Mouse cDNA microarray analysis uncovers Slug targets in mouse embryonic fibroblasts

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Abstract

There is a need to reveal mechanisms that account for maintenance of the mesenchymal phenotype in normal development and cancer. Slug (approved gene symbol Snai2), a member of the Snail gene family of zinc-finger transcription factors, is believed to function in the maintenance of the nonepithelial phenotype. This study identified candidate Slug target genes linked to Slug gene suppression in primary mouse embryonic fibroblasts. Expression analyses were performed with a mouse cDNA microarray (Mousechip-CNIO) containing 15,000 clones. A total of 15 novel Slug target species were validated by real-time PCR or Western analyses. These included self-renewal genes (Bmi1, Nanog, Gfi1), epithelial–mesenchymal genes (Tcfe2a, Ctnb1, Sin3a, Hdac1, Hdac2, Muc1, Cldn11), survival genes (Bcl2, Bbc3), and cell cycle/damage genes (Cdkn1a, Rbl1, Mdm2). Expression patterns were studied in wild-type MEFs and Slug-deficient MEFs. Slug-complementation studies recovered aberrant gene expression in cells lacking Slug, indicating that these genes were regulated directly by Slug. These results highlight their potential roles in mediating Slug function in mesenchymal cells and may help to identify novel therapeutic biomarkers in cancers linked to Slug.

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Keywords: Slug; MEFs; Microarray analysis; Mesenchymal phenotype

 Slug is a member of the Snail family of zinc-finger transcription factors that share an evolutionarily conserved role in mesoderm formation in invertebrates and vertebrates [1–4]. In human and mouse, much of the knowledge regarding the function of Snai2 has been derived both from analysis of loss-of-function mutations [5–8] and from insights into the interaction of Snai2 with specific oncogenes in human cancer [9–11].

In the mouse Slug (approved gene symbol Snai2) is not implicated in epithelial–mesenchymal transitions (EMT) [2,3], while the Snail gene has been shown to trigger EMT, an important pathway to acquisition of the invasive phenotype in epithelial solid tumors [12,13]. Our data support this observation, with neither benign epithelial proliferative lesions nor carcinomas in Slug-expressing mice [11].

SLUG (approved gene symbol SNAI2) expression is being increasingly recognized as an alteration in human cancer [9–11,14], suggesting that SNAI2 may be a critical factor in the maintenance of the nonepithelial phenotype during tumor progression [11]. Moreover, the specific expression of Slug in migratory neural crest and mesodermal cells of the mouse embryo [2,12,15] supports its involvement in the maintenance of the mesenchymal phenotype during development. These observations emphasize the need to understand how Slug exerts its biological effects.

To identify the precise network of Slug-mediated signals involved in the maintenance of the nonepithelial phenotype, we investigated Slug target genes linked to Slug gene suppression in primary mouse embryonic fibroblasts (MEFs) by microarray analyses that can globally measure gene expression. Identification of Slug target genes that are differentially regulated in normal cells versus Slug-deficient cells could reveal specific pathways involved in Slug mesenchymal phenotype in normal development and cancer [16]. As SNAI2 is considered a marker of malignancy and an attractive target for therapeutic modu-
lation of invasiveness in human cancer [11], these genes might represent novel pharmacological targets or surrogate markers of Slug clinical response. For these reasons, this study was undertaken to uncover Slug targets linked to Slug gene suppression in MEFs.

Results

To investigate the role of Slug in mesenchymal phenotype, we used primary MEFs derived from Slug−/− embryos. Four independent mouse cDNA microarrays were used to search for Slug-regulated species in MEFs. Differential expression of the novel candidate Slug target genes was examined in these MEFs by quantitative real-time polymerase chain reaction assays or Western analyses. Complementation studies in Slug-deficient MEFs confirmed this regulation.

Identification of Slug target genes by mouse cDNA microarray analysis

To obtain a global view of the number of genes regulated by Slug, we hybridized differentially labeled RNA from control MEFs versus the Slug-deficient MEFs to a mouse cDNA microarray containing 15,000 clones. Overall the expression of the majority of the spotted genes was not altered in Slug-deficient cells. The expression of both the related transcription factor Snail and the previously implicated Slug target gene E-cadherin [21,22] was not significantly modulated.

Analysis of the expression microarray data revealed 15 candidate sequences with a more than twofold change in Slug-deficient cells (Tables 1 and 2). Modulated genes are classified in Table 3 on the basis of the biological or pathological function of the encoded protein. These genes modulated by Slug belong mainly to the following categories: self-renewal, epithelial–mesenchymal transition, survival genes, and cell cycle/DNA damage control.

