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An epistatic mini-circuitry between the transcription factors Snail and HNF4α controls liver stem cell and hepatocyte features exhorting opposite regulation on stemness-inhibiting microRNAs

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Preservation of the epithelial state involves the stable repression of epithelial-to-mesenchymal transition program, whereas maintenance of the stem compartment requires the inhibition of differentiation processes. A simple and direct molecular mini-circuitry between master elements of these biological processes might provide the best device to keep balanced such complex phenomena. In this work, we show that in hepatic stem cell Snail, a transcriptional repressor of the hepatocyte differentiation master gene *HNF4α*, directly represses the expression of the epithelial microRNAs *(miRs)-200c* and *-34a*, which in turn target several stem cell genes. Notably, in differentiated hepatocytes HNF4α, previously identified as a transcriptional repressor of *Snail*, induces the *miRs-34a* and *-200a, b, c* that, when silenced, causes epithelial dedifferentiation and reacquisition of stem traits. Altogether these data unveiled Snail, HNF4α and miRs-200a, b, c and -34a as epistatic elements controlling hepatic stem cell maintenance/differentiation.
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Cellular differentiation implies an orchestrated sequence of events guiding stem cells/precursors toward specialized cell types based on the contemporary and strictly correlated phenomena of loss of stemness and acquisition of histotypic markers and functions. The homeostasis of the stem cell compartment requires mechanisms actively counteracting differentiation;[1] similarly, the maintenance of the differentiated state involves a stable repression of elements capable to induce morphological transition and dedifferentiation.[2] The observation that a number of stem cells are restricted to a specific differentiation fate suggests that elements pivotal for the coordinated execution of the opposite processes could be tissue-specific. Considering that stem cell compartments are rare and give rise to a heterogeneous cellular population capable to reversibly shift among different states,[3] the availability of a stable stem cell line executing specific differentiation programs discloses an unique possibility to investigate mechanisms regulating alternative cellular choices. A simple and direct molecular mini-circuitry of master elements of mutually exclusive biological processes, also able to reciprocally influence their own expression, may provide the theoretically best device to trigger such complex phenomena.

We previously characterized a number of stable liver stem cell lines named RLSCs (from resident liver stem cells) that spontaneously acquire an epithelial morphology and differentiate into hepatocytes (named RLSCdH from RLSC-derived hepatocytes). Notably, RLSCs were also proved to recapitulate the hepatocyte post-differentiation patterning defined as 'zonation': their spontaneous differentiation, in fact, generates periportal hepatocytes that may be induced to switch into perivenular hepatocytes by means of the convergence of Wnt signaling on the HNF4α-driven transcription.[4] Furthermore, we identified a simple cross-regulatory circuitry between HNF4α (master regulator of hepatocyte differentiation) and Snail (master regulator of the epithelial-to-mesenchymal transition, EMT), whose expression is mutually exclusive because of their direct reciprocal transcriptional repression.[2,5] These findings, relevant for the comprehension of the EMT and of the reverse process mesenchymal-to-epithelial transition (MET), have been demonstrated pivotal also for the maintenance of a stable epithelial phenotype.[2] Notably, EMT/MET dynamics are proposed to be relevant in the reacquisition of stem cell features from differentiated cells. In particular, a pioneering work of Mani *et al.*[6] provided evidence that untransformed human mammary epithelial cells acquire stem cell-like characteristics through an EMT induced by ectopic expression of Twist or Snail transcription factors and that the EMT promotes the generation of cancer stem cells

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Abbreviations: BMI1, BMI1 polycomb ring finger oncogene; ChIP, chromatin immunoprecipitation; CNN1, calponin 1; E-CAD, E-cadherin; EMT, epithelial to mesenchymal transition; FSP1, fibroblast-specific protein-1; FOXA1, forkhead box protein A1; HNF4α, hepatocyte nuclear factor 4 alpha; KLF4, Krüppel-like-4; KO mouse, knock out mouse; MET, mesenchymal to epithelial transition; NES, nestin; RLSCs, resident liver stem cells; RLSCdH, resident liver stem cells-derived hepatocytes; RUNX2, runt-related transcription factor 2; SCA1, stem cell antigen 1; SOX2, Sex Determining Region Y-box 2; SIRT1, sirtuin 1; ZEB, zinc finger E-box-binding homeobox
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from more differentiated neoplastic cells. More recently, MET was shown as an essential step for the nuclear reprogramming of mouse fibroblasts in induced pluripotent stem cells via exogenous transcription factors.[7,8]

In this work, starting from the finding that Snail is expressed in RLSCs, we demonstrate its positive role in stemness markers expression. This observation, unexpected considering that the transcriptional repression is the only function so far attributed to Snail, prompted us to investigate on other factors integrating/mediating Snail activity. Mirror observations made in RLSCs and RLSCdH allowed us to conclude that (1) in RLSC Snail inhibits the hepato-specific program through direct repression of *HNF4α* gene and of the epithelial microRNAs *(miR)-200c* and *-34a*, (2) in RLSCdH HNF4α, together with a direct repression of *Snail* gene, directly upregulates miR-200 family members (*200a, b* and *c*) and *miR-34a* transcription, thus further stabilizing the hepatocytic phenotype. Altogether these data unveiled Snail, HNF4α and miRs-200a, b, c and -34a as epistatic elements controlling hepatic stem cell maintenance/differentiation.

Results

The transcriptional repressor Snail positively controls the expression of stemness markers. Our analysis evidenced as RLSCs differentiation, underscored by morphological modifications and changes in Snail/HNF4α expression (Figures 1a and b), is accompanied by a negative regulation of several stemness markers (i.e. the endoderm fork head DNA-binding protein *FOXA1*, *RUNX2*, the thin filament-associated protein calponin1 (*CNN1*), the intermediate filament protein nestin (*NES*), the stem cell antigen 1 (*SCA1*), the polycomb ring fingers member *BMI1* and the transcription factor Sex Determining Region Y-box 2 (*SOX2*)) (Figures 1b–d).

