Editorial

Twist1 activated circRNA-10720 is a new player in hepatocellular carcinoma metastasis

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Comment on: Meng J, Chen S, Han JX, *et al.* Twist1 Regulates Vimentin through Cul2 Circular RNA to Promote EMT in Hepatocellular Carcinoma. Cancer Res 2018;78:4150-62.

Submitted Oct 24, 2018. Accepted for publication Nov 30, 2018.
doi: 10.21037/tcr.2018.12.01
View this article at: http://dx.doi.org/10.21037/tcr.2018.12.01

Epithelial-mesenchymal transition (EMT) is a process that allows an epithelial cell to acquire a mesenchymal-like phenotype that enhances migration capacities. One of the first steps in the EMT process is the loss of cell-cell contacts, mediated in part by the loss of E-cadherin and other epithelial markers. Moreover, the acquisition of mesenchymal markers, such as Vimentin, is crucial in this process (1). EMT was first described in embryogenesis, where it plays a role in the formation of various tissues and organs, and later was related to oncology and the metastatic process (2). Twist1, a transcription factor first studied in embryonic development, where it acts as a morphogen regulating mesodermal differentiation (3), was first identified as an EMT gene by Yang *et al.* in 2004 (4). Twist1 acts as a transcriptional repressor of E-cadherin and has also been found to induce the expression of mesenchymal markers, such as Vimentin, Fibronectin and N-cadherin, during EMT (5). The role of Twist1 on EMT through upregulation of Vimentin levels in hepatocellular carcinoma (HCC) has been known since 2009 (6). However, the exact mechanism associated with the upregulation of Vimentin has not been elucidated, although transcriptional regulation has been ruled out since no binding site for Twist1 on the Vimentin promoter has been identified.

The recent study by Meng *et al.* (7) deciphers one of the mechanisms involved in the upregulation of Vimentin during the EMT process that enhances metastasis in HCC. The authors demonstrated that Twist1 activates the transcription of a *CUL2*-derived circular RNA (circRNA) that is involved in the absorption of the microRNAs (miRNAs) targeting Vimentin mRNA, hence producing a rise in Vimentin protein levels (*Figure 1*). CircRNAs are covalently closed single-strand RNA transcripts produced from a pre-mRNA mainly by two different mechanisms, back-splicing and exon-skipping (8). Their functions are mostly unknown, but some of them have been reported to regulate gene transcription and mRNA splicing and to act as a sponge for miRNAs affecting mRNA translation. In HCC, most of the circRNAs described to date have been shown to participate in the titration of miRNAs to regulate cell proliferation, apoptosis, cell cycle, different signaling pathways, invasion and metastasis (summarized in *Table 1*). However, the work of Meng *et al.* is the first that demonstrates *in vitro* and *in vivo* that a circRNA can regulate EMT in HCC through regulation of key molecules of the process such as Vimentin.

To explore how Twist1 promotes EMT and specifically Vimentin upregulation, the authors performed chip-seq to identify Twist1 transcriptionally regulated genes and detected a binding site in the promoter of *CUL2*. *CUL2* is a tumor suppressor gene involved in the ubiquitination and degradation of HIFα during normal vasculogenesis (36). When they analyzed the *CUL2* levels in patient samples, no differences were observed between metastatic and non-metastatic patients, indicating that Twist1 action over *CUL2* to promote EMT was not likely related to the coding form of the gene. Using a prediction model, seven potential circRNAs were identified that could be generated by back-splicing of CUL2 pre-mRNA, but only one—circ-10720—showed significant differences in expression between metastatic and non-metastatic HCC patients. Circ-10720 was significantly overexpressed in metastatic patients and its expression correlated with Twist1. Moreover, its expression

