Crif1 is Required for Proper Mesenchymal to Epithelial Transition

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ABSTRACT

The epithelial to mesenchymal and the mesenchymal to epithelial transitions (EMT and MET) are fundamental developmental processes required for shaping the embryo but are also hijacked by cancer cells during metastasis. The regulation of EMT is very well studied, and major regulators have been identified. A similar understanding of the regulation of MET is needed. Recently we and others have described essential factors for the initiation and progression of MET; among them is the ETS transcription factor Elf3. Recent reports showed that Crif1 is critical for the function of Elf3, yet the influence on MET has not been reported. Here, we studied the involvement of Crif1 in MET using a loss of function approach in NMuMG cells. We found that the depletion of Crif1 resulted in an impaired MET. We have also noticed that Cdh1 mRNA and protein expression was not affected; instead, E-cadherin, the protein product of Cdh1, was localized to the cytoplasm. These results are in agreement with our previous findings following the depletion of Elf3. In conclusion, Crif1 was essential for the mesenchymal to epithelial transition, and it may exert its function in cooperation with Elf3.

Keywords: EMT, Elf3, Crif1,

Introduction

The epithelial to mesenchymal transition (EMT) is a very well studied developmental process [1]. It is essential for proper gastrulation as well as many developmental stages, including organogenesis and embryo shaping [2, 3]. This process is also hijacked by tumor cells for dissemination into distant sites during metastasis [4-6]. The cells undergoing EMT establish a new non-polarized phenotype and motile capacity due to changes in gene expression of key EMT-related genes [7, 8]. The most notable changes include the downregulation of Cdh1 and the upregulation of Cdh2 (the cadherin switching) [9].

On the other hand, the mesenchymal to epithelial transition (MET) is also a developmental process; it is critical for cell fate determination and during organogenesis [2]. MET is the reversal of EMT because the cells will gain epithelial characteristics. Still, recent research suggested that MET is not simply the opposite of EMT, considering the differences in the regulatory cues controlling these transitions [10-12]. Despite the well-characterized EMT, a similar understanding of MET and its regulation is lagging.

The regulation of MET has gained attention in recent years, and novel mechanisms appear to impact its control. One of the earlier reports suggested that MET is required to reprogram induced pluripotent stem cells (iPSCs) [12]. MET activation is initiated by the activation of Cdh1 in response to exogenous Klf4 leading to the activation of the core pluripotency network [12]. More recently, Grhl2 and Grhl3 have been shown to regulate MET by activating Cdh1 expression [10, 13]. Other factors such as Ovol2 have also been attributed to safeguarding the epithelial state by sustaining MET [14, 15]. Finally, the Ets transcription factor Elf3 was described as an essential requirement for proper MET, which may be due to its role in regulating the transcription of Grhl3 [16]. The accumulated evidence on the regulation of MET is still limited to a few transcription factors and pathways, limiting the understanding of MET in general and negatively impacting the understanding of metastasis. A better knowledge of MET is of utmost importance for knowing the associated disease and understanding basic concepts in developmental biology.

Recently, the CR6-interacting factor 1 or Crif1 was described as an essential factor for the proper function of Elf3 during intestinal development. Crif1 is better known as mitochondrial protein taking part in the large mitochondrial ribosomal subunit [17]. But other reports showed a nuclear expression of Crif1 acting as a transcriptional repressor of the orphan nuclear receptor NR4A1 [18]. Despite the diverse roles of Crif1 in controlling different aspects of cellular metabolism, the relationship with Elf3 was of significant interest to us. We have recently shown a fundamental requirement of Elf3 during MET [16], which makes it reasonable to ask whether Crif1 is also assisting Elf3 during its role on MET. To this end, we hypothesized that Crif1 could be a component of the regulatory circuit of MET, and to test this hypothesis, we applied a loss of function approach using the well-known NMuMG cells.
Materials and Methods

**Cell Line**

NMuMG, normal murine mammary gland cell line, was obtained from ATCC. In general, cells were cultured at 37°C in an incubator supplied with 10% CO₂. The culture medium was DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS), 10 U ml⁻¹ Penicillin/Streptomycin, 0.1 mM non-essential amino acids, 2 mM L-glutamine (Gibco), and 10 µg ml⁻¹ insulin (Sigma). Culture media were replenished every couple of days. Cell passaging was performed at 80-90% confluence.