<table>
<thead>
<tr>
<th>Table 1 summary of downregulated genes in MEFs lacking Slug</th>
<th>Gene symbol</th>
<th>GeneID</th>
<th>Microarray fold change (average)</th>
<th>Real-Time PCR validation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Bmi1</td>
<td>12151</td>
<td>4.4</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>2. Nanog/ENK</td>
<td>71950</td>
<td>4.1</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>3. Sin3a/Sin3</td>
<td>20466</td>
<td>3.8</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>4. Hdac1/HD1/RPD3</td>
<td>15181</td>
<td>3.8</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>5. Hdac2/D10Wsu1</td>
<td>15182</td>
<td>3.5</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>79e/YAF1/Yy1bp/mRPD3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Muc1/CD227/EMA/Muc-1</td>
<td>17829</td>
<td>3.2</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>7. Claudin-1/Cldn1</td>
<td>12737</td>
<td>3.0</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>8. Bcl2/Bcl-2</td>
<td>12043</td>
<td>2.7</td>
<td>-(western)</td>
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<tr>
<td>9. Bbc3/PUMA/JFY1</td>
<td>170770</td>
<td>2.3</td>
<td>1.8</td>
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<tr>
<td>10. Cdkn1a/CAP20/CDK1/CIPI/Cdkn1/P21/SDII</td>
<td>12575</td>
<td>2.1</td>
<td>-(western)</td>
<td></td>
</tr>
</tbody>
</table>

Genes that are downregulated more than 2.0-fold in response to the absence of Slug by mouse cDNA microarray analysis are listed. Genes were identified as unique as mentioned in the GenBank and are sorted in descending order. (*) The mean Ct value of triplicate assays is presented. (-) Validation was done by Western analysis.

Validation of the mouse cDNA microarray assay using quantitative real-time PCR and Western blot analysis

To validate the gene expression level of specific Slug target genes, quantitative real-time PCR and Western blot analysis were used. A panel of 12 genes was analyzed by quantitative real-time PCR. Quantitative real-time PCR of Bmi1, Nanog, Sin3a, Hdac1, Hdac2, Muc1, Cldn1, Bbc3, Mdm2, Ctnb1, Gfi1, and Tcfe2a confirmed the microarray changes (Tables 1 and 2). Genes highly regulated in microarray analysis such as Bmi1 and Mdm2 showed changes of comparable intensity in real-time PCR assays. Genes still modulated in microarray analysis, but at a lower extent, such as Bbc3 and Tcfe2a, also showed a comparable change in real-time PCR analysis (Tables 1 and 2).

Of the 15 transcriptionally modulated genes that were identified by microarray analysis, 3 genes were studied by Western blot analysis. Modulation of genes Bcl2, Cdkn1a, and Rb1l was confirmed by Western analyses as shown in Fig. 1.

Slug-complementation studies

Our microarray analysis revealed that 15 genes were modulated by Slug and these data were confirmed by

<table>
<thead>
<tr>
<th>Table 2 Summary of genes induced in MEFs lacking Slug</th>
<th>Gene symbol</th>
<th>GeneID</th>
<th>Microarray fold change (average)</th>
<th>Real-Time PCR validation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Mdm2/Mdm-2</td>
<td>17246</td>
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<td>3.1</td>
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<tr>
<td>2. Rbl1/p107/PRB1</td>
<td>19650</td>
<td>4.7</td>
<td>-(western)</td>
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<tr>
<td>3. Ctnb1/Ctnb1/beta-catenin</td>
<td>12387</td>
<td>4.1</td>
<td>3.2</td>
<td></td>
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<td>4. Gfi1/Pal-1/Pal1</td>
<td>14581</td>
<td>2.7</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>5. E2A/Tcfe2a/A1/ALF2/Pan1/Pan2/E12/TCF3</td>
<td>21423</td>
<td>2.2</td>
<td>2.1</td>
<td></td>
</tr>
</tbody>
</table>

Genes that are upregulated more than 2.0-fold in response to the absence of Slug by mouse cDNA microarray analysis are listed. Genes were identified as unique as mentioned in the GenBank and are sorted in descending order. (*) The mean Ct value of triplicate assays is presented. (-) Validation was done by Western analysis.

