FEBS Letters 589 (2015) 1249–1256

FEBS Letters

journal homepage: www.FEBSLetters.org

Snail-mediated Cripto-1 repression regulates the cell cycle and epithelial–mesenchymal transition-related gene expression

Vijaya S. Pilli, K. Gupta, Bhanu P. Kotha, Gopala Krishna Aradhyam *

Department of Biotechnology, Bhupat & Jyoti Mehta School of Bioscience, Indian Institute of Technology Madras, Chennai 600036, India

ARTICLE INFO

Article history:
Received 28 November 2014
Revised 23 March 2015
Accepted 5 April 2015
Available online 15 April 2015

Edited by Zhijie Chang

Keywords:
Snail
Cripto-1
Epithelial–mesenchymal transition
Transcription

ABSTRACT

Transcription factor Snail mediates epithelial to mesenchymal transitions (EMT) by coordinate repression of epithelial markers, facilitating mass cell movement during germ layer formation. Aberrant reprogramming in its signaling pathways causes metastatic cancer. Snail's involvement in "fate-changing" decisions is however not understood. Cripto-1 shares a common temporal expression pattern with Snail during development. While Cripto-1 is required for mammary morphogenesis and hematopoietic stem cell renewal, its unregulated expression causes metastatic cancers. Therefore, we suspected that Snail regulates the expression of Cripto-1 controlling decisions such as motility, transformation and differentiation. We demonstrate that Snail represses Cripto-1 gene by direct transcriptional interaction and propose a novel mechanism by which it co-ordinately regulates cell fate decisions during development and could be causal of cancers.

1. Introduction

Cripto-1/teratoma derived growth factor-1 (TDGF-1) is a membrane anchored/secretory protein that functions as a co-receptor for the TGF-beta ligands, Activin and Nodal [1]. Although it is expressed at various stages of embryonic development including gastrulation, its molecular role in cellular physiology remains unclear (e.g., in facilitating the specification of the anterior–posterior axis) [2–4]. In adults, its tissue restricted expression occurs to support hematopoietic stem cell renewal in the bone marrow and for mammary gland morphogenesis [5–7]. In addition to the early identification of Cripto-1 as a cofactor in the TGF-beta pathway, it is now reported to be involved in the Wnt/Beta-catenin, Notch and Akt pathways [8–11].

Involvement of Cripto-1 in myriad pathways controlling growth and differentiation has led to its identification as an oncogene and a biomarker of several cancers including metastatic breast cancer and neuroblastomas [10–12]. Some of the mechanisms by which Cripto-1 directly increases cell proliferation are (i) enhanced ligand binding activities for growth differentiating factors, GDF1 and GDF3 in the TGF-beta pathway, (ii) activation of MAPK/c-src/PI3k/protein kinase B, and (iii) activation of the EGF-receptor [10]. Concomitantly, inhibition of Cripto-1 activity either by tumor suppressors like caveolin-1 or through antibody-mediated-depletion, reduces cell proliferation [13,14].

Snail is a zinc finger, helix-loop-helix containing transcription factor involved in specification of the mesodermal cell lineage by orchestrating favorable molecular programs like repression of epithelial markers, changes in the cell cycle, increased survival, among others [15–18]. Absence of Snail leads to the loss of left–right axis asymmetry in mice, either independently or as a result of the pro-mesenchymal properties of Snail activation [19]. Cancers, in which Snail is atypically expressed are often associated with increased metastasis, in part due to increased motility conferred by Snail-mediated repression of E-cadherin [4,20]. Further, Snail is also known to arrest cell cycle while inducing motility and enhancing cell survival [17,21].

Based on the involvement of Snail and Cripto-1 in embryonic development, promoting motility of cancer cells and seemingly antagonistic roles of these proteins in cell-cycle progression and differentiation, we hypothesize that Snail (being a transcription factor) might affect/regulate Cripto-1 expression. Our in vitro and in vivo data on binding analysis demonstrates that Snail directly binds to the E-Box element on the 'Cripto-1 promoter' and represses its expression, also correlating with changes to the EMT and the cell-cycle. Together, our data elucidate an important molecular interaction, physiologically designed for cell fate specification but aberrantly utilized by cancer for successful metastasis and growth.

Author contributions: Pilli VS, Gupta K and Aradhyam GK designed the experiments; Pilli VS, Gupta K and Kotha BP performed the experiments; Pilli VS, Gupta K and Aradhyam GK wrote the manuscript.

* Corresponding author.
E-mail address: agk@iitm.ac.in (G.K. Aradhyam).

http://dx.doi.org/10.1016/j.febslet.2015.04.005
0014-5793/

2. Materials and methods

2.1. Culture of mammalian cell lines

HEK293, IMR-32 and MCF 7 were maintained in DMEM with high glucose (Gibco-USA), supplemented with 1X antibiotic (Gibco-USA), 10% FBS at 37 °C and 5% CO_2.

2.2. Plasmid constructs

pGL4.20 plasmid and pGL4.20 plasmid containing Cripto-1 promoter were kind gift from Dr. Salomon (National Cancer Institute, USA) [22]. pCDNA3-Flag Snail was a kind gift from Dr. Weiss (University of Michigan, USA) [23], pEF Cripto-1 was a kind gift from Dr. Bianco C (National Cancer Institute, USA) [24]. Snail shRNA (Sc-38398) and control shRNA (Sc-108060) plasmids were purchased from Santa Cruz Biotechnology, β-gal plasmid was a kind gift from Dr. Mahapatra (IITM, India) [25].

2.3. Promoter activity analysis by reporter gene (luciferase) assays

HEK293 cells were seeded at 40–50% confluency in 12 well plate on day 1 and 24 h later cells were transfected with pGL4.20, pGL4.20-Cripto-1 promoter (200 ng per well) and varying concentrations of pCDNA3-Flag Snail by calcium phosphate method. 24 h post transfection, cells were washed with ice cold PBS and lysed in 200 μl of lysis buffer consisting of 0.1 M phosphate buffer (pH 7.4), 0.1% Triton X-100 and 1 mM DTT. 50 μl lysate was used for luciferase assay with 1 mM luciferin sodium salt (Sigma Aldrich), 3 mM ATP (Sigma Aldrich), 15 mM $MgSO_4$ and 30 mM HEPES (pH 7.8). Luciferase readings were normalized against transfection control by β-galactosidase assay.