**Initiation of EMT and MET**

NMuMG cells were treated with TGFβ3 (5 ng/ml, PeproTech) for 3 days. For the induction of MET, TGFβ withdrawal was applied by washing the cells two times with PBS and incubating for an additional 3 days in fresh DMEM.

**siRNA Transfection**

For loss of function experiments, 100000 cells were seeded in 6-well plates and transfected with 50 nM siRNAs targeting Elf3 (Qiagen), Crif1 (Qiagen), or a non-targeting control siRNA (Ambion) using Lipofectamine RNAi Max (Invitrogen). After transfection, cells were incubated for 3 days and then collected for RNA isolation or immunofluorescence labeling.

**mRNA Expression Level Analysis**

Total RNA was prepared from treated and control cells using the Nucleospin RNA II kit (Macherey-Nagel). Changes in mRNA levels were measured from isolated RNA by qPCR. Generally, 1 µg total RNA was used for cDNA synthesis using Maxima First Strand cDNA Synthesis Kit (Thermo). qPCR analysis and quantification were performed using the ΔΔCt method. Primer sequences are available upon request.

**Western Blot Analysis**

For gene expression changes at the protein level, cultured cells were collected with the aid of a cell lifter (Corning). Cells were then lysed in cell lysis buffer (50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 0.1% Nonidet P-40, and 1X protease inhibitor cocktail (Roche)). Protein quantification was performed with Bradford assay. 50 µg total lysate was loaded on a 10% acrylamide gel transferred onto PVDF membranes (Millipore). The membranes were blocked in Blotto containing 5% nonfat milk for 1 h at room temperature. The membranes were then incubated with mouse monoclonal anti-E-cad (1:1000, BD), anti-Vimentin (1:1000), or anti-Gapdh (1:1000, SantaCruz) for 1h at RT, washed three times with Blotto and incubated with peroxidase-conjugated secondary antibodies for 1h. Detection of protein bands was done with ECL-plus (Amersham). The membranes were exposed to X-ray films (AGFA) for 1 min. The films were then developed using a hyper-processor developer (Amersham).

**Immunofluorescence Labeling and Confocal Microscopy**

Cells on coverslips were washed two times with PBS and fixed with 4% formaldehyde for 10 min. The cells were then washed two times with PBS and permeabilized with 0.25% Triton X-100 for 5 min. After washing twice with PBS, the cells were incubated with anti-E-cadherin antibodies (BD Bioscience) in PBS (1:200) for 2 h at room temperature. Next, the cells were washed twice with PBS and incubated with Alexa594-conjugated secondary antibodies together with Phalloidin (Invitrogen) for 1 h. Nuclei were stained with DAPI (1:1000, Invitrogen), and the slides were mounted with Citifluor antifadant solution. Immunostained cells were imaged with a Zeiss LSM780 confocal microscope powered by the ZEISS software (Zeiss).

**Statistical Analysis**

qPCR experiments were performed in triplicates. Statistical evaluation of the results was performed using the student’s t-test; a 95% confidence interval was applied. All experiments were performed at least three times. Error bars depict “standard error of the mean”, * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001.

**Results**

**EMT and MET in NMuMG Cells**

We used NMuMG cells to study the role of Crif1 during MET. We first confirmed the transition to the mesenchymal and epithelial states by monitoring the cellular morphology changes associated with each transition, followed by immunofluorescent staining with E-cadherin-specific antibodies. The results revealed, as expected, a successful EMT and MET (Figure 1A). The reduction of E-cadherin expression and its disappearance from the plasma membrane during EMT is accompanied by the rearrangement of actin filaments (visualized by phalloidin staining) as stress fibers (Figure 1A - middle). E-cadherin expression reappears at the plasma membrane after the completion of MET (Figure 1A - right). These changes are also associated with a typical cadherin switching, where Cdh1 expression is lost in mesenchymal cells and restored during MET (Figure 1B).