<table>
<thead>
<tr>
<th>Table 3 Classification of Slug-target genes regulated by category</th>
<th>Category</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Self-renewal</td>
<td>Bmi1</td>
</tr>
<tr>
<td></td>
<td>Nanog</td>
<td>Gfi1</td>
</tr>
<tr>
<td>Epithelial-Mesenchymal</td>
<td>Tcfe2a</td>
<td>Sin3a</td>
</tr>
<tr>
<td></td>
<td>Hdac1</td>
<td>Hdac2</td>
</tr>
<tr>
<td></td>
<td>Muc1</td>
<td>Cldn1</td>
</tr>
<tr>
<td>Survival genes</td>
<td>Bcl2</td>
<td>Bbc3</td>
</tr>
<tr>
<td>Cell cycle/damage</td>
<td>Cdkn1a</td>
<td>Rb1l</td>
</tr>
<tr>
<td></td>
<td>Mdm2</td>
<td></td>
</tr>
</tbody>
</table>

The analysed genes are classified on the basis of established biological or pathological functions of the encoded proteins.
quantitative real-time PCR and Western blot analysis, suggesting an interesting link between these genes and Slug. To confirm this transcriptional regulation we reintroduced wild-type Slug into Slug-deficient MEFs by retroviral transduction (Fig. 2) and 24 h after virus infection the expression levels of the genes Bcl2, Cdkn1a, and Rbl1 were evaluated by Western analyses. Retrovirus-mediated expression of Slug in Slug-deficient MEFs reestablished the aberrant expression of Bcl2, Cdkn1a, and Rbl1 genes as shown in Fig. 1. The demonstration that Slug was sufficient to recover their aberrant expression fully in cells lacking Slug further indicates that these genes are regulated directly by Slug.

Discussion

During the past 10 years, different studies have indicated the involvement of Slug in the mesenchymal phenotype in normal development and cancer [1–4,6–11,14–16,23–25]. These studies emphasize the need to understand how Slug exerts its biological effects. Nevertheless, the molecular mechanisms by which Slug participates in these biological processes are not yet clear.

Recently our group demonstrated that Slug is required for cancer development in mice [11]. These data strongly suggest the gene therapeutical potential of Slug in cancer. In this view, the identification of genes modulated by Slug expression could serve as a valuable tool in identifying new targets for therapy in cancer as well as to understand better the molecular mechanisms behind biological processes regulated by Slug. For these reasons, we decided to analyze the Slug-deficient MEFs.

In our study we performed a mouse cDNA microarray analysis to measure and evaluate the gene’s expression profile. We identified 15 genes that were modulated more than twofold in Slug-deficient cells. Regulated genes were summarized in four categories as shown in Table 3. We applied a cut-off ratio of 2.0 that has been commonly used in many microarray data analyses previously developed [26,27]. These genes were independently confirmed as Slug target genes by quantitative real-time PCR analysis or by Western blot analyses (Tables 1 and 2 and Fig. 1). The mouse cDNA microarray is a powerful tool for analyzing global gene expression. Yet, as limitations in this technology exist, all Slug-regulated genes in this microarray were confirmed by other assays. Thus, Slug regulated the expression of a limited set of mRNAs in MEFs.

The zinc-finger protein Slug is considered a transcriptional repressor. In agreement with this idea, five genes, Mdm2, Rbl1, Ctnb1, Gfi1, and Tcfe2a, were induced in MEFs lacking Slug (Table 2). Nevertheless, the majority of Slug-target genes were downregulated in MEFs lacking Slug (Table 1). These results could suggest that Slug can also behave as a positive transcriptional regulator. However, it is clear that microarray analysis gives an overview of the final expression profile that can be mediated by both direct and indirect gene expression regulation.

Among previously identified Slug-regulated species, the related transcription factor Snail was reported as Slug-induced in Xenopus [28]. However, Slug does not influence the expression of Snail in MDCK cells [21], as shown in this study. Similarly, the expression of the previously implicated Slug target gene E-cadherin [21,22], although it is not clear

![Fig. 1. Western blot confirmation of highlighted Slug targets in control MEFs, Slug−/− MEFs, and Slug-virus-infected Slug−/− MEFs. Western blot analysis of Rbl1, Bcl2, and Cdkn1a proteins in control MEFs, Slug−/− MEFs, and Slug-virus-infected Slug−/− MEFs is shown. Actin was included as a loading control.](image)

![Fig. 2. Retrovirus-mediated overexpression of Slug in Slug−/− MEFs. Northern blot analysis of Slug in control MEFs and Slug-virus-infected Slug−/− MEFs is shown. Actin was included as a loading control.](image)
how this transcriptional activity is implicated in vivo, was not significantly modulated in Slug-deficient MEFs. This lack of effect on E-cadherin expression can be explained by the known expression of Snail, which is not altered in these cells.