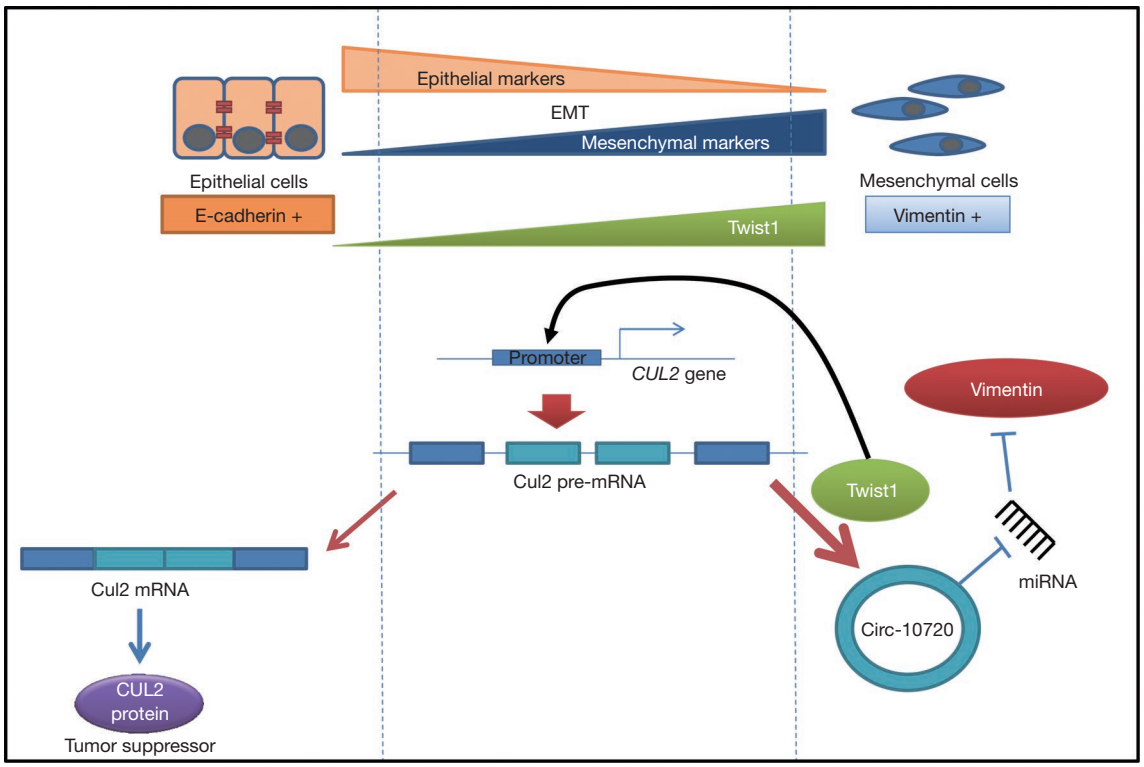

Figure 1 In EMT, epithelial markers decrease and mesenchymal markers, including Twist1, increase. In epithelial cells, *CUL2* transcription produced a mRNA that will be translated to Cul2 protein, while in mesenchymal cells, the overexpression of Twist1 promoted the transcription of circ-10720 through direct binding of Twist1 to the promoter region of *CUL2*. The CUL2-derived circ-10720 regulated the translation of Vimentin, thus inhibiting Vimentin-targeting miRNAs. EMT, epithelial-mesenchymal transition.

was associated with disease stage and pathological grade, where advanced stages/grades showed higher levels, and also higher levels were found in patients with high fetoprotein (AFP) levels and with the hepatitis B marker. The expression of this CUL2-derived circRNA was clearly associated with a more aggressive phenotype in HCC patients and the patients showing high levels of circ-10720 had shorter overall survival.