Although a role in the reacquisition of stemness features by differentiated epithelial cells has been recently attributed to Snail,[6] the influence of this factor on the stable maintenance of stem cell properties is yet unknown. We knocked down endogenous *Snail* expression in RLSC and overexpressed it in RLSCdH. As shown in Figure 2a, *Snail* silencing in RLSC

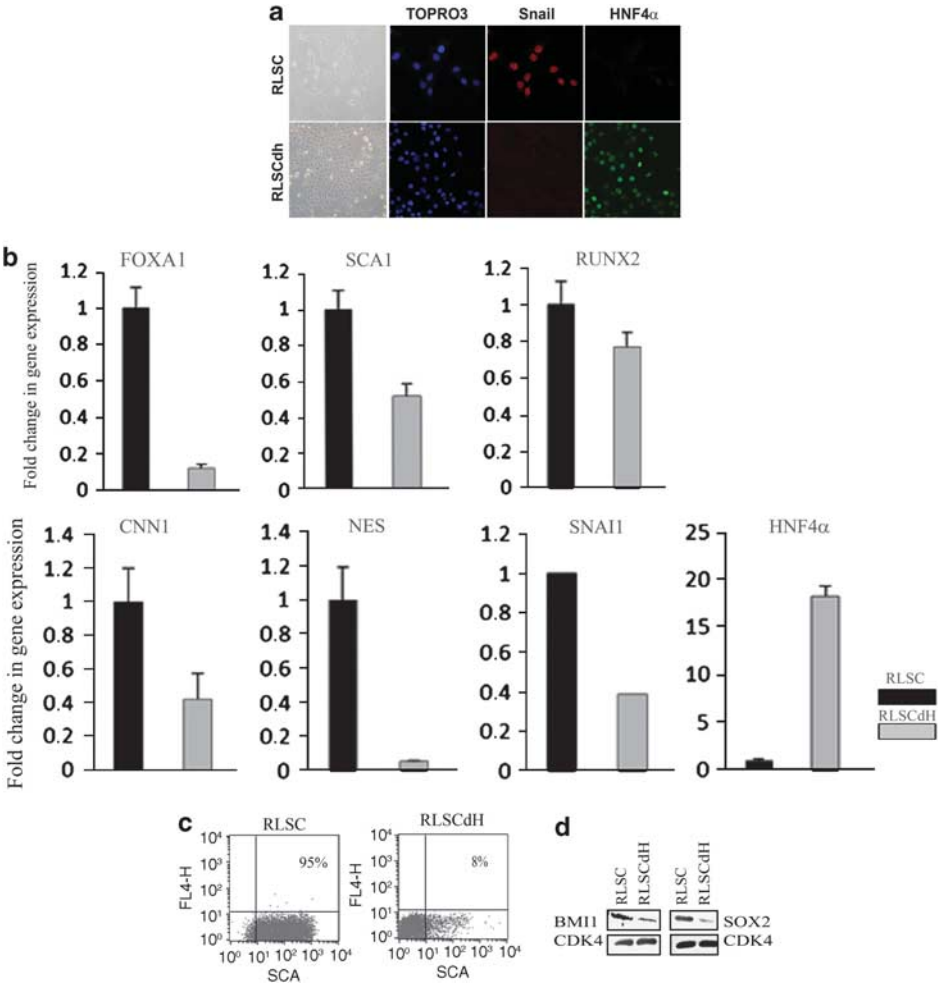


Figure 1 RLSC differentiation in RLSCdH is underlined by the negative regulation of Snail and stemness markers. (**a**) Phase-contrast micrographs magnification × 20, nuclei (TOPRO3) and immunofluorescence staining for Snail and HNF4α in RLSC and RLSCdH, examined with a Leica TCS2 confocal microscope (Leica Microsystems, Mannheim, Germany) magnification × 64. (**b**) RT-qPCR analysis on RLSC and RLSCdH cells for the indicated markers. Values are expressed as fold change in gene expression with means ± S.D. for triplicate samples ($\Delta\Delta C_t$ method). (**c**) FACS analysis for SCA1-positive cells on RLSC and RLSCdH. (**d**) Western blot analysis of BMI1, SOX2 and, as control, CDK4 in RLSC and RLSCdH cells