Slug expression confers resistance to programmed cell death, a function shared by Snail [29], elicited either by growth factor [6] or by DNA damage [16,22,23]. In this sense, it has been recently shown that Slug induced Bcl2 expression [30]. Bcl2 in our microarray analysis showed a reduction of expression in Slug-deficient cells with respect to the control.

Fourteen novel candidate Slug target genes were identified: Bmi1, Nanog, Gfi1, Tce2a, Cnb1, Sin3a, Hdac1, Hdac2, Muc1, Cldn1, Bbc3, Cdkn1a, Rbl1, and Mdm2. Slug induced Muc1 and claudin-1 expression in MEFs. However, Snail represses both Muc1 [31] and claudin-1 [32,33] expression during the epithelium–mesenchyme transition. Thus, Slug seems to overcome the role of Snail with respect to these targets. This expression pattern supports the view that epithelial–mesenchymal transitions in the mouse are carried out by Snail and not by Slug [2,3], and suggests that Slug would not behave not only as a repressor of the epithelial phenotype, but also as an inducer of the mesenchymal phenotype. Our previous data also support this observation, with neither epithelial alterations nor carcinomas in Slug-expressing mice [11]. Our microarray analysis also identified Cdkn1a as a gene downregulated in Slug-deficient cells. Conversely, Cdkn1a is a gene inhibited by Snail [34]. These results may reflect that Slug and Snail carry out different functions in normal development and cancer.

Recent work implicated several Slug targets, Sin3a, Hdac1, and Hdac2, as mediators of the gene regulation induced by Snail [35]. Their induction could indicate that genes modulated by Slug require the same complexes. Tce2a family members have been implicated in EMTs [21]. Their induction in Slug-deficient cells could be important to maintain the mesenchymal properties of Slug-deficient MEFs. However, the model used in this study being a classical knockout, one can always think that another protein can try to compensate for the Slug deficiency.

Among the novel Slug targets, Rbl1 is a gene implicated in regulating cell differentiation [36]. Its induction could indicate a cellular differentiation role.

Several of these Slug targets, Bmi1, Nanog, Gfi1, and Cnb1, have been implicated in regulating self-renewal [37–41]. It has been previously demonstrated that Slug is induced by Cnb1 [42]. The regulation of these genes by Slug could be important in preserving the integrity of stem cells and in tumorigenesis. Additionally, Mdm2 overexpression is frequently seen in cancer and its induction in Slug-deficient cells could indicate a predisposition to cancer development.

In summary, this study used mouse cDNA microarray analyses to identify candidate Slug target genes in MEFs. Several highlighted genes were implicated in prior work, while others have not been previously recognized. Expression of some of these candidate Slug target genes was reestablished in Slug-deficient cells. This highlighted their potential roles in mediating Slug function in mesenchymal cells. As Slug can be considered both a marker of malignancy and an attractive target for therapeutic modulation of invasiveness in the treatment of human cancer [11], this study highlights candidate Slug target genes that could represent novel pharmacological targets in cancer and may help to identify novel therapeutic biomarkers in cancers linked to Slug. Future studies will explore the functional role of Slug with regard to these candidate target genes.

Methods

Culture of MEFs

Heterozygous Slug+/- mice [5] were crossed to obtain wild-type and null Slug−/− embryos. Primary embryonic fibroblasts were harvested from 13.5-dpc embryos. Head and organs of day 13.5 embryos were dissected; fetal tissue was rinsed in PBS, minced, and rinsed twice in PBS. Fetal tissue was treated with trypsin/EDTA and incubated for 30 min at 37°C and subsequently dissociated in medium. After removal of large tissue clumps, the remaining cells were plated out in a 175-cm² flask. After 48 h, confluent cultures were frozen down. These cells were considered as being passage 1 MEFs. For continuous culturing, MEF cultures were split 1:3. MEFs and the ßNX ecotropic packaging cell line were grown at 37°C in Dulbecco’s modified Eagle’s medium (Boehringer Ingelheim) supplemented with 10% heat-inactivated FBS (Boehringer Ingelheim). All the cells were negative for mycoplasma (MycoAlert Mycoplasma Detection Kit, Cambrex).