2.4. Analysis of protein expression

Transfected cells were lysed in 'RIPA' buffer, supplemented with 1X protease and phosphatase inhibitor. Total protein concentration was estimated by BCA method (PI-23221, Thermo scientific, USA). 50 μg of protein was loaded on SDS PAGE, transferred onto nitrocellulose membrane and blocking was carried out for 20 min at room temperature with 5% skimmed milk in TBST. Membrane was probed with primary antibody at 4 °C over night, washed three times with TBST and probed with secondary antibody at room temperature for 1 h and blots were developed with luminol (Sigma # 8511) using Versadoc. Primary antibodies used in this study are β-Actin (Santa Cruz Biotechnologies # sc-47778), Snail (Santa Cruz Biotechnologies # sc-10432), E-Cadherin (BD Bioscience # 610181), Vimentin (Santa Cruz Biotechnologies # sc-373717), p21 (AB biotech # 251259), Cyclin D1 (BD Biosciences # 556470), V5 antibody (Invitrogen # R-960-25). Anti mouse and anti rabbit secondary antibodies are purchased from Santa Cruze Biotechnologies (# sc-358914and # sc-2357).

2.5. Gene-expression analysis by qPCR

MCF7 and IMR-32 cells were seeded at a confluency of 30–40% on day 1 and cells were transfected on day 2 with Snail. Snail shRNAs treated cells were lysed in Trizol, 24 h after transfection (#10296010, Life technologies, USA) and total RNA was isolated. 1 μg of total RNA was converted into cDNA using 'transcriptor high fidelity cDNA synthesis kit' (05091284001 Roche, Germany). Relative mRNA levels were analyzed by qPCR with Syber-green master mix (DyNAmo color Flash SYBR Green qPCR Kit #F-416L, Thermo scientific, USA) using Human Cripto-1 (F: 5′-CACGATGTGCGCAAAGAGAA-3′; R: 5′-TGACCGTGCCAGCATTTACA-3′), Snail (F: 5′ ACCACTATGCCGCGCTCTT 3′; R: 5′ GGTCGTAGGGC TGCTGGAA 3′) and Actin (F: 5′ TCATGAAGATCCTCACCGAG 3′; R: 5′ TTGCCAATGGTGATGACCTG 3′) specific primers.

2.6. Electrophoretic mobility shift assay (EMSA)

In order to identify the in vitro interaction of Cripto-1 promoter with Snail, a 33 bp oligo nucleotide corresponding to Cripto-1 promoter region (Wild type F: 5′-GGTGGGCGTCCCGCCCACCTGA-AA GGTCTCCCC-3′, R: 5′-GGGGAGACCTTTCAGGTGGGCGGGACGC CCACC-3′ and Snail binding site mutant oligos: 5′-GGTGGG CGTCCCGCCCCCGCGA AAGGTCTCCCC-3′, R: 5′-GGGGAGACCT TTCGCGGGGGCGGGACGCCCACC-3′), with the Snail binding site in the center. We followed the protocol of incubating constant amount of oligos (a final concentration of 50 pM) with varying concentrations of nuclear protein extract (NPE) (2 and 4 μg).

Binding reactions were performed in a total volume of 20 μl consisting of 50 pM oligonucleotides, 20 mM HEPES (pH 7.4), 80 mM NaCl, 20 mM KCl, 0.2 μl of 100X protease inhibitor cocktail and 1-4 μg of total nuclear protein extract (NPE). The mixture was incubated for 60 min at room temperature and complexes were resolved by non-denaturing PAGE in 0.5X TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA). For super shift experiments, additional 1-2 μg of Snail antibody (# ab85931, Abcam, UK) was added and incubated for 4 h at 4 °C before the addition of oligonucleotides. Gels were soaked in TBE supplemented with 10 μM ethidium bromide and incubated for 20–30 min prior to visualization.

2.7. Chromatin immuno-precipitation assay (ChIP)

Chromatin of 100% confluent IMR-32 cells was cross linked with proteins in 1% formaldehyde. Nuclear content was extracted and chromatin was fragmented to a size of ~400 bp by sonication. Chromatin was, then immunoprecipitated by Snail antibody (# ab85931 Abcam, UK) and an IgG control (Sigma). Immunoprecipitated chromatin was reverse cross linked by heating at 60 °C overnight with 200 mM NaCl. Denatured protein was precipitated by phenol chloroform (25:1) and DNA fragments were eluted by PCR purification kit (Biobasic, Canada). Presence of Cripto-1 promoter fragments was analyzed with Cripto-1 gene promoter specific primers F: 5′-*CCGAAAAGAGTACCTCTGATC*-3′, R: 5′-CTTTCGGCCTTGGACGTTTTGACC-3′. Further, amplification-specificity was confirmed by nucleotide sequencing of the DNA.

3. Results

3.1. Identification of putative Snail-binding elements on Cripto-1 promoter

Cripto-1 is an oncofetal protein vital for embryonic development. In humans the gene is located at the chromosomal locus 3p21.31 [26]. It is known to stabilize the transcription factor Snail, which in turn promotes cell migration by down regulating genes involved in cell proliferation and ceasing cell cycle. Though the Cripto-1 stabilized Snail acts antagonistic to Cripto-1 in regulating cell proliferation, the impact of Snail on Cripto-1 expression is not yet elucidated. In order to understand this regulatory phenomenon, Cripto-1 promoter sequence was analyzed for the presence of Snail-binding elements (SBE: 5′-CANNTG-3′) by transcription factor binding analysis tool Consite (http://consite.genereg.net). This prediction revealed presence of the four putative Snail-binding sites (SBE1 from −294 to −288; SBE2 from −234 to −228; SBE3 from −1429 to −1423 and SBE4 from −1945

to −1939) in the Cripto-1 promoter (From −2450 to +8 bp of transcription start site) (Fig. 1A).

3.2. Snail represses Cripto-1 promoter activity

Luciferase assays were performed to understand the impact of Snail on the expression of Cripto-1, as described earlier [27]. Briefly, Cripto-1 promoter (−2489 to +8 bp) was cloned into pGL4.20 vector that bears a luciferase gene (Fig. 1A). Upon expression of the putative transcription-factor, the luciferase expression is read-out and is representative of the promoter-strength. Co-transfection of Snail with Cripto-1 promoter drastically reduces the luciferase activity in a dose-dependent manner, indicating that Snail negatively regulates Cripto-1 promoter (Fig. 1B). The estimation of efficiency of transfection is normalized by a control transfection of β-gal and normalized by a colorimetric method (Fig. 1B). The dose dependent increase in the Snail transfection is additionally confirmed by western blot (Fig. 1C).