In contrast, Cdh2 expression is upregulated during EMT and then downregulated in MET (Figure 1C). The mesenchymal transition can also be confirmed by the elevated Vim and Zeb1 expression (Figure 1C). The expression pattern of Crif1 during EMT and MET resembled that of Elf3 (Figure 1B).
Depletion of Crif1 Results in an Impaired MET

We extended the correlation of expression studies to loss of function experiments. For this purpose, we utilized siRNAs against Crif1 and transfected NMuMG cells undergoing MET with either siCrif1 or siCntrl siRNAs at the time of TGFβ withdrawal (i.e., at the onset of MET). The cells were monitored daily, and changes in morphological appearance were recorded and were discernible using phase-contrast microscopy (Figure 2A). After 72 hours of treatment, the cells were prepared for confocal microscopy imaging by immunofluorescent staining as described in the methods section. The morphological changes resulting from Crif1 depletion were reminiscent of the changes previously described in response to Elf3 depletion (Figure 2A, B). The absence of Elf3 expression resulted in an impaired MET [16]. We also noticed that the E-cad expression was not reduced but rather present in the cytoplasm (Figure 2B), leading to rearrangement of actin filaments as stress fibers, similar to what was observed in the Elf3 depleted cells (Figure 2B).

To confirm the observed morphological changes, we studied the expression of key markers of EMT and MET in response to siCrif1 treatment. NMuMG cells were prepared and transfected as described in the previous section. 72 hours later, the RNA was isolated, and changes in Crif1 and Cdh1 mRNA levels were determined by qPCR and western blot analyses (Figure 3).
First, we examined the knock-down efficiency by both siCrif1 and siElf3. Both genes were significantly downregulated upon siRNA treatment (Figure 3A, C). The downregulation of Crif1 did not impact the expression levels of Cdh1. Significant expression of Cdh1 was observed compared to the control-treated sample (Figure 3A). This result confirmed our confocal imaging results, in which E-cad expression was detected but not localized to the plasma membrane (Figure 2B).

The downregulation of Crif1 was also accompanied by elevated levels of key mesenchymal markers such as Snai1, Snai2, Zeb1, and Twist (Figure 3B). Finally, the levels of E-cad and Vimentin were studied by western blot. The results indicated the presence of E-cad protein and the increased Vim levels (Figure 3E, F).

**Figure 3. Gene expression changes in response to Crif1 depletion. A and C) qPCR results of Crif1 and Elf3 depleted cells showing the knock-down efficiency and the expression of Cdh1. B and D) the expression of key mesenchymal genes in response to Crif1 and Elf3 downregulation. qPCR results represent 3 independent experiments. **: P<.01, ***: P<.001. E and F) Western blot analysis of E-cadherin and Vimentin in response to Crif1 and Elf3 depletion. Gapdh was used as an internal control.

**Discussion**

In this study, we examined the effects of Crif1 depletion on the progression of MET in NMuMG cells. Crif1 was selected for this study based on previous reports showing its importance for the function of Elf3 [19]. We have previously reported the importance of Elf3 for the progression of MET [16], so it was plausible to hypothesize a functional impact of Crif1 on MET.

We selected the normal murine mammary gland cell line NMuMG, which is well known for its ability to switch between epithelial and mesenchymal states in response to TGFβ treatment and withdrawal [10, 16]. Besides, these cells are isolated from normal mammary tissue; thus, their lack of chromosomal abnormalities makes them a perfect cellular model for studying the biology of EMT and MET.

