression is known to be expressed at higher levels during embryonic development and then gradually decreases after birth and becomes restricted at a low level in a few tissues (Auden et al., 2006). The expression of Grhl3 in cancer cells is different and was found elevated in some cancer types (Frisch et al., 2017; Mlacki et al., 2015; Xu et al., 2014; Zhao et al., 2016). In a previous study, we observed a high correlation of Grhl3 and Cdh1 expression in human breast cancer data sets (Alotaibi et al., 2015). The status of Cdh1 or Grhl3 expression in cancer cells is not entirely in accordance with that in normal cells, i.e., in contrast between the mesenchymal and epithelial states (Zhao et al., 2016). This might explain the expression of Grhl3 in the Cdh1 negative Hepa1-6 cells. Nevertheless, the other cancer cells used in this study (CSG and CMT) showed a positive correlation between Cdh1 and Grhl3 in agreement with the other cell lines under study.

The regulation of Cdh1 expression has been the focus of several studies in recent years. The repression of Cdh1 expression by the EMT inducers is well known and is essential for the progression of EMT (Brabletz, 2012; Kalluri and Weinberg, 2009). It was also clear that the activation of Cdh1 expression cannot be simply explained by the absence of the repressors (Alotaibi et al., 2015; Stemmler et al., 2005; Stemmler et al., 2003). On the other hand, the activation of Cdh1 expression has been explained by the direct involvement of a distinct set of transcription factors other than the EMT inducers (Alotaibi et al., 2015; Li et al., 2010; Werth et al., 2010). Besides Klf4 (Li et al., 2010) and Hnf4a (Alotaibi et al., 2015), the grhl family of transcription factors appears to be the major Cdh1 regulators (Alotaibi et al., 2015; Werth et al., 2010). This may not be a surprise considering the regulation of the Cdh1 ortholog in Drosophila (Shotgun) by Grainyhead (Grh) (Almeida and Bray, 2005). Using cell lines of several origins provides the advantage of discovering new cellular models to study Cdh1 regulation. We have previously used NMuMG cells to study Cdh1 regulation during MET (Alotaibi et al., 2015). The implications of modulating Cdh1 expression go beyond MET and pluripotency; thus, exploring its regulation in different cellular models is of utmost importance. Our results indicate that CSG cells are a good candidate cell line to study different aspects of Cdh1 regulation; the previously annotated enhancer (Alotaibi et al., 2015) within the Cdh1 intron is active and can be regulated by Grhl3.

Moreover, P19 cells also show activation of both Cdh1 promoter and enhancer. In addition to these results, the activation of Cdh1 expression in Hepa1-6 cells is of great importance; The Cdh1 promoter and the intronic enhancer in these cells were found responsive to the Grhl3 induction; this was accompanied by an increase in Cdh1 mRNA levels. This observation needs to be further investigated to better understand the capacity of Grhl3 to initiate MET in other cellular models of mesenchymal origin, such as in mouse embryonic fibroblasts or cancer cells of mesenchymal background. Research in this field is growing to identify new therapeutics for the management of advanced cancer and metastasis.

CONCLUSION

In this study, we investigated the regulatory potential of Grhl3 on the activation of Cdh1. We used several cell lines of different backgrounds to assess the diversity of the regulation. The overall results obtained here are in agreement with the current literature; they also provide new insights on the ability of Grhl3 to initiate Cdh1 transcription in mesenchymal cells.

In summary, we have studied the regulatory potential of Grhl3 in different mouse cell lines. One of the major findings of this study is the capacity of Grhl3 to reactivate Cdh1 expression in Cdh1 negative...
cells. These results will serve as the basis for future studies where modulation of Cdh1 expression is required and eventually pave the way for developing novel therapeutics.

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Conflict of Interest

The article's authors declare that there is no conflict of interest between them.

Author’s Contributions

The authors declare that they have contributed equally to the article.

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