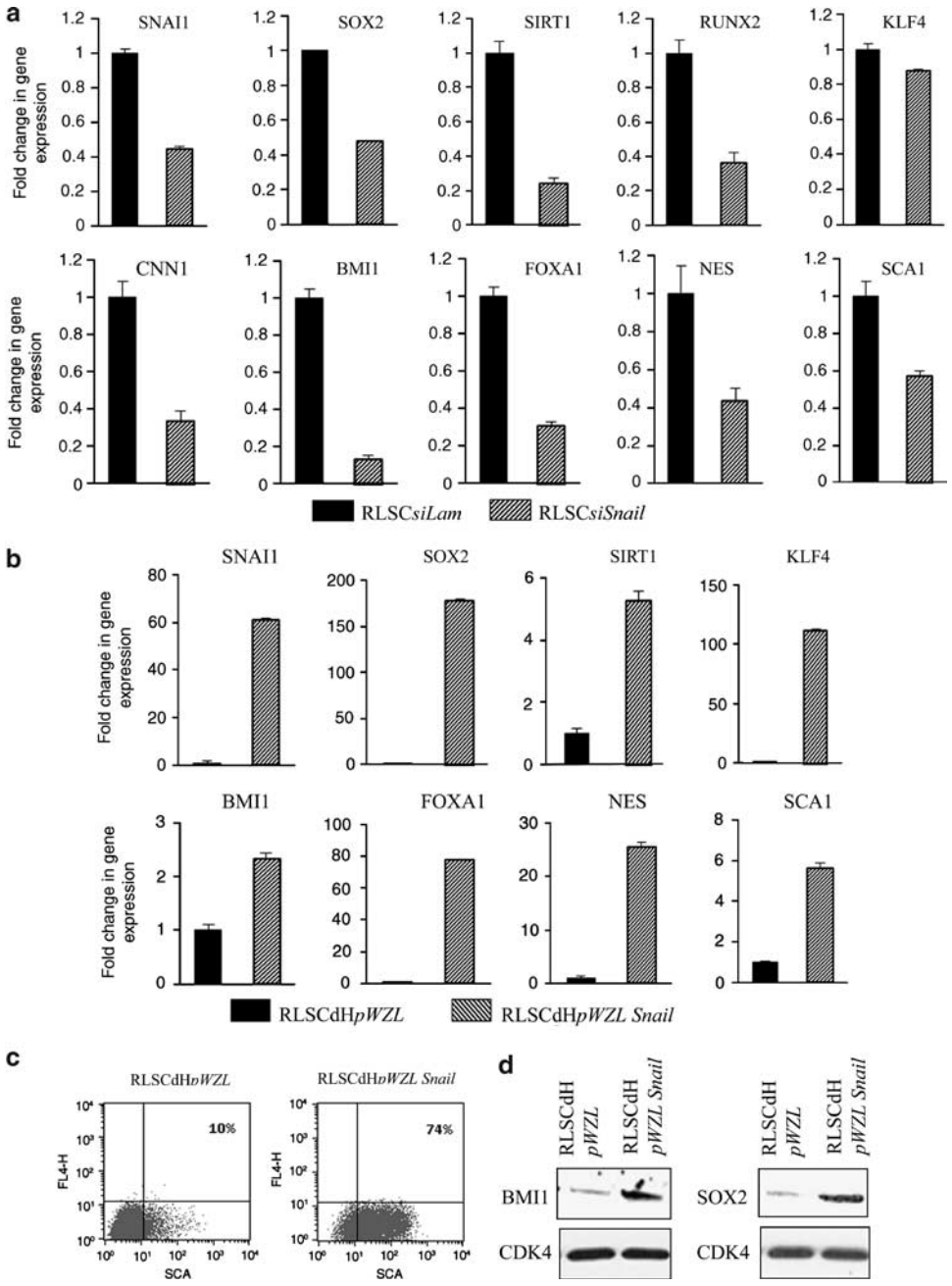


Figure 2 The transcriptional repressor Snail positively controls the expression of stemness markers. (**a**) RT-qPCR analysis on RLSCsiLam, as control, and on RLSCsiSnail for the indicated markers. Values are expressed as fold change in gene expression with means ± S.D. for triplicate samples ($\Delta\Delta C_t$ method). (**b**) RT-qPCR analysis on RLSCdHpWZL, as control, and on RLSCdHpWZL Snail cells for the indicated markers. Values are expressed as fold change in gene expression with means ± S.D. for triplicate samples ($\Delta\Delta C_t$ method). (**c**) FACS analysis for SCA1-positive cells on RLSCdHpWZL, as control, and on RLSCdHpWZL Snail cells. (**d**) Western blot analysis of BMI1, SOX2 and CDK4 on RLSCdHpWZL, as control, and on RLSCdHpWZL Snail cells

resulted in the negative regulation of stem cell factors such as SOX2, the deacetylase Sirtuin 1 (*SIRT1*), *RUNX2*, Krüppel-like-4 (*KLF4*), *CNN1*, *BMI1*, *FOXA1*, *NES* and *SCA1*. Consistently, in a mirror experiment, the stable overexpression of *Snail* in RLSCdH determined a significant positive regulation of *SOX2*, *SIRT1*, *KLF4*, *BMI1*, *FOXA1*, *NES* and *SCA1* transcription (Figure 2b), the appearance of a large SCA^+ cell population (Figure 2c) and the increase of BMI1 and SOX2 protein expression (Figure 2d).

Altogether these data indicate that Snail has a significant and yet unveiled role in the maintenance of stemness traits in RLSC, whereas in differentiated hepatocytes it causes the upregulation of a broad repertoire of stem markers.

The stemness inhibiting *miR-200c* and *miR-34a* are transcriptional targets of Snail. The described results designate for Snail, so far characterized only as a

transcriptional repressor, a positive role on the transcription of several genes belonging to the 'stemness' functional category. Bioinformatics search by MatInspector (http://www.genomatix.de) failed to find putative binding sites for Snail on the promoter regions of *SOX2*, *RUNX2*, *KLF4*, *SIRT1*, *BMI1*, *FOXA1*, *CNN1*, *NES* and *SCA1*, suggesting that the influence of Snail on the transcription of these genes is more likely an indirect one. Thus, we hypothesized that the observed Snail-induced positive regulation of stem markers expression might be mediated by stemness inhibitors miR-200 family members and miR-34a. MicroRNAs-200 are in fact known to suppress the expression of *BMI1*, *SOX2*, *KLF4*[9] and *SIRT1*,[10] whereas miR-34a to target *SIRT1*.[11] Moreover, our bioinformatics research by TargetScan (http://www.targetscan.org/) indicated the other stem cell markers we analyzed *SCA1*, *FOXA1* and *SIRT1* as putative targets of miR-200 family, and *KLF4* as a target of miR-34a.

The robustness of our hypothesis was emphasized by the strong upregulation of *miR-200a*, *b* and *c* and *miR-34a* we observed along with the differentiation of RLSC (Figure 3a). Nevertheless, as a bioinformatics search by MatInspector analysis identified Snail putative consensus sites on *miR-200c* and *miR-34a* promoters but not on the promoter shared by *miR-200a* and *miR-200b*, we first verified the involvement of the first two miRs, overexpressing them in RLSC. As shown in Figure 3b, transient transfection of hepatic stem cells with a miR-34a precursor correlated with the negative regulation of expression of the stemness markers *FOXA1*, *SCA1*, *SIRT1*, *KLF4*, *BMI1* and of the mesenchymal/stemness genes *Desmin* and *Vimentin*. Notably, the repertoire of stem cell markers we empirically found downregulated by *miRNA-34a* overexpression was broader than what predicted by Targetscan.

The overexpression of the miR-200c precursor correlated with the negative regulation of the stemness markers *SIRT1*, *KLF4*, *BMI1* and *Vimentin* expression. Co-expression of the two miRs enhances the downregulation of all the targets analyzed, with the exception of *BMI1* and *SCA1* (this last, in particular, appearing to be regulated only by miR-34a).

To validate the hypothesis that Snail positively controls the stemness markers through repression of these stemness inhibiting miRs, we therefore monitored *miR-200c* and *-34a* expression in RLSCdH overexpressing *Snail*. As shown in Figure 3c, *Snail* overexpression caused a significant downregulation of endogenous *miR-200c* and *-34a*. Notably, TGF-β treatment, that triggers EMT and Snail expression in hepatocytes,[5,12] caused a transcriptional downregulation of these miRs in RLSCdH (Figure 3c).

The causal correlation between Snail expression and inhibition of miRs transcription has been explored by chromatin immunoprecipitation (ChIP). Figure 3d shows the direct recruitment of endogenous Snail on the *miR-200c* and *-34a* genes both in RLSCs and in TGF-β-treated RLSCdH.

Overall, (1) the ectopic expression of *miR-200c* and *-34a* in RLSC recapitulated features of Snail silencing, and (2) *miR-200c* and *-34a* are direct targets of Snail repression in RLSC and in hepatocytes induced to EMT by TGF-β.