The successful EMT and MET transitions are visible through the changes in cellular morphology and the changes in the expression of key epithelial or mesenchymal markers. For example, the cadherin switch [9] is the hallmark for a bona fide EMT since E-cad and N-cad are expressed in a mutually exclusive manner, although they share about 70% homology [20]. Elf3 is a
known epithelial transcription factor [21], its expression was found essential for MET [16], yet its expression is not diminished in cells undergoing EMT. This may relate to a specific function of Elf3 in the mesenchymal cells. A similar expression pattern for Crif1 can also be observed during EMT and MET; its expression is not changed during the transitions, which provides evidence for the correlation of Elf3 and Crif1 expression. This correlation supports the possible dependency of Elf3 on Crif1; as suggested by published research, Crif1 was reported as an essential factor for the function of Elf3 during intestinal development [19].

Depleting Crif1 during MET resulted in an aberrant transition; cells could not progress to the epithelial state and retained the expression of mesenchymal markers. The reduction of E-cadherin expression and its disappearance from the plasma membrane is accompanied by the rearrangement of actin filaments (visualized by phalloidin staining) as stress fibers. These changes are also associated with cadherin switching [9]; epithelial cells lose Cdh1 expression and gain Cdh2 expression. These changes in morphology and gene expression were also visible in response to the loss of other key regulators of MET, such as Grhl3 and Hnf4α [10] and, more recently, Elf3 [16]. In fact, having a similar phenotype due to the depletion of several transcription factors can only suggest a common mechanism controlled by different factors in the form of a regulatory network. Growing evidence suggests the involvement of other transcription factors within this network, such as Ovol2 [15, 22, 23].

The disrupted localization of E-cadherin away from the plasma membrane is as critical to the cells as its reduced mRNA levels (during EMT, for example). The impact of E-cad loss has many outcomes, it is a key pluripotency marker [20], and its loss results in a disturbed epithelial state, with indications of EMT [24].

Crif1 was previously associated with functions in the mitochondria and the nucleus. It is a component of the mitochondrial large ribosomal subunit [17, 25]. The mitochondrial function is not limited to the ribosome since Crif1 has been reported to interact with key mitochondrial DNA replication components such as ATAD3A and ATAD3B [26]. In the nucleus, Crif1 was found to interact with DNA damage sensors such as GADD45A, GADD45B, and GADD45G [27] and also with the nuclear receptor NR4A1 via the NR4A1 AB domain [18]. Crif1 negatively impacts the progression of the cell cycle; its overexpression led to latency in the G1 phase, while its inhibition accelerated the cell cycle by interacting with CDK2 [28].

The regulation of MET and the mechanisms governing its initiation and progression are poorly understood compared to EMT. The impaired MET observed here suggests a cell cycle defect, marked by the enlarged cells and the presence of multinucleation. It is reasonable to assume that the function of Crif1 during MET is, in part, to regulate the cell cycle through its interaction with Cdk2. The increasing evidence elucidating the regulation of MET will increase our knowledge about this vital process and widen our perspective for the search for novel therapeutics for the management of advanced cancer and metastasis.

Conclusion

In this study, we examined the effects of Crif1 loss on MET. We used the well-known EMT/MET cellular model, NMuMG cells, for their well-documented cellular plasticity and responsiveness to TGFβ. The overall results obtained here are in agreement with the current literature and with our previous research; they also provide new insights into the functional requirement of Crif1 during the initiation of MET. We showed for the first time a novel function for Crif1 during MET; Crif1 played a critical role within the transcriptional regulatory network regulating MET. This is of particular importance not only for understanding the basics of EMT/MET from the regulatory point of view but also for translational implications, which would help design novel therapeutics for the management of metastasis.

Acknowledgment

This work was supported by TÜBİTAK Grant 219Z034.

Conflicts of interest

The author states no conflict of interests.