3.3. Snail causes repression of Cripto-1 expression

Snail-mediated repression of Cripto-1 promoter was further confirmed by estimating the changes in relative mRNA levels of Cripto-1 by transfecting Snail in increasing amounts, in two cancer cell lines. MCF-7 is a Snail deficient cell line [28], whereas IMR-32 expresses Snail endogenously (Fig. 2A). Though the endogenous Cripto-1 mRNA levels in MCF-7 and IMR-32 do not vary much (Data not shown), the relative levels of Cripto-1 mRNA are decreased in both MCF-7 and IMR-32 with increase in the amount of recombinant Snail transfected (Fig. 2C). Snail mediated repression is also confirmed by shRNA-mediated depletion of Snail. Snail shRNA causes specific reduction of Snail protein in IMR-32 and recovery of Cripto-1 mRNA levels to baseline, whereas the shRNA against Snail elicited no significant effect on MCF-7 cells, as expected (Fig. 2B and D).

3.4. In vitro analysis of direct interaction of Snail and Cripto-1 promoter

To monitor the physical interaction between Snail and Cripto-1 promoter, we selected the third putative Snail-biding site (with maximum cut off value) on Cripto-1 promoter. Incubation of 33 base pair oligos representing this site with nuclear protein extracts (NPE) of IMR-32 cells lead to a shift in migration and (the level of migration as estimated by the intensity of the band) is dependent on the amount of NPE used (Fig. 3B, lanes 2 and 3). Incubation of control or mutant oligos with the NPE failed to elicit any difference in migration (Fig. 3B, lanes 1, 5 and 6). Similarly, incubation of NPE

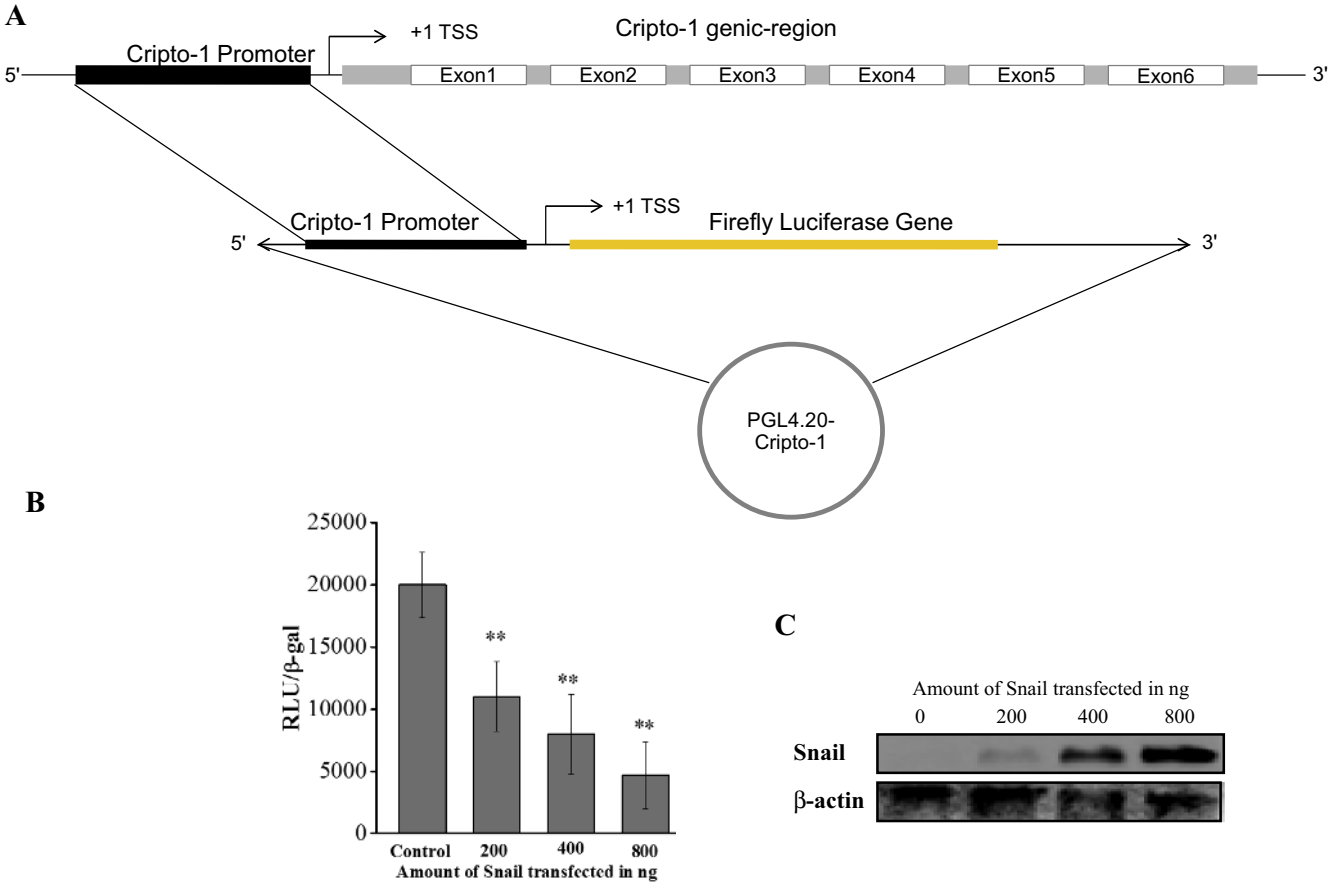

Fig. 1. Cripto-1 promoter activity in cells over expressing Snail. (A) Schematic overview representing the intron–exon organization of Cripto-1. The Cripto-1 gene is organized into 6 exons along the short arm of chromosome 3. The cloning of the Cripto-1 promoter region upstream of the luciferase gene is schematically displayed. The clone was used to ascertain the strength of the promoter and its regulation by Snail. (B) Construct expressing Snail was transfected at indicated increasing concentrations in cells along with reporter constructs harboring the Cripto-1 promoter. Empty reporter vector is used as vector control, pCDNA3 is used as control for Snail transfection and β-Galactosidase construct is utilized to ensure equal transfection efficiency. The relative luciferase activity is plotted as a function of increasing Snail expression. Experiments are carried out in triplicates and repeated at least 3 times. ** Indicates *P*-value < 0.05 compared to control cells. (C) Over-expressing Snail levels are measured by Western blot analysis from the same batch of transfected cells, to confirm increasing Snail levels upon increasing amounts of transfected Snail gene containing vector. Equal loading is confirmed by β-actin. The blot is representative of 3 experiments ($n = 3$).