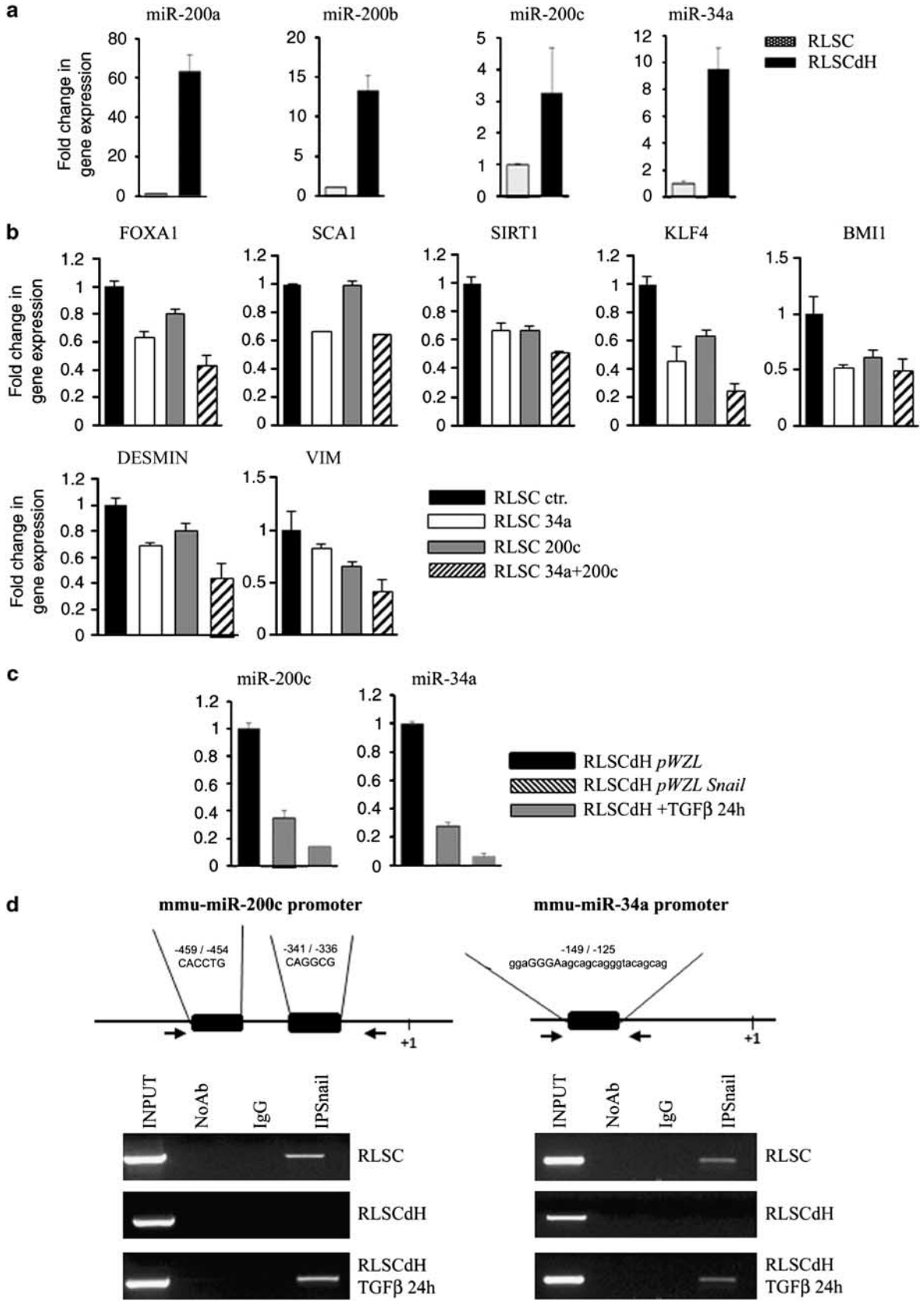
HNF4α is required for the expression of *miR-200a*, *b*, *c* and *-34a*. Given both the positive regulation of *miR-200* family members and *miR-34a* during hepatocyte differentiation and our recent findings that unveiled a circuitry of reciprocal repression between Snail and HNF4α,[2] we next asked whether these miRNAs are regulated by HNF4α. Notably, our bioinformatics analysis revealed putative consensus for HNF4α on all these miRNA promoters.

Indeed, silencing of HNF4α in RLSCdH significantly decreased *miR-200a*, *b*, *c* and *-34a* expression (Figure 4a). Moreover, it resulted in the (1) positive regulation of the miR-200 putative targets *SCA-1* and *FOXA1* (Figure 4b), (2) acquisition of SCA$^+$ cells (Figure 4c) and (3) positive regulation of BMI1 and SOX2 protein expression (Figure 4d). Notably, as shown in Figure 4e, ChIP assay demonstrated the recruitment of the endogenous transcriptional factor on all promoters of these miRNAs. These data are strongly supported by the '*in vivo*' analysis; in the liver of *hnf4α*-specific knockout (KO) mice,[13] miRs-200 and -34a were undetectable compared with controls (Figure 4f), whereas a positive modulation of *SCA-1* and *FOXA1* was observed (Figure 4g). Notably, ChIP analysis showed the recruitment of endogenous HNF4α on all promoters of these miRNAs in WT murine livers (Figure 4h).

miR-200 family maintains the hepatocytic epithelial phenotype. Finally, the effects of the inhibition of endogenous miR-34a or miRs-200 in RLSCdH have been analyzed at the morphological, transcriptional and immunophenotypical levels. Although reiterated rounds of miR-34a inhibitor transfection up to 2 weeks did not alter the hepatocyte-differentiated phenotype (data not shown), a combination of miR-200a, b and c inhibitors led RLSCdH cells to adopt a mesenchymal-like morphology with delocalization of E-CAD and acquisition of mesenchymal marker FSP1 (Figure 5a); qRT-PCR analysis revealed downregulation of epithelial (*E-CAD*, *Occludin*) and upregulation of mesenchymal (*α-SMA*, *N-CAD*) markers expression (Figure 5b). Furthermore, an increase in the number of cells expressing the stem marker SCA1 was observed (Figure 5c).

Notably, these results largely mimicked those obtained with *HNF4α* knockdown (compare Figures 4 and 5).

Figure 3 The stemness inhibiting *miR-200c* and *-34a* are targets of Snail. (**a**) RT-qPCR analysis on RLSC and RLSCdH cells for the indicated miRs. Values are expressed as fold change in gene expression with means ± S.D. for triplicate samples ($\Delta\Delta C_t$ method). (**b**) RT-qPCR analysis for the indicated markers on RLSC expressing a miR precursor as negative control, RLSC overexpressing pre-miR 34a, RLSC overexpressing pre-miR 200c and RLSC overexpressing both pre-miR 34a and pre-miR 200c. Values are expressed as fold change in gene expression with means ± S.D. for triplicate samples ($\Delta\Delta C_t$ method). (**c**) RT-qPCR analysis for the indicated miRs on RLSCdHpWZL as control, RLSCpWZLSnail and RLSCdH treated for 24 h with TGF-β. Values are expressed as fold change in gene expression with means ± S.D. for triplicate samples ($\Delta\Delta C_t$ method). (**d**) PCR analysis of an anti-Snail ChIP assays on RLSC, RLSCdH and RLSCdH treated for 24 h with TGF-β. Murine miR-200c promoter consensus sites for Snail (from −459 to −454 and from −341 to −336 with respect to the transcriptional start +1),[33] (left panel) and murine miR-34a promoter consensus site for Snail (from −149 to −125 with respect to the transcriptional start +1; right panel), are schematically depicted as black boxes; the regions amplified are depicted as arrows. As controls, ChIPs were also performed without antibody (no Ab) or with unrelated IgG (IgG). A 1:10 dilution of starting chromatin DNA was used as PCR template for input normalization