References

[1] Chen T., You Y., Jiang H., Wang Z.Z., Epithelial-mesenchymal transition (EMT): A biological process in the development, stem cell differentiation, and tumorigenesis, J. Cell Physiol., 232(12) (2017) 3261-3272.
[2] Chen J., Han Q., Pei D., EMT and MET as paradigms for cell fate switching, J. Mol. Cell Biol., 4(2) (2012) 66-69.
[3] Kim D.H., Xing T., Yang Z., Dudek R., Lu Q., Chen Y.H., Epithelial Mesenchymal Transition in Embryonic Development, Tissue Repair and Cancer: A Comprehensive Overview, J. Clin. Med., 7(1) (2017).
[4] Banyard J., Bielenberg D.R., The role of EMT and MET in cancer dissemination, Connect Tissue Res., 56(5) (2015) 403-413.
[5] Brabletz T., Kalluri R., Nieto M.A., Weinberg R.A., EMT in cancer, Nat Rev Cancer, 18(2) (2018) 128-134.
[6] Chaffer C.L., San Juan B.P., Lim E., Weinberg R.A., EMT, cell plasticity and metastasis, Cancer Metastasis Rev., 35(4) (2016) 645-654.
[7] Kalluri R., EMT: when epithelial cells decide to become mesenchymal-like cells, J. Clin. Invest, 119(6) (2009) 1417-1419.
[8] Sayan A.E., Tumour-promoting role of EMT-inducing transcription factor ZEB1 in mantle cell lymphoma, Cell Death Differ, 21(2) (2014) 194-195.
[9] Wheelock M.J., Shintani Y., Maeda M., Fukumoto Y., Johnson K.R., Cadherin switching, J. Cell. Sci., 121 (Pt 6) (2008) 727-735.
[10] Alotaibi H., Basilicata M.F., Shehwana H., Kosowan T., Schreck I., Braeutigam C., Konu O., Brabletz T., Stemmler M.P., Enhancer cooperativity as a novel mechanism underlying the transcriptional regulation of E-cadherin during mesenchymal to epithelial transition, *Biochimica Et Biophysica Acta-Gene Regulatory Mechanisms*, 1849(6) (2015) 731-742.

[11] Li Q., Hutchins A.P., Chen Y., Li S., Shan Y., Liao B., Zheng D., Shi X., Li Y., Chan W.Y., Pan G., Wei S., Shu X., Pei D., A sequential EMT-MET mechanism drives the differentiation of human embryonic stem cells towards hepatocytes, *Nat. Commun.*, 8 (2017) 15166.

[12] Li R., Liang J., Ni S., Zhou T., Qing X., Li H., He W., Chen J., Li F., Zhuang Q., Qin B., Xu J., Li W., Yang J., Gan Y., Qin D., Feng S., Song H., Yang D., Zhang B., Zeng L., Lai L., Esteban M.A., Pei D., A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts, *Cell Stem Cell*, 7(1) (2010) 51-63.

[13] Werth M., Walentin K., Aue A., Schonheit J., Wuebken A., Pode-Shakked N., Villanovitch L., Erdmann B., Dekel B., Bader M., Barasch J., Rosenbauer F., Luft F.C., Schmidt-Ott Pode-Shakked N., Vilianovitch L., Erdmann B., Dekel B., Bader M., Barasch J., Rosenbauer F., Luft F.C., Schmidt-Ott M.P., Enhancer cooperativity as a novel mechanism underlying the transcriptional regulation of E-cadherin, *Biochimica Et Biophysica Acta-Genetic Regulatory Mechanisms*, 1849(6) (2015) 731-742.

[14] Goshima N., Watanabe A., Okita K., Kawasaki S., Ueno M., Kinoshita S., Masui S., OVOL2 -interacting factor 1 and ORC1 cooperate to maintain epithelial identity, *Science*, 370(6510) (2020) 922-926.

[15] Kinoshita S., Masui S., OVOL2 Maintains the Epithelial Specific Ets Transcriptional Program of Human Corneal Epithelium by Suppressing Epithelial-to-Mesenchymal Transition, *Cell Rep.*, 15(6) (2016) 1359-1368.

[16] Brembeck F.H., Opitz O.G., Libermann T.A., Rugta A.K., Dual function of the epithelial specific ets transcription factor, ELF3, in modulating differentiation, *Oncogene*, 19(15) (2000) 1941-1949.