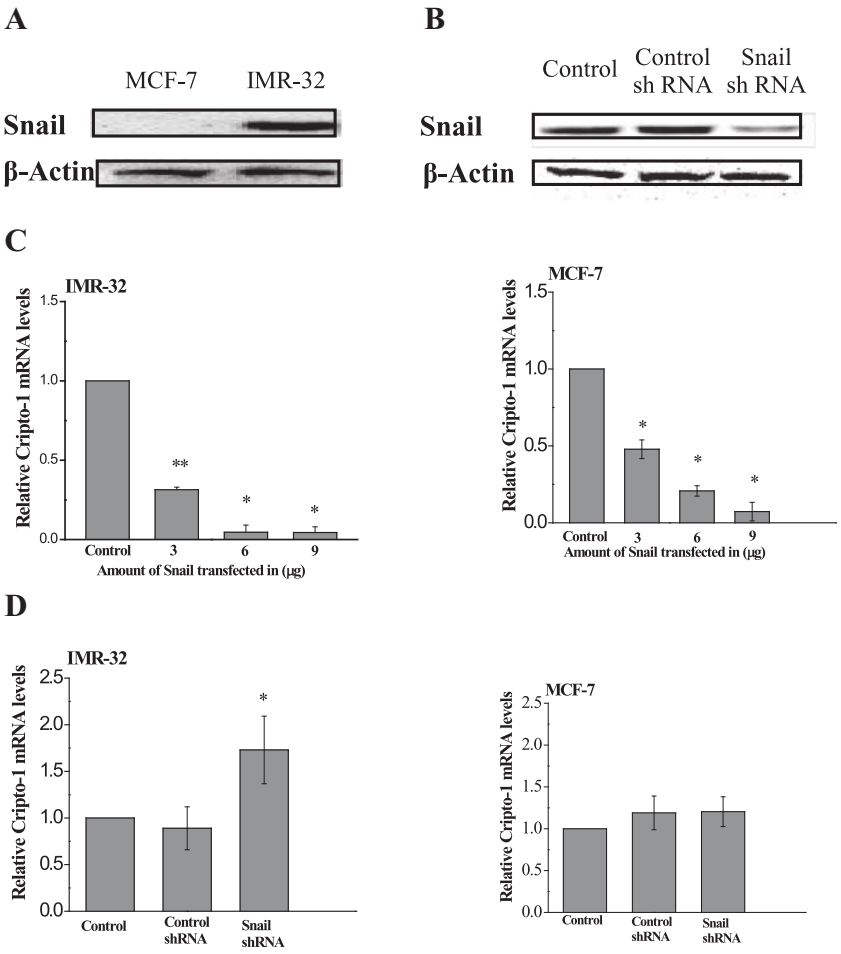

Fig. 2. Alteration of Cripto-1 expression in response to Snail. (A) Analysis of endogenous Snail expression in the human breast cancer cell line MCF-7 and the human neuroblastoma cell line-IMR-32. (B) MCF-7 and IMR-32 cells were transfected with increasing amounts of Snail expressing construct and RT-qPCR was performed to measure the levels of Cripto-1 mRNA. (C) shRNA construct or a control vector was transfected in MCF-7 and IMR-32 cells to silence Snail and the change in the levels of Cripto-1 mRNA levels was assayed by RT-qPCR. Experiments were carried out in triplicates and repeated at least 3 times. ** Indicates *P*-value < 0.05 compared to control cells. (D) Snail levels are measured by Western blotting from the same batch of transfected cells to confirm Snail-expression as well as knock-down in Snail transfected, control shRNA construct and shRNA-Snail constructs. Equal loading is confirmed by β-actin. The blot is representative of 3 experiments ($n = 3$).

with Snail antibody followed by interaction of the oligos reduced the intensity of migrated oligos band but not by control IgG, indicating that this migration is specific to the Snail protein interaction with its binding site on the oligos (Fig. 3A and C). Further, we confirmed the specificity of interaction between Snail and Cripto-1 promoter using MCF-7 NPE as a negative control (Fig. 3D). To further validate that the interaction of Snail with the Cripto-1 promoter is specific to IMR-32 cells, we perform EMSA with the NPE of MCF-7 and IMR-32 cells. We observe that there is no interaction of the oligonucleotides with the MCF-7 NPE at various NPE amounts whereas in IMR-32 cells there was a clear shift, demonstrating specificity in interaction.

3.5. In vivo analysis of direct Snail and Cripto-1 promoter interaction

In vivo interaction of Snail with Cripto-1 promoter is demonstrated by the chromatin immunoprecipitation (ChIP) assay. Briefly, DNA bound proteins in cells were cross-linked and followed up by antibody mediated precipitation, using a transcription factor specific antibody or a control IgG antibody. The relative abundance of the transcription factor-bound promoter fragment is then assayed in the immuno-precipitate using PCR, where primers are designed to span the predicted transcription factor binding site. If the transcription factor is indeed bound to the DNA, it will be amplified in the PCR reaction. In our study, we found that the predicted SBEs on the Cripto-1 promoter were amplified only when Snail-specific antibody is used for pull down, demonstrating the specificity of Snail binding to Cripto-1 promoter. Equal loading is confirmed using total input DNA (Fig. 4).

3.6. Snail mediated repression of Cripto-1 alters EMT and cell-cycle related gene expression

Based on the observation that Snail causes direct repression of Cripto-1 activity, we ascertained whether this repression also influences the EMT markers as well as the changes to the cell cycle by expressing Snail both exogenously in IMR-32 cells (Fig. 5A) and ectopically in MCF-7 cells (Fig. 5B). We observe that in both cases cells begin to undergo EMT transition upon expression of Snail due to a reduction in the levels of E-cadherin and increased levels of Vimentin. Interestingly, Snail strongly represses the levels of Cyclin D1 levels in agreement with its role in suppressing the cell-cycle but promoting EMT, while its expression correlates with the induction of p21 in agreement with the negative effect of Snail on the cell cycle (Fig. 5A and B). The exogenous expression of Cripto-1 collaborates with Snail and strongly represses E-cadherin. Interestingly Vimentin appearing late during EMT is affected in snail over-expressing cells only.