a
miR-200a
miR-200b
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Fold change in gene expression
RLSC
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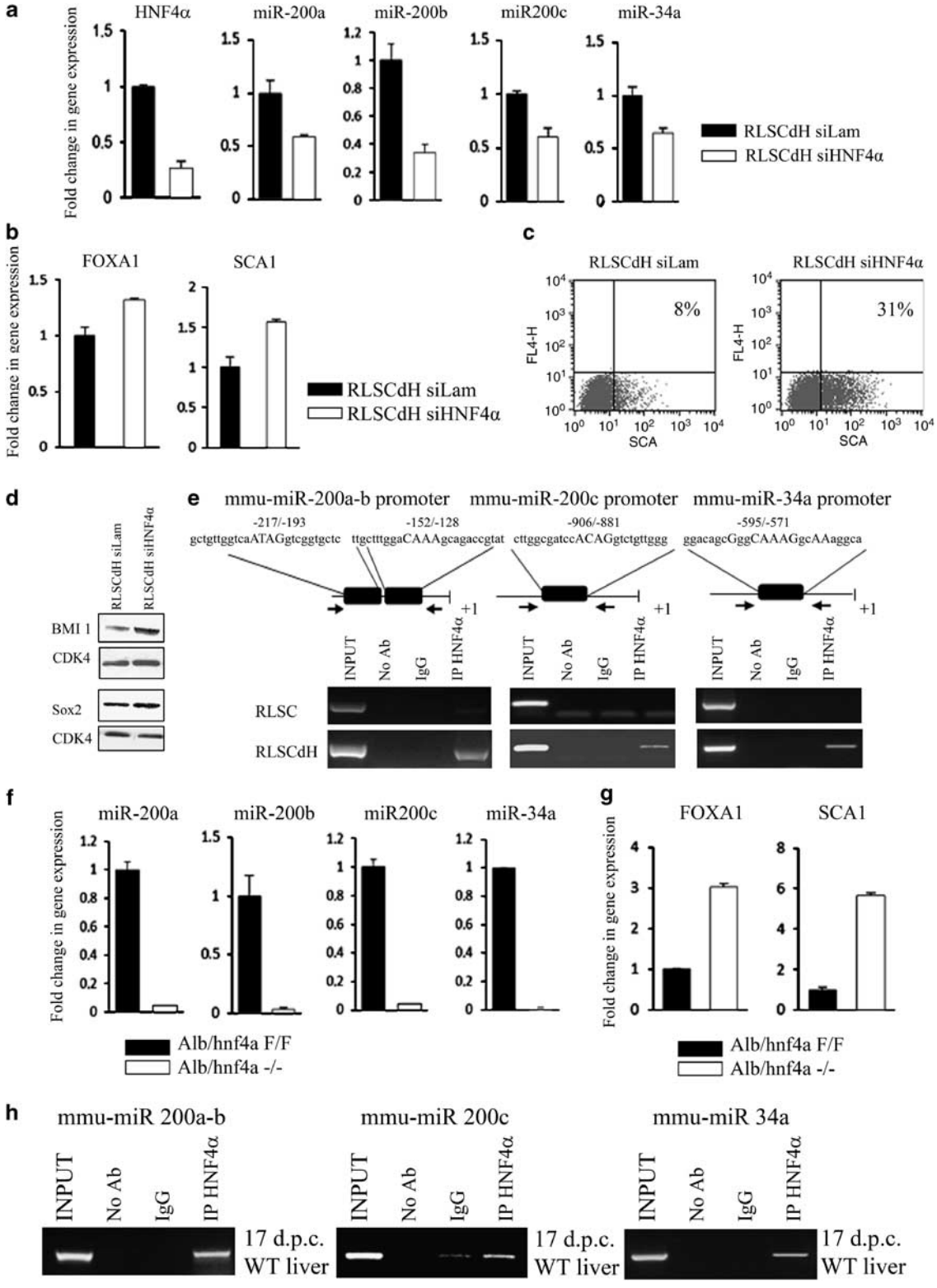
b
FOXA1
SCA1
SIRT1
KLF4
BMI1
DESMIN
VIM
Fold change in gene expression
RLSC ctr.
RLSC 34a
RLSC 200c
RLSC 34a+200c
c
miR-200c
miR-34a
RLSCdH pWZL
RLSCdH pWZL Snail
RLSCdH +TGFβ 24h
d
mmu-miR-200c promoter
mmu-miR-34a promoter
-459 / -454
CACCTG
-341 / -336
CAGGCG
-149 / -125
ggaGGGAagcagcagggtacagcag
+1
INPUT
NoAb
IgG
IPSnail
RLSC
RLSCdH
RLSCdH
TGFβ 24h

Overall, we unveiled an epistatic mini-circuitry between the transcription factors Snail and HNF4α that, exhorting opposite regulation on stemness-inhibiting miRs, controls liver stem cell and hepatocyte features (Figure 6).

Discussion

In our work we highlighted a network involving regulatory elements previously, otherwise extensively, characterized for their biological properties: (1) Snail, a transcription factor



known to repress the epithelial program in EMT;[5] (2) HNF4α, an orphan nuclear receptor key factor in hepatocyte differentiation that both activates epithelial program and represses the mesenchymal one;[14,15] (3) stemness and proliferation inhibiting miRs-200 and -34a that reinforce epitheliality.[16] The major contribution of this work has been to ascribe to these elements a role in the control of hepatic stem properties and to place them in new epistatic relationships.

The transcriptional repressor Snail, such us other members of the Snail family, has long been associated to EMT and cancer metastasis mainly for its E-cadherin-suppressive activity. Moreover, beyond this function, recent findings demonstrate that Snail functions in opposition to miRs-200 to regulate EMT and germ-layer fate restriction in differentiating ES cells.[17] The transcription factor HNF4α1 orchestrates the expression of several epithelial markers in