The authors verified *in vitro* that Twist1 overexpression produces an increase of CUL2 pre-mRNA and circ-10720 levels, while CUL2 mRNA and protein levels were significantly reduced. The study of the potential oncogenic role of circ-10720 showed that after its *in vitro* upregulation, morphological changes could be observed in the cells with the acquisition of a mesenchymal-like phenotype and with upregulation of Vimentin and downregulation of E-cadherin levels, while the silencing of circ-10720 produced the reverse effect. Moreover, the upregulation of circ-10720 was associated with increased proliferation, migration and invasion. To study, how the CUL2-derived circRNA was regulating EMT, the authors then analyzed the potential role of this circRNA in miRNA titration. They showed that circ-10720 functions as a miRNA sponge and identified 14 miRNAs that bound to the circRNA, three of which (miR-1246, miR-578, miR-490-5p) had Vimentin as a target and were expressed in HCC, according to TCGA data. The upregulation of circ-10720 inhibits the miRNA repressive action over Vimentin mRNA translation, allowing upregulation of Vimentin protein levels, which are crucial for EMT. Moreover, the upregulation of circ-10720 produced higher molecular changes in the HCC cells, with an increase in cancer-associated and VEGF-associated genes in addition to the upregulation of EMT-associated genes and the downregulation of cell adhesion-related genes.

The authors performed several loss-of-function experiments to verify the Twist1—circ-10720 relation. In HCC cells overexpressing Twist1, which produces upregulation of Vimentin, the silencing of circ-10720 was associated with downregulation of Vimentin levels, which impacted proliferation, migration and invasion.

Table 1 circRNAs reported in hepatocellular carcinoma with experimentally validated functions

circRNA (ref)	circBase ID	Position (strand)	Host gene	HCC level	miRNA sponge in HCC	Function
circ-10720 (7)	hsa_circ_0018189	chr10:35321362-35338693 (–)	*CUL2*	Up	miR-1246, miR-127-5p, miR-331-5p, miR-1200, miR-888, miR-587, miR-656, miR-890, miR-490-5p, miR-1238, miR-548g, miR-513a-3p, miR-521	Proliferation; Metastasis; EMT
ciRS-7/CDR1as (9)	hsa_circ_0001946	chrX:139865339-139866824 (+)	*CDR1*	Up	hsa-miR-7	Proliferation; Metastasis
circHIPK3 (10,11)	hsa_circ_0000284	chr11:33307958-33309057 (+)	*HIPK3*	Up	miR-124, miR-152, miR-193a, miR-29a, miR-29b, miR-338, miR-379, miR-584, miR-654	Proliferation; Metastasis
hsa_circ_0000673 (12)	hsa_circ_0000673	chr16:11940357-11940700 (–)	*RSL1D1*	Up	miR-767-3p	Proliferation; Metastasis
hsa_circ_0067934 (13)	hsa_circ_0067934	chr3:170013698-170015181 (+)	*PRKC1*	Up	miR-1324	Proliferation; Metastasis
hsa_circ_0016788 (14)	hsa_circ_0016788	chr1:228581376-228594517 (–)	*TRIM11*	Up	miR-486	Proliferation; Apoptosis; Metastasis
circRBM23 (15)	hsa_circ_0004137	chr14:23375403-23378804 (–)	*RBM23*	Up	miR-138	Proliferation; Metastasis
hsa_circ_100338 (16)	–	chr1:151638888-151639119 (+)	*SNX27*	Up	miR-141	Metastasis
hsa_circ_000839 (17)	hsa_circ_0000497	chr13:78293666-78327493 (+)	*SLAIN1*	Up	–	Metastasis
hsa_circ_SLAIN1 (18)	hsa_circ_0100929	chr13:78293666-78335245 (+)	*SLAIN1*	Up	miR-375	Proliferation; Apoptosis
hsa_circ_0005075 (19)	hsa_circ_0005075	chr1:21377358-21415706 (–)	*EIF4G3*	Up	miR-23b-5p, miR-93-3p, miR-581, miR-23a-5p	Proliferation; Metastasis
hsa_circ_0103809 (20)	hsa_circ_0103809	chr15:51242247-51250991 (+)	*AP4E1*	Up	miR-490-5p	Proliferation; Apoptosis; Metastasis
circFBLIM1 (21)	hsa_circ_0010090	chr1:16084668-16113084 (+)	*FBLIM1*	Up	miR-346	Proliferation; Apoptosis; Metastasis
circ-ZEB1.33 (22)	hsa_circ_0004907	chr10:31749965-31791437 (+)	*ZEB1*	Up	miR-200a-3p	Proliferation
circRNA-101368 (23)	hsa_circ_0003028	chr14:66028054-66028484 (+)	*FUT8*	Up	miR-200a	Metastasis
hsa_circ_0078710 (24)	hsa_circ_0078710	chr6:169625239-169654137 (–)	*THBS2*	Up	miR-31	Proliferation; Metastasis
circRNA_104075 (25)	hsa_circ_0075736	chr6:17669523-17669777 (–)	*NUP153*	Up	miR-582-3p	Proliferation
circSMAD2 (26)	hsa_circ_0000847	chr18:45391429-45423180 (–)	*SMAD2*	Down	miR-629	Metastasis; EMT