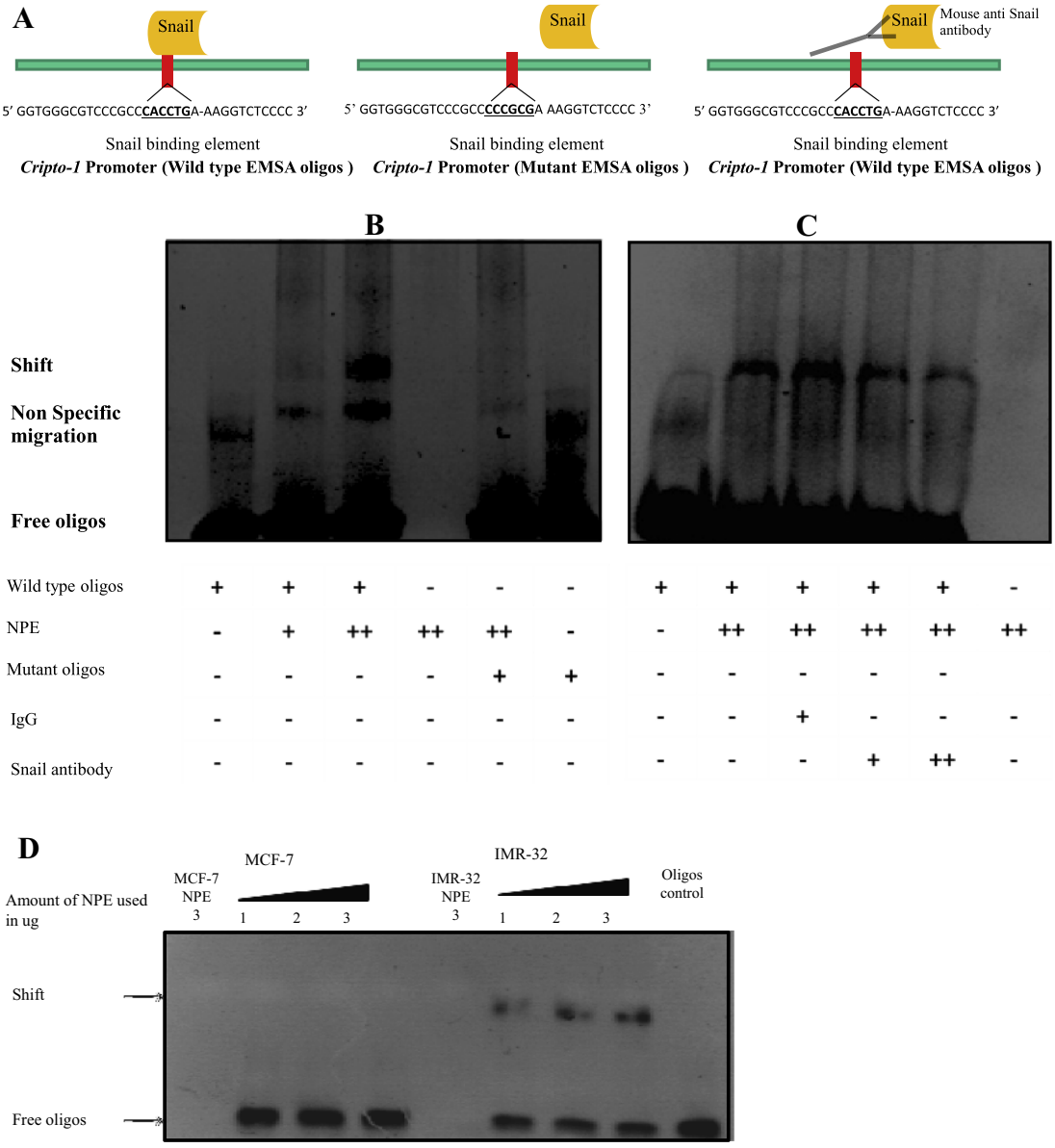

Fig. 3. Interaction of Cripto-1 promoter region with endogenous Snail in vitro in IMR-32 cells nuclear lysates. Total nuclear protein extract (NPE) was isolated from IMR-32 cells that express Snail endogenously. (A) Cripto-1 promoter region spanning the Snail binding element was synthesized, along with ±15 bp as a duplex and annealed (for measuring shift). (B) *Lane 1* represents Oligos control. *Lane 2* represents Oligos with 2 μg NPE. *Lane 3* represents oligos with 4 μg NPE. *Lane 4* represents NPE control. *Lane 5* represent mutant oligos incubated with 4 μg NPE. *Lane 6* represents free mutant oligos. (C) In order to confirm the specificity of Snail mediated shift, NPE was incubated with Snail antibody (Abcam) and a control IgG before performing EMSA. *Lane 1* represents Oligos control. *Lane 2* represents Oligos with 4 μg NPE. *Lane 3* represents oligos with 4 μg NPE and 2 μg IgG antibody. *Lane 4* represents oligos with 4 μg NPE and 1 μg Snail antibody. *Lane 5* represents oligos with 4 μg NPE and 2 μg Snail antibody. (D) Total NPE was isolated from IMR-32 cells and MCF-7 cells. Lane 1 represents NPE of MCF7 Lane 2, 3 and 4 represent increasing concentration of MCF-7 NPE. Lane 5 represents NPE of IMR-32. Lane 6, 7 and 8 represent increasing concentration of IMR-32 NPE. Lane 9 represents oligo control.

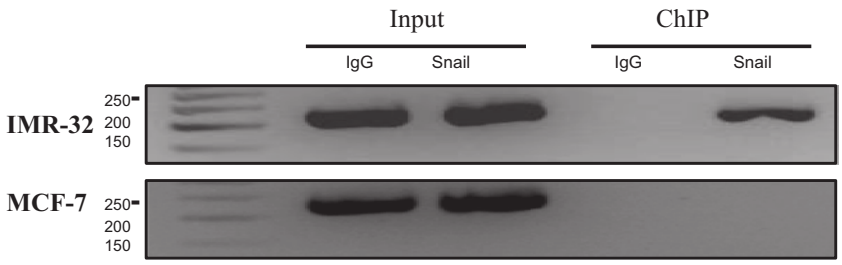

Fig. 4. Interaction of Snail with the Cripto-1 promoter in vivo. Chromatin immunoprecipitation (ChIP) was performed in IMR-32 cells for the putative Snail-binding sites. The cells expressing endogenous Snail were cross linked using formaldehyde followed by shearing and immunoprecipitation using a Snail specific or IgG control antibody. The resulting chromatin was reverse-cross linked and amplified using the primers flanking the two putative Snail-binding sites. Equal loading was confirmed by the amplification of input chromatin. The resulting blot is representative of 3 experiments ($n = 3$).