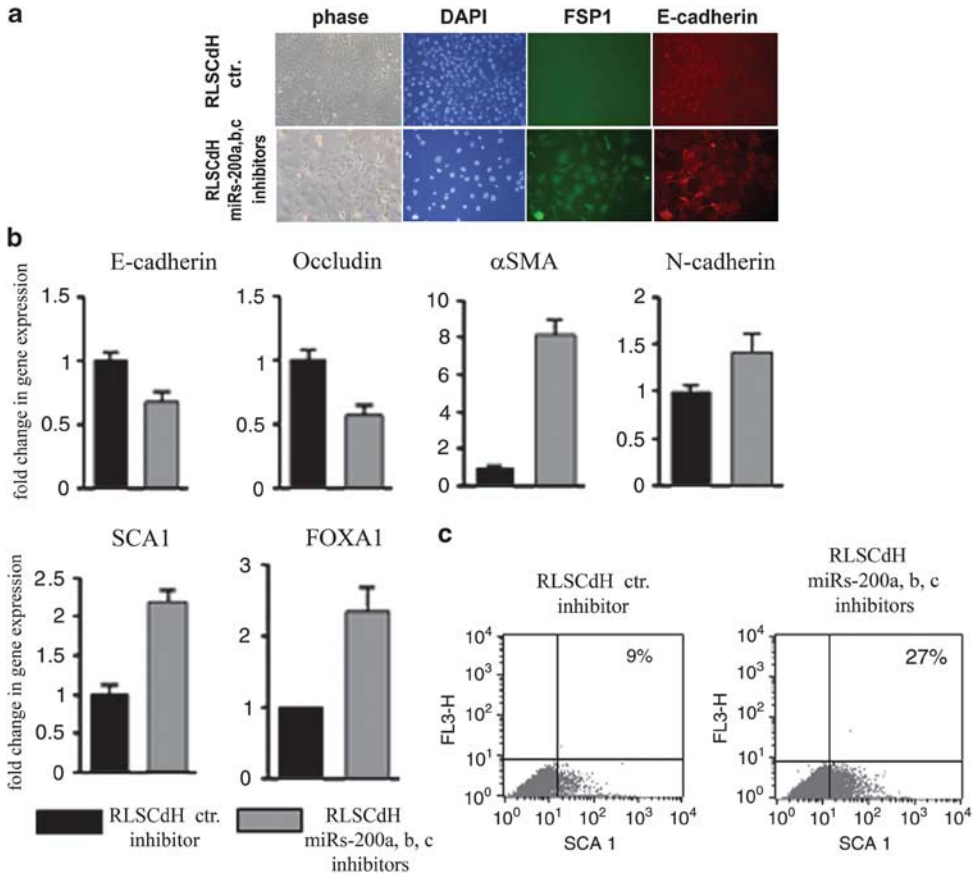


Figure 5 The miRs-200 family maintains the hepatocytic epithelial phenotype. (**a**) Phase-contrast micrographs, nuclei (DAPI) and immunofluorescence staining for FSP1 and E-Cadherin in RLSCdH transfected with the pool of inhibitors against miR-200 family members (200a, b and c) or the control negative inhibitor, examined with a Leica TCS2 confocal microscope magnification × 64. (**d**) RT-qPCR analysis on RLSCdH transfected with the pool of inhibitors against miR-200 family members (200a, b and c) or the control negative inhibitor for the indicated markers. Values are expressed as fold of change in gene expression with means ± S.D. for triplicate samples ($\Delta\Delta C_t$ method). (**e**) FACS analysis for SCA1-positive cells in RLSCdH transfected with the pool of inhibitor against miR-200 family members (200a, b and c) or the control negative inhibitor

Figure 4 HNF4α positively regulates the expression of *miRs-200* and *-34a*. (**a**) RT-qPCR analysis on RLSCdHsiLam, as control, and RLSCdHsiHNF4α cells for the indicated miRs. Values are expressed as fold change in gene expression with means ± S.D. for triplicate samples ($\Delta\Delta C_t$ method). (**b**) RT-qPCR analysis on RLSCdHsiLam, as control, and RLSCdHsiHNF4α cells for the indicated markers. Values are expressed as fold change in gene expression with means ± S.D. for triplicate samples ($\Delta\Delta C_t$ method). (**c**) FACS analysis for SCA1-positive cells in RLSCdHsiLam and RLSCdH siHNF4α cells. (**d**) Western blot analysis of BMI1, SOX2 and, as control, CDK4 in RLSCdHsiLam and RLSCdH siHNF4α cells. (**e**) PCR analysis of an anti-HNF4αChIP assays on RLSC and RLSCdH. Murine *miR-200a*, *b* promoter consensus sites for HNF4α (from −217 to −193 and from −152 to −128 with respect to the transcriptional start +1; left panel), murine *miR-200c* promoter consensus sites for HNF4α (from −906 to −881 with respect to the transcriptional start +1; middle panel) and murine *miR-34a* promoter consensus site for HNF4α (from −595 to −571 with respect to the transcriptional start +1; right panel), are schematically depicted as black boxes; the regions amplified are depicted as arrows. As controls, ChIPs were also performed without antibody (no Ab) or with unrelated IgG (IgG). A 1:20 dilution of starting chromatin DNA was used as PCR template for input normalization. (**f**) RT-qPCR analysis on liver from HNF4α KO mice (*Alb/hnf4α−/−*) and control mice (*Alb/hnf4α F/F*) for the indicated miRs. Values are expressed as fold change in gene expression with means ± S.D. for triplicate samples ($\Delta\Delta C_t$ method). (**g**) RT-qPCR analysis on liver from HNF4α KO mice (*Alb/hnf4α−/−*) and control mice (*Alb/hnf4α F/F*) for the indicated markers. Values are expressed as fold change in gene expression with means ± S.D. for triplicate samples ($\Delta\Delta C_t$ method). (**h**) PCR analysis of an anti-HNF4αChIP assays on WT 17 d.p.c. murine liver; *miR-200a-b* promoter (left panel), *miR-200c* promoter (middle panel) and murine *miR-34a* promoter (right panel) consensus sites for HNF4α are as in the above panel **e**. As controls, ChIPs were also performed without antibody (no Ab) or with unrelated IgG (IgG). A 1:10 dilution of starting chromatin DNA was used as PCR template for input normalization

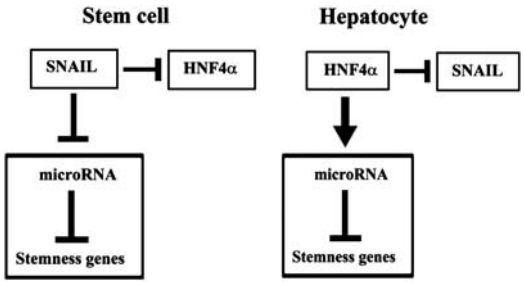


Figure 6 Schematic representation of the epistatic relationship among elements controlling stemness and differentiation of liver cells. Snail and HNF4α, regulating in opposite manner stemness-inhibiting miRs expression, control liver stem cell and hepatocyte features. Arrows and end-blocked lines indicate positive and negative regulation, respectively

hepatocytes,[18,19] re-establishes a differentiated phenotype to invasive hepatocellular carcinoma[19] and, as recently we demonstrated, controls the hepatic epithelial phenotype by direct repression of master EMT regulators including Snail.[2] MiRs-200 induce epithelial differentiation of cancer stem cells by direct targeting the transcriptional repressors of E-cadherin *ZEB1* and *ZEB2*, and other stemness genes.[9,20,21] *MiR-34a*, first identified as direct transcriptional target of p53,[22] has been found to regulate apoptosis,[23,24] expression of metabolic enzymes in the liver[16] and embryonic stem cells differentiation.[11]