Retroviral infection

MEFs were infected with high-titer retrovirus stocks produced by transient transfection of ßNX cells [17]. The efficiency of infection was always >80% (data not shown). The day before the infection, cells were plated at 2 × 10⁶ cells per 10-cm dish. Infected MEFs were selected for 3 days with 2.5 μg/ml Puromycin (Sigma) and replated for the corresponding assay. The mouse Slug cDNA was subcloned in the pQCXIIP retrovirus (obtained from T. Jacks, Massachusetts Institute of Technology).

RNA extraction

Total RNA was isolated in two steps using TRIzol (Life Technologies, Inc., Grand Island, NY, USA) followed by RNeasy Mini-Kit (Qiagen, Inc., Valencia, CA, USA) purification following the manufacturer’s RNA clean-up protocol with the optional on-column DNase treatment. The integrity and quality of the RNA were verified by electrophoresis and its concentration was measured.

Microarray procedures

Thirty micrograms of total RNA from each sample was labeled directly with cyanine 3-conjugated dUTP (Cy3), whereas 30 μg of RNA from the Universal Mouse Reference RNA (Stratagene) was labeled with cyanine 5-conjugated dUTP (Cy5) as reference. For all of the microarray studies the CNIO MouseChip was used [18]. Hybrizations were performed as described [18]. After being washed, the slides were scanned using a Scanarray 5000 XL (GSI Lumonics, Kanata, ON, Canada) and images were analyzed with the GenePix 4.0 program (Axon Instruments, Inc., Union City, CA, USA). All experiments were repeated four times, using cells from different embryos. All microarray experiments were done using passage 2 MEFs.

Data analysis

Data obtained from each hybridization were stored in a database for analysis. The Cy3:Cy5 ratios were normalized to the median ratio value of all of the spots in the array. After normalization, spots with intensities for both channels (sum of medians) less than that of the local background were discarded. The ratios of the remaining spots were log transformed (base 2), and...
duplicated spots on the MouseChip were averaged to the median. To obtain the expression profile of Slug\(^{−/−}\) MEFs, we referred the ratios of the Slug-deficient cells to the controls.

**Real-time PCR quantification**

Real-time quantitative PCR [19] was developed and carried out in control and Slug\(^{−/−}\) MEFs for the detection and quantitation of the following mouse genes: Bmi1, Nanog, Gli1, Tfe3a, Cnb1, Sin3a, Hdad1, Hdad2, Mac1, Cdkn1b, Bbc3, and Mdm2. The PCRs were set up in a reaction volume of 50 \(\mu\)l using the TaqMan PCR Core Reagent kit (PE Biosystems). PCR primers were synthesized by Isogen. Each reaction contained 5 \(\mu\)l of 10× buffer, 300 nM each amplification primer, 200 \(\mu\)M each dNTP, and 1.25 U AmpliTaq Gold, 2 mM MgCl\(_2\), and 10 ng cDNA. cDNA amplifications were carried out in a 96-well reaction plate format in a PE Applied Biosystems 5700 sequence detector. Thermal cycling was initiated with a first denaturation step of 10 min at 95°C. The subsequent thermal profile was 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 1 min. Multiple negative water blanks were tested and a calibration curve was determined in parallel with each analysis.

**Northern blot analysis**

Total cytoplasmic RNA (10 \(\mu\)g) of control MEFs and Slug\(^{−/−}\) MEFs transduced with pQCXIP+mSlug was glyoxylated and fractionated in 1.4% agarose gels in 10 mM Na\(_2\)HPO\(_4\) buffer (pH 7.0). After electrophoresis, the gel was blotted onto Hybond-N (Amersham), UV cross-linked, and hybridized to a \(^{32}\)P-labeled mouse Slug cDNA probe. Loading was monitored by reprobing the filter with a mouse \(\beta\)-actin cDNA probe.

**Western blot analysis**

Western blot analysis of control MEFs, Slug\(^{−/−}\) MEFs, and Slug\(^{+/−}\) MEFs transduced with pQCXIP+mSlug was carried out essentially as described [20]. Extracts were normalized for protein content by Bradford analysis (Bio-Rad Laboratories, Inc., Melville, NY, USA) and Coomassie blue gel staining. Western blot analysis of control MEFs, Slug\(^{−/−}\) MEFs, and Slug\(^{+/−}\) MEFs transduced with pQCXIP+mSlug was carried out in parallel with each analysis.

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