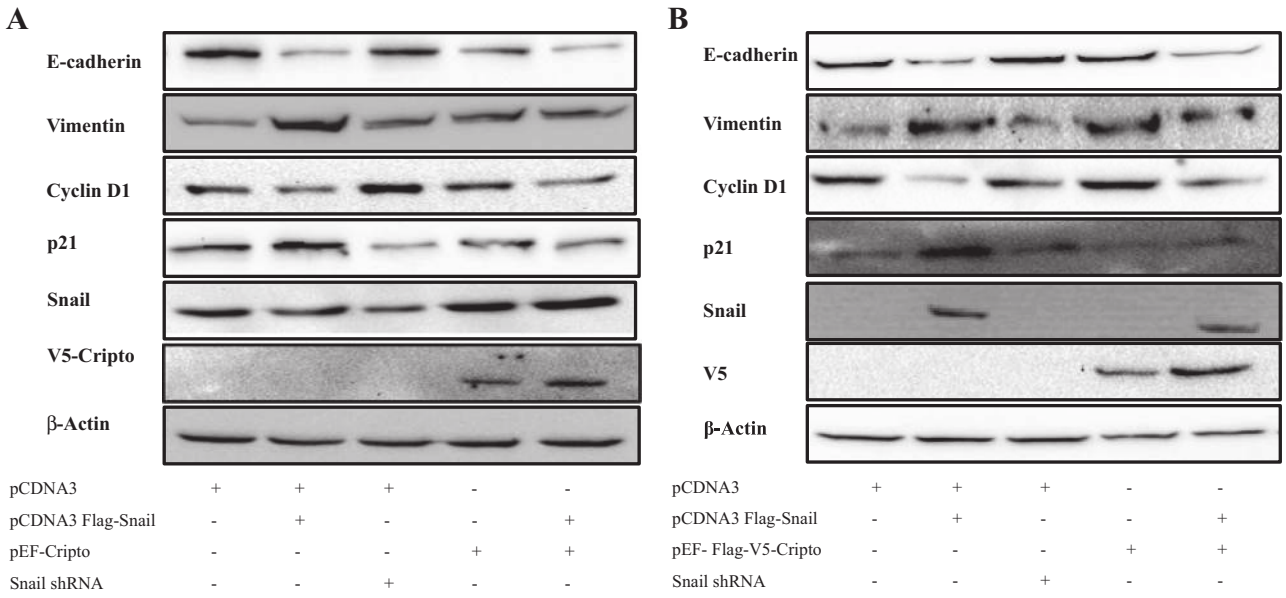

Fig. 5. Snail and Cripto-1 exogenous expression causes alteration in EMT and cell-cycle markers. Levels of EMT markers E-cadherin and Vimentin and cell cycle markers Cyclin D1 and p21 are assayed by western blotting upon exogenous expression of the constructs, as indicated, for (A) IMR-32 and (B) MCF-7 cells. Equal lading is confirmed by β-Actin. The blot is representative of 3 experiments ($n = 3$).

4. Discussions

Snail is an important, if not the only converging point for coordinating the temporal regulation of genes that help cells egress from the epithelial lineage [15]. In the current work, we identify Cripto-1 promoter as the specific transcriptional target of Snail and report a repressive mode of regulation that might facilitate the entry of cells into mesenchymal lineage. A bioinformatics based screen for putative transcription factor binding sites on the Cripto-1 promoter led to the identification of Snail as a probable transcription factor. We followed this by reporter assays that demonstrate reduced Cripto-1 promoter activity upon increasing Snail transfection. Further, we validate the reduced promoter activity by quantifying the mRNA levels and observe a similar trend of reduced Cripto-1 mRNA levels with increasing Snail. Subsequent to our finding that Snail represses Cripto-1 promoter activity and expression, we ascertained whether the observed reduction in Cripto-1 transcript (and promoter activity with increasing Snail levels) is a result of direct transcriptional interaction. We performed promoter binding studies in vivo and also in vitro. We observed that reconstituted interaction of Snail containing NPE of IMR-32 cells and oligonucleotides of Cripto-1 promoter sequence (containing SBE) leads to a specific interaction as demonstrated by our EMSA data. In addition, ChIP analysis demonstrated that the promoter binding by Snail also happens in cells (in vivo). Taken together, the standard assays for transcription factor with its cognate site on the DNA interaction demonstrate a direct interaction of Snail with Cripto-1 promoter and functional assays suggest a repressive mode of regulation. We demonstrate that the repression of Cripto-1 by Snail causes changes in the early markers of EMT by suppressing epithelial markers and promoting mesenchymal markers, as well as alterations in cell-cycle progression through suppression of cyclin D1 and increased p21 expression. Importantly, the interaction is specific to IMR-32 cells that express endogenous Snail but is recapitulated by ectopic expression of Snail in MCF-7 cells.

Indirect evidences have previously demonstrated that interactions between Snail and Cripto-1 are required for the positive regulation of EMT [11]. Therefore, our observation of Snail-mediated Cripto-1 repression is physiologically insightful. Limited studies on the interaction of Snail and Cripto-1 in past reports have demonstrated that Cripto-1 facilitates the stabilization of Snail at the protein level, which is in agreement to their collaborative roles in promoting EMT [29].

Our novel observation of a repressive mode of regulation might relate to the numerous instances where Cripto-1 and Snail play antagonistic roles and therefore, Snail-mediated repression of Cripto-1 assumes special importance. For instance, it is now well established that Cripto-1 provides growth-advantage to cells by participating in key pathways involved in growth and self-renewal [11]. Further, Strizzi et al. have reported that Cripto-1 is associated with increased cell-cycling activities through Cyclin D1 activation in mammary tumors [29]. In contrast, Snail is involved in arresting the cell-cycle in various contexts, including breast cancers [17,21]. In addition, Cripto-1 provides cells with increased self-renewal potential and maintenance of stemness, whereas Snail is actively involved in the differentiation of cells [5,30–32]. Taken together, Snail and Cripto-1 are also involved in antagonistic processes of growth and differentiation. Our data provides a molecular basis for these observations – Snail-mediated repression of Cripto-1 expression.

Based on previous reports, we suspect that Cripto-1 repression by Snail is context specific and highlight that transcriptional activators directly upregulate Cripto-1 during EMT transitions [11]. In this regard, we observed that the mRNA levels of Cripto-1 did not vary significantly in IMR-32 and MCF-7 cells despite varying Snail expression. It has been reported that Cripto-1 is quite dynamically regulated in IMR-32 cells, as neural transcription factors like N-myc promotes expression of TCF3 which inturn promotes Cripto-1 expression. This might restore Cripto-1 expression even in the presence of Snail [11,33]. Also signaling pathways like anaplastic lymphoma kinase (ALK)/β-catenin/Cripto-1 specific to IMR-32 cells might upregulate Cripto-1 in MCF-7 [11,34,35]. In addition complex regulatory network of transcription factors may cause differential Cripto-1 expression. For example, Msx2, a homeobox domain containing transcription

factor, was previously reported to promote EMT by upregulating Cripto-1 expression [36]. Also, a short form of Cripto-1 (lacking the first two exons) is found to be upregulated by Wnt pathway owing to the binding of the beta-Catenin/TCF/LEF complex to the cis-elements on the Cripto-1 promoter [37]. Also, Cripto-1 inturn induce Snail expression. It has recently been found that a Snail-related transcription factor, Twist, is associated with a significant fraction of genes that Snail binds [38]. In contrast to the repressive role of Snail, the binding of Twist promotes the transcription of the target gene [39]. Therefore, Cripto-1 could be an interesting target gene that is co-regulated by Snail and Twist, along with other transcription factors promoting EMT. A definitive knowledge of the upstream regulators of Cripto-1 that promote EMT is now beginning to emerge.