We gathered a number of evidences regarding Snail: (1) hepatic stem cells constitutively express *Snail*; (2) their spontaneous differentiation into hepatocytes is underlined by negative regulation of *Snail* expression; (3) *Snail* silencing causes downregulation of stemness markers; (4) its ectopic expression in hepatocytes is sufficient both to restore expression of several stemness markers and (5) to repress *miR-200c* and *-34a*. This latter activity is probably due to a direct mechanism as suggested by the binding of endogenous Snail to *miR-200c* and *-34a* promoters in RLSC and in RLSCdH induced to EMT by TGF-β. In terms of conceptual advances, our data allow to extend the role of Snail from EMT inducer to stemness stabilizer. We addressed our observation also in the light of the previously demonstrated reciprocal repression between Snail and HNF4α. We found the requirement of HNF4α for *miR-200a*, *b* and *c*, and *-34a* expression in hepatocytes: *HNF4α* silencing in RLSCdH and its targeting in KO mouse models correlates with a strong downregulation of their expression. This is probably due to a direct mechanism as suggested by the fact that endogenous HNF4α was found recruited on *miR-200a*, *b*, *c* and *-34a* promoters in both RLSCdH and mouse liver. Notably, in HNF4α KO mouse models miRs downregulation correlates to a strong upregulation of the stemness markers SCA1 and FOXA1. Thus, HNF4α, first identified as a positive regulator of hepatocyte differentiation and recently located at the crossroad of other cellular functional categories (i.e. cell cycle, apoptosis, stress response),[25,26] appears to participate also in the active repression of stemness. In partial functional analogy with our data, HNF4α has been identified as transcriptional activator of *miR-122*[27] that in turn targets the inhibitor of terminal differentiation CUTL1.[28]

Regarding miRs, we highlighted as the inhibition of the 200a, b, c members of miR-200 family (and not of miR-34a) is sufficient to induce the mesenchymal phenotype in hepatocytes. Our data are in line with previous evidences indicating these miRs as central factors of the regulation of epithelial phenotype.[19]

Our results suggest the epistatic relationship depicted in Figure 6 in which transcriptional and post-transcriptional regulators are involved. The proposed mechanism implies that the execution of a stemness program requires the active repression of a differentiation program, whereas the maintenance of the hepatocyte one requires the active repression of stemness traits. Our observations, focusing on epithelial differentiation, are centered on HNF4α/Snail/epithelial-miRs circuitry; however, we believe conceivable that other differentiation paths may be regulated by similar mechanisms. In this light Snail can probably be considered as a general factor counteracting (and counteracted by) tissue-specific regulators. This is further suggested by observations indicating that Snail family members repress the expression of tissue-specific inducers as the pro-neural genes *sim* and *rho*[29] and the skeletal muscle master regulator *MyoD*.[30]

Materials and Methods

Cell cultures and transfections. RLSCs were grown in DMEM with 10% FBS (Cambrex BioSciences, Verviers, Belgium), 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and antibiotics. RLSCdH hepatocytes were grown in RPMI 1640 with 10% FBS (GIBCO, Grand Island, NY, USA), 50 ng/ml EGF, 30 ng/ml IGF II (PeproTech Inc., Rocky Hill, NJ, USA), 10 μg/ml insulin (Roche, Mannheim, Germany) and antibiotics, on collagen I (Upstate Biotechnology, Waltham, MA, USA)-coated dishes. When indicated, cells were treated with 2 ng/ml TGF-β (PeproTech Inc.). pWZL RLSCdH and pWZLSnail RLSCdH cells were generated by stable transfection by Lipofectamine 2000 (Invitrogen, San Diego, CA, USA), respectively, with empty or Snail carrying pWZL-bast vectors, as described previously.[5] Cells were selected in 5 μg/ml blasticidin for a week.

For RNA interference, RLSCdH cells were transfected with siRNA oligos against mouse HNF4α, or LaminA/C (5′-GGUGGUGACGAUCUGGGCUUUTT-3′) using ON-TARGET plus SMARTpool siRNA (J-065463-05/06/07/08 Dharmacon, Lafayette, CO, USA), by Lipofectamine 2000 (Invitrogen, San Diego, CA, USA). RLSC cells were transfected with siRNA oligos against mouse Snail (sense 5′-GCUCCUUCGU CCUUCUCCU-3′; antisense: 5′-AGGAGAAGGACGAAGGACG-3′; Sigma-Aldrich), or LaminA/C by Lipofectamine 2000 (Invitrogen).

RNA extraction, reverse transcription, PCR and real-time quantitative PCR (RT-qPCR). Total RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA) or NucleoSpin RNA II kit (Macherey–Nagel, Düren, Germany) and reverse transcribed with MMLV-reverse transcriptase (Promega, Milan, Italy). For miRs retrotrancription, single-stranded cDNA was obtained by reverse transcription of 200 ng of total RNA using Superscript kit reaction (Invitrogen, Carlsbad, CA, USA). RT-qPCR was performed using Bio-Rad Miniopticon with KAPA SYBR Green FAST qPCR mix (Kapa Biosystems, Woburn, MA, USA). Relative amounts were obtained with $2^{-\Delta\Delta C_t}$ method and normalized to β-actin. Primers are listed in Table 1.

Immunofluorescence and FACS analysis. For indirect immunofluorescence analysis, cells were grown on collagen I-coated dishes, fixed and treated as described previously.[31] The antibodies were used at the following dilutions: anti-Snail rabbit polyclonal (ab85931 Abcam, Cambridge, UK) 1 : 500, goat polyclonal anti-HNF4α (C-19 sc-6556, Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1 : 50, rabbit polyclonal anti-FSP1 (S100A4 Abcam) 1 : 50; mouse monoclonal anti-E-cadherin (Transduction Laboratories, BD Biosciences Pharmingen, Palo Alto, CA, USA) 1 : 50. Secondary antibodies (anti-goat Alexa-Fluor 488, anti-mouse Alexa-Fluor 594, anti-rabbit Alexa-Fluor 594 diluted 1 : 1000) were from Molecular Probes (Eugene, OR, USA). Preparations were examined with a Zeiss Axiophot microscope (Carl Zeiss Microscopy, Thornwood, NY, USA). Flow cytometry was undertaken using a FACS Calibur