[17] Kitazawa A., Hikichi T., Nakamura T., Mitsunaga K., Tanaka A., Nakamura M., Yamakawa T., Furukawa S., Takasaka M., Goshima N., Watanabe A., Okita K., Watanabe K., Liu Y., Noguchi S., Murray M., Chang J.C., Ramakrishnan V., Structures of the human mitochondrial ribosome, *Science*, 348(6230) (2015) 95-98.

[18] He J., Cooper H.M., Reyes A., Di Re M., Sembongi H., Litwin T.R., Gao J., Neuman K.C., Fearnley I.M., Spinazzola A., Walker J.E., Holt I.J., Mitochondrial nucleoid interacting proteins support mitochondrial protein synthesis, *Nucleic Acids Res.*, 40(13) (2012) 6109-6121.

[19] Kwon M.C., Koo B.K., Kim Y.Y., Lee S.H., Kim N.S., Kim J.H., Kong Y.Y., Essential role of CR6-interacting factor 1 (Crif1) in E74-like factor 3 (ELF3)-mediated intestinal development, *J Biol Chem.*, 284(48) (2009) 33634-33641.

[20] Bedzhov I., Alotaibi H., Basilicata M.F., Ahlborn K., Liszewska E., Brabletz T., Stemmler M.P., Adhesion, but not a specific cadherin code, is indispensable for ES cell and induced pluripotency, *Stem. Cell Res.*, 11(3) (2013) 1250-1263.

[21] Brembeck F.H., Opitz O.G., Libermann T.A., Rugta A.K., Dual function of the epithelial specific ets transcription factor, ELF3, in modulating differentiation, *Oncogene*, 19(15) (2000) 1941-1949.

[22] Kitazawa A., Hikichi T., Nakamura T., Mitsunaga K., Tanaka A., Nakamura M., Yamakawa T., Furukawa S., Takasaka M., Goshima N., Watanabe A., Okita K., Watanabe K., Liu Y., Noguchi S., Murray M., Chang J.C., Ramakrishnan V., Structures of the human mitochondrial ribosome, *Science*, 348(6230) (2015) 95-98.

[23] Rocca H., Hernandez J., Weidner S., McCaich R.C., Fuller D., Sud S., Schumann T., Wilkinson J.E., Zaslavsky A., Li H., Maher C.A., Daignault-Newton S., Healy P.N., Pienta K.J., Transcription factors OVOL1 and OVOL2 induce the mesenchymal to epithelial transition in human cancer, *PloS One*, 8(10) (2013) e76773.

[24] Alotaibi H., Transcriptional Impact of E-cadherin Loss on Embryonic Stem Cells, *Jordan Journal of Biological Sciences*, 14(5) (2021) 1035-1043.

[25] Amunts A., Brown A., Toots J., Scheres S.H.W., Ramakrishnan V., Ribosome. The structure of the human mitochondrial ribosome, *Science*, 348(6230) (2015) 95-98.

[26] He J., Cooper H.M., Reyes A., Di Re M., Sembongi H., Litwin R.T., Gao J., Neuman K.C., Fearnley I.M., Spinazzola A., Walker J.E., Holt I.J., Mitochondrial nucleoid interacting proteins support mitochondrial protein synthesis, *Nucleic Acids Res.*, 40(13) (2012) 6109-6121.

[27] Chung H.K., Yi Y.W., Jung N.C., Kim D., Suh J.M., Kim H., Park K.C., Song J.H., Kim D.W., Hwang E.S., Yoon S.H., Bae Y.S., Kim J.M., Bae I., Shong M., CR6-interacting factor 1 interacts with Gadd45 family proteins and modulates the cell cycle, *J. Biol. Chem.*, 278(30) (2003) 28079-28088.

[28] Ran Q., Hao P., Xiao Y., Xiang L., Ye X., Deng X., Zhao J., Li Z., CRIF1 interacting with CDK2 regulates bone marrow microenvironment-induced G0/G1 arrest of leukemia cells, *PloS One*, 9(2) (2014) e85328.