Past studies have also demonstrated the involvement of other transcription factors in activating Cripto-1 expression that are related to EMT in less direct manner [11]. Some of these include those proteins that are more directly associated with the overlapping functions of controlling self-renewal as well as motility. For example, the hypoxia-inducible factor 1a (Hif1α) was reported to activate Cripto-1 expression and is also known to promote EMT [22]. In addition, Cripto-1 promoter is bound by Nanog and is also predicted to bind to Oct3/4, thereby correlating with the self-renewal and pluripotency-potential of Cripto-1 [11].

Therefore, while a number of positive-regulators of Cripto-1 are already described in over-lapping contexts of promoting EMT and potential in providing stemness, the simultaneous retention of the EMT function and oncogenic role in maintenance of undifferentiated state was unknown. Our finding of a novel, negative regulator of Cripto-1 expression, therefore, provides an understanding of how Cripto-1 expression may also be negatively modulated for successful development and oncogenesis.

Acknowledgements

Financial support for this work from IIT Madras, Department of Biotechnology, Department of Science and Technology, Council of Scientific and Industrial Research and Indian Council for Medical Research (all Govt. of India organizations) is acknowledged. SSVP and KG thank IIT Madras for fellowship and BVSSPKK thanks IIT Madras for funding under the 'innovative idea' scheme.

References

[1] Yan, Y.T., Liu, J.J., Luo, Y., Chaosu, E., Haltiwanger, R.S., Abate-Shen, C. and Shen, M.M. (2002) Dual roles of Cripto as a ligand and coreceptor in the nodal signaling pathway. Mol. Cell. Biol. 22, 4439–4449.
[2] D'Andrea, D., Liguori, G.L., Le Good, J.A., Lonardo, E., Andersson, O., Constam, D.B., Persico, M.G. and Minchiotti, G. (2008) Cripto promotes A-P axis specification independently of its stimulatory effect on Nodal autoinduction. J. Cell Biol. 180, 597–605.
[3] Ding, J., Yang, L., Yan, Y.T., Chen, A., Desai, N., Wynshaw-Boris, A. and Shen, M.M. (1998) Cripto is required for correct orientation of the anterior–posterior axis in the mouse embryo. Nature 395, 702–707.
[4] Jin, J.Z. and Ding, J. (2013) Cripto is required for mesoderm and endoderm cell allocation during mouse gastrulation. Dev. Biol. 381, 170–178.
[5] Miharada, K., Karlsson, G., Rehn, M., Rorby, E., Siva, K., Cammenga, J. and Karlsson, S. (2011) Cripto regulates hematopoietic stem cells as a hypoxic-niche-related factor through cell surface receptor GRP78. Cell Stem Cell 9, 330–344.
[6] Salomon, D.S., Bianco, C. and De Santis, M. (1999) Cripto: a novel epidermal growth factor (EGF)-related peptide in mammary gland development and neoplasia. Bioessays 21, 61–70.
[7] Wechselberger, C., Strizzi, L., Kenney, N., Hirota, M., Sun, Y., Ebert, A., Orozco, O., Bianco, C., Khan, N.I., Wallace-Jones, B., Normanno, N., Adkins, H., Sanicola, M. and Salomon, D.S. (2005) Human Cripto-1 overexpression in the mouse mammary gland results in the development of hyperplasia and adenocarcinoma. Oncogene 24, 4094–4105.
[8] Barrallo-Gimeno, A. and Nieto, M.A. (2005) The Snail genes as inducers of cell movement and survival: implications in development and cancer. Development 132, 3151–3161.
[9] Bianco, C., Rangel, M.C., Castro, N.P., Nagaoka, T., Rollman, K., Gonzales, M. and Salomon, D.S. (2010) Role of Cripto-1 in stem cell maintenance and malignant progression. Am. J. Pathol. 177, 532–540.
[10] Nagaoka, T., Karasawa, H., Castro, N.P., Rangel, M.C., Salomon, D.S. and Bianco, C. (2012) An evolving web of signaling networks regulated by Cripto-1. Growth Factors 30, 13–21.
[11] Rangel, M.C., Karasawa, H., Castro, N.P., Nagaoka, T., Salomon, D.S. and Bianco, C. (2012) Role of Cripto-1 during epithelial-to-mesenchymal transition in development and cancer. Am. J. Pathol. 180, 2188–2200.
[12] Bianco, C., Strizzi, L., Normanno, N., Khan, N. and Salomon, D.S. (2005) Cripto-1: an oncofetal gene with many faces. Curr. Top. Dev. Biol. 67, 85–133.
[13] Adkins, H.B., Bianco, C., Schiffer, S.G., Rayhorn, P., Zafari, M., Cheung, A.E., Orozco, O., Olson, D., De Luca, A., Chen, L.L., Miatkowski, K., Benjamin, C., Normanno, N., Williams, K.P., Jarpe, M., LePage, D., Salomon, D. and Sanicola, M. (2003) Antibody blockade of the Cripto CFC domain suppresses tumor cell growth in vivo. J. Clin. Investig. 112, 575–587.
[14] Bianco, C., Strizzi, L., Mancino, M., Watanabe, K., Gonzales, M., Hamada, S., Raafat, A., Sahlah, L., Chang, C., Sotgia, F., Normanno, N., Lisanti, M. and Salomon, D.S. (2008) Regulation of Cripto-1 signaling and biological activity by caveolin-1 in mammary epithelial cells. Am. J. Pathol. 172, 345–357.
[15] Muqbil, I., Wu, J., Aboukameel, A., Mohammad, R.M. and Azmi, A.S. (2014) Snail nuclear transport: the gateways regulating epithelial-to-mesenchymal transition? Semin. Cancer Biol. 27, 39–45.
[16] Scherbakov, A.M., Stefanova, L.B., Sorokin, D.V., Semina, S.E., Berstein, L.M. and Krasil'nikov, M.A. (2013) Snail/beta-catenin signaling protects breast cancer cells from hypoxia attack. Exp. Cell Res. 319, 3150–3159.
[17] Vega, S., Morales, A.V., Ocana, O.H., Valdes, F., Fabregat, I. and Nieto, M.A. (2004) Snail blocks the cell cycle and confers resistance to cell death. Genes Dev. 18, 1131–1143.
[18] Yasui, K., Shimamura, M., Mitsutake, N. and Nagayama, Y. (2013) SNAIL induces epithelial-to-mesenchymal transition and cancer stem cell-like properties in aldehyde dehydroghenase-negative thyroid cancer cells. Thyroid 23, 989–996.
[19] Murray, S.A. and Gridley, T. (2006) Snail1 gene function during early embryo patterning in mice. Cell Cycle 5, 2566–2570.
[20] Chen, D., Zheng, X., Jiao, X., Gao, Y., Zhang, K. and Liang, J. (2012) Transcriptional repressor snail and metastasis in hepatocellular carcinoma. Hepatogastroenterology 59, 1359–1365.
[21] Hu, C.T., Wu, J.R., Chang, T.Y., Cheng, C.C. and Wu, W.S. (2008) The transcriptional factor Snail simultaneously triggers cell cycle arrest and migration of human hepatoma HepG2. J. Biomed. Sci. 15, 343–355.
[22] Bianco, C., Cotten, C., Lonardo, E., Strizzi, L., Baraty, C., Mancino, M., Gonzales, M., Watanabe, K., Nagaoka, T., Berry, C., Arai, A.E., Minchiotti, G. and Salomon, D.S. (2009) Cripto-1 is required for hypoxia to induce cardiac differentiation of mouse embryonic stem cells. Am. J. Pathol. 175, 2146–2158.
[23] Yook, J.I., Li, X.Y., Ota, I., Fearon, E.R. and Weiss, S.J. (2005) Wnt-dependent regulation of the E-cadherin repressor snail. J. Biol. Chem. 280, 11740–11748.
[24] Sun, Y., Strizzi, L., Raafat, A., Hirota, M., Bianco, C., Feigenbaum, L., Kenney, N., Wechselberger, C., Callahan, R. and Salomon, D.S. (2005) Overexpression of human Cripto-1 in transgenic mice delays mammary gland development and differentiation and induces mammary tumorigenesis. Am. J. Pathol. 167, 585–597.
[25] Sasi, B.K., Sonawane, P.J., Gupta, V., Sahu, B.S. and Mahapatra, N.R. (2014) Coordinated transcriptional regulation of Hspa1a gene by multiple transcription factors: crucial roles for HSF-1, NF-Y, NF-kappaB, and CREB. J. Mol. Biol. 426, 116–135.
[26] Dono, R., Montuori, N., Rocchi, M., De Ponti-Zilli, L., Ciccodicola, A. and Persico, M.G. (1991) Isolation and characterization of the CRIPTO autosomal gene and its X-linked related sequence. Am. J. Hum. Genet. 49, 555–565.
[27] S.T. Smale, Luciferase assay, Cold Spring Harbor protocols, 2010 (2010) pdb prot5421.
[28] Dhasarathy, A., Phadke, D., Mav, D., Shah, R.R. and Wade, P.A. (2011) The transcription factors Snail and Slug activate the transforming growth factor-beta signaling pathway in breast cancer. PLoS ONE 6, e26514.
[29] Strizzi, L., Bianco, C., Normanno, N., Seno, M., Wechselberger, C., Wallace-Jones, B., Khan, N.I., Hirota, M., Sun, Y., Sanicola, M. and Salomon, D.S. (2004) Epithelial mesenchymal transition is a characteristic of hyperplasias and tumors in mammary gland from MMTV-Cripto-1 transgenic mice. J. Cell. Physiol. 201, 266–276.
[30] Batlle, R., Alba-Castellon, L., Loubat-Casanovas, J., Armenteros, E., Franci, C., Stanisavljevic, J., Banderas, R., Martin-Caballero, J., Bonilla, F., Baulida, J., Casal, J.I., Gridley, T. and Garcia de Herreros, A. (2013) Snail1 controls TGF-beta responsiveness and differentiation of mesenchymal stem cells. Oncogene 32, 3381–3389.
[31] Cicchini, C., Filippini, D., Coen, S., Marchetti, A., Cavallari, C., Laudadio, I., Spagnoli, F.M., Alonzi, T. and Tripodi, M. (2006) Snail controls differentiation of hepatocytes by repressing HNF4alpha expression. J. Cell. Physiol. 209, 230–238.
[32] Spike, B.T., Kelber, J.A., Booker, E., Kalathur, M., Rodewald, R., Lipianskaya, J., La, J., He, M., Wright, T., Klemke, R., Wahl, G.M. and Gray, P.C. (2014) CRIPTO/GRP78 signaling maintains fetal and adult mammary stem cells ex vivo. Stem Cell Rep. 2, 427–439.
[33] Zheng, X., Naiditch, J., Czurylo, M., Jie, C., Lautz, T., Clark, S., Jafari, N., Qiu, Y., Chu, F. and Madonna, M.B. (2013) Differential effect of long-term drug selection with doxorubicin and vorinostat on neuroblastoma cells with cancer stem cell characteristics. Cell Death Dis. 4, e740.