Table 1 Murine oligonucleotides used for quantitative real-time RT-PCR

Gene (*Mus musculus)*	Accession number	RT-qPCR primers
Bmi1	NM_007552	*For* 5′-CACAGTTCCCTCACATTTCC-3′ *Rev* 5′-AGATGAAGTTGCTGATGACC-3′
CNN1	NM_009922	*For* 5′-AAACAAGAGCGGAGATTTGAG-3′ *Rev* 5′-CCAGTTTGGGATCATAGAGG-3′
Foxa1	NM_008259	*For* 5′-GACCCGTGCTAAATACTTCC-3′ *Rev* 5′-TATGTGGTTGGTTTGGTGTG-3′
HNF4α	NM_008261	*For* 5′-ATCTTCTTTGATCCAGATGCCA-3′ *Rev* 5′-GTTGATGTAATCCTCCAGGC-3′
Klf4	NM_010637	*For* 5′-CCACACTTGTGACTATGCAG-3′ *Rev* 5′-GTAAGGTTTCTCGCCTGTG-3′
Mmu-miR-200a	NR_029723	*For* 5′-TAACACTGTCTGGTA-3′ *Rev* 5′-GGCCGAACGGAAGGTCGCTCG-3′ RT 5′-GGCCGCCCGGAAGGTCGCTCGACATCGT-3′
Mmu-miR-200c	NR_029792	*For* 5′-TAATACTGCCGGGTA- 3′ *Rev* 5′-AACTGCGGCTTATCGAACTAT-3′ RT 5′-AATGCGGATTATCGAACTATTCCATCA-3′
Mmu-miR-34a	NR_029751	*For* 5′-TGGCAGTGTCTTAGC-3′ *Rev* 5′-AATCAATCGTCCGGTGA-3′ RT 5′-AATCAATAGTCCGGTGATCGAACAACCA-3′
Nestin	NM_016701	*For* 5′-ATCCGCGCTTACCAAGCCT-3′ *Rev* 5′-GTCTCCAGTGATTCTATGTTCTC-3′
Runx2	NM_001145920	*For* 5′-TCCTATGACCAGTCTTACCC-3′ *Rev* 5′-CTCAGTGAGGGATGAAATGC-3′
Sca1	NM_009124	*For* 5′-AGAAAGAGCTCAGGGACTG-3′ *Rev* 5′-CAATATTAGGAGGGCAGATGG-3′
Sirt1	NM_019812	*For* 5′-CCAGAACAGTTTCATAGAGCC-3′ *Rev* 5′-ACTTGGAATTAGTGCTACTGG-3′
Snail (Snail 1)	NM_011427	*For* 5′-CCACTGCAACCGTGCTTTT-3′ *Rev* 5′-CACATCCGAGTGGGTTTGG-3′
Sox2	NM_011443	*For* 5′-AGGATAAGTACACGCTTCCC-3′ *Rev* 5′-TCCTGCATCATGCTGTAGC-3′
U6	NM_026309	*For* 5′-GCTTCGGCAGCACATATACT-3′ *Rev* 5′-GAATTTGCGTGTTCATCCTTG-3′ RT 5′-GAATTTGCGTGTTCATCCTTG-3′
β-actin	NM_007393	*For* 5′-ACCACACCTTCTACAATGAG-3′ *Rev* 5′-AGGTCTCAAACATGATCTGG-3′

(Becton Dickinson, Franklin Lakes, NJ, USA) using a monoclonal FITC-conjugated anti-Sca1 purchased from BD Pharmingen (Erembodegem, Belgium).

Bioinformatics analysis. Regulatory sequences (up to 1 kb upstream of transcription start site) of murine miR-200a-b (ENSMUSG00000065400), miR-200c (ENSMUST00000083528) and miR-34a (ENSMUST00000083559), were obtained from ENSEMBL (http://www.ensembl.org) and submitted to MatInspector Professional (release 8.0, Genomatix, Munchen, Germany), using the vertebrate matrix library and optimized thresholds, to identify putative Snail and HNF4α consensus binding sites (see Figures 3 and 4). Bioinformatics search of putative miR-200 family and miR-34a targets has been performed by TargetScan (http://www.targetscan.org/).

Chromatin immunoprecipitation assay. ChIP analysis were performed according to the Upstate protocol with slight modifications, as described previously.[2] For *in-vivo* ChIP analysis, livers from seven mouse embryos were obtained at 17 d.p.c., cut with a razor blade and mechanically disaggregated with 15 strokes of a glass Potter–Elvehjem homogenizer in ice-cold PBS/protease inhibitors before fixation in 1% formaldehyde. Cross-linked cells were dounced to release nuclei. Immunoprecipitations were performed with anti-HNF4α (sc 8987), anti-SNAI1 H130, (sc-28199) and the negative control rabbit IgG (sc-2027) from Santa Cruz Biotechnology. A 1 : 10 dilution of starting chromatin DNA was used as template for input amplification. Primers are reported: miR-200c consensus Hnf4α For 5′-CTG CCATCTCAGGGTAAAACCAAAGA-3′, Rev 5′-CAGACAGGGCCGGGCAACA A-3′; miR-200c consensus Snail For 5′-CCTCAAGAGGAGGTGAATCC-3′, Rev 5′-AGGACCCCCCTGATCGGTGG-3′; miR-34a consensus Hnf4α For 5′-GAG GGAAGGTCGCTGTTGTT-3′, Rev 5′-AGTTACAGGGACTCTGACAC-3′; miR-34a consensus Snail For 5′-TGTCCCTCCAGCTGAATCC-3′, Rev 5′-CCGCAG TCACAGGAAGATGG-3′; miR-200a-b consensus Hnf4α For 5′-GGGATACC TTACCTTCCTGC-3′, Rev 5′-CCAGCAGAGATGTTGACCCT-3′.

Western blots. Cells were lysed in RIPA buffer containing protease inhibitors (Roche, Monza, Italy). Protein concentrations were determined by use of the Bio-Rad assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Blots were blocked in 5% non-fat milk prepared in TBST and incubated overnight with the primary antibody. The antibodies were used in the following dilutions: anti-BMI (Millipore) 1 : 1000; anti-SOX2 (R&D Systems, Minneapolis, MN, USA) 1 : 2500. Blots were incubated with HRP-conjugated species-specific secondary antibodies (Bio-Rad), followed by enhanced chemiluminescence reaction (Pierce Chemical, Rockford, IL, USA).

Transfection of miR precursors and inhibitors treatments. For miR overexpression, RLSC cells were transiently transfected with synthetic oligonucleotides for mmu-miR-200c precursor (PM12741 Ambion/Applied Biosystems, Austin, TX, USA) or mmu-miR-34a precursor (PM11030 Ambion/Applied Biosystems) at a final concentration of 60 pmol or with a cocktail of 60 nM each of both precursors using Lipofectamine 2000 (Invitrogen, San Diego, CA, USA). A miRNA precursor negative control (Ambion/Applied Biosystems) was used at the same concentrations. RNAs were collected for assay 2 days post transfection and miR-200c and -34a expressions were confirmed by qPCR.

For miR interference, RLSCdH cells were transfected by Lipofectamine 2000 (Invitrogen, San Diego, CA, USA) with a mixture of 200 nM miR-200c inhibitor (AM12741, Ambion/Applied Biosystems, Austin USA), 200 mM miR-200a inhibitor and 200 mM miR-200b inhibitor[32] or 600 nM of an Anti-miR miRNA Inhibitor Negative Control (AM17010Ambion/Applied Biosystems). After 3 days of transfection, cells were split and re-transfected with the same miR inhibitors. This process has been repeated every 3–4 days for up to a total of 19 days before analysis.

Conflict of Interest

The authors declare no conflict of interest.

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