[34] Fragoso, M.A., Patel, A.K., Nakamura, R.E., Yi, H., Surapaneni, K. and Hackam, A.S. (2012) The Wnt/beta-catenin pathway cross-talks with STAT3 signaling to regulate survival of retinal pigment epithelium cells. PLoS ONE 7, e46892.
[35] Passoni, L., Scardino, A., Bertazzoli, C., Gallo, B., Coluccia, A.M., Lemonnier, F.A., Kosmatopoulos, K. and Gambacorti-Passerini, C. (2002) ALK as a novel lymphoma-associated tumor antigen: identification of 2 HLA-A2.1-restricted CD8+ T-cell epitopes. Blood 99, 2100–2106.
[36] di Bari, M.G., Ginsburg, E., Plant, J., Strizzi, L., Salomon, D.S. and Vonderhaar, B.K. (2009) Msx2 induces epithelial–mesenchymal transition in mouse mammary epithelial cells through upregulation of Cripto-1. J. Cell. Physiol. 219, 659–666.
[37] Hamada, S., Watanabe, K., Hirota, M., Bianco, C., Strizzi, L., Mancino, M., Gonzales, M. and Salomon, D.S. (2007) Beta-Catenin/TCF/LEF regulate expression of the short form human Cripto-1. Biochem. Biophys. Res. Commun. 355, 240–244.
[38] Rembold, M., Ciglar, L., Yanez-Cuna, J.O., Zinzen, R.P., Girardot, C., Jain, A., Welte, M.A., Stark, A., Leptin, M. and Furlong, E.E. (2014) A conserved role for Snail as a potentiator of active transcription. Genes Dev. 28, 167–181.
[39] Leptin, M. (1991) Twist and snail as positive and negative regulators during Drosophila mesoderm development. Genes Dev. 5, 1568–1576.