Table 1 (*continued*)

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circRNA (ref)	circBase ID	Position (strand)	Host gene	HCC level	miRNA sponge in HCC	Function
circC3P1 (27)	–	chr19:10073090-10074135 (+)	*C3P1*	Down	miR-4641	Proliferation; Metastasis
hsa_circ_0005986 (28)	hsa_circ_0005986	chr1:14057494-14068652 (+)	*PRDM2*	Down	miR-129	Proliferation
cSMARCA5 (29)	hsa_circ_0001445	chr4:144464661-144465125 (+)	*SMARCA5*	Down	miR-17, miR-181b	Proliferation; Apoptosis; Metastasis
circMTO1 (30)	hsa_circ_0007874	chr6:74175931-74176329 (+)	*MTO1*	Down	miR-9	Proliferation; Apoptosis; Metastasis
circARSP91 (31)	hsa_circ_0085154	chr8:101721360-101721451 (–)	*PABPC1*	Down	–	Proliferation; Metastasis
circCDK13 (32)	hsa_circ_0001699	chr7:40027197-40041630 (+)	*CDK13*	Down	–	Metastasis
circZKSCAN1 (33)	hsa_circ_0001727	chr7:99621041-99621930 (+)	*ZKSCAN1*	Down	–	Proliferation; Metastasis
hsa_circ_0001649 (34)	hsa_circ_0001649	chr6:146209155-146216113 (–)	*SHPRH*	Down	–	Proliferation; Apoptosis; Metastasis
circADAMTS14 (35)	hsa_circ_001866	chr7:38295937-38305279 (–)	*TARP*	Down	miR-572	Proliferation; Apoptosis; Metastasis

Using patient-derived tumor xenografts (PDTX) models, the authors monitored the growth of the primary tumors from different patients after *in vivo* modification of circ-10720 levels. The tumors were classified according to low or high Twist1 expression. In the group with low Twist1 levels, overexpression of circ-10720 through lentiviral transfection was associated with increased tumor volume. Inversely, in the group with high Twist1 levels, the silencing of circ-10720 was linked to reduced tumor growth. In both cases, the circRNA levels were correlated with the Vimentin levels.

Twist1 had previously been shown to participates in the EMT process, specifically by enhancing the intravasation step of metastasis, and the loss of Twist1 expression was directly related to a reduction of the number of circulating cells and a decrease in metastasis (4). Meng *et al.* (7) used a TetOn-Twist1 mouse model to evaluate the role of circ-10720 in metastasis. After activation of Twist1 overexpression, the mice first produced HCC tumors and later, after a long period of Twist1 activation, distant metastases emerged. Intravenous treatment with a siRNA against circ-10720 produced a reduction in metastasis. This model demonstrated the role of circ-10720 in metastasis, explained why the metastatic HCC patients had higher levels of circ-10720, and suggested a potential therapeutic use of this circRNA for the treatment of HCC.

Finally, the authors showed that circ-10720 can also be used as a prognostic biomarker in HCC. Detection of circ-10720 by FISH, together with Vimentin positivity, was an indicator of a more aggressive phenotype, higher risk of metastasis, and shorter overall survival. However, these results were obtained in a small cohort of 75 HCC patients and further investigation in an independent and larger cohort of patients is warranted to validate these findings.

In summary, this study is a good example of how circRNA expression can be regulated by cell type-specific mechanisms, as occurs during EMT when several circRNAs are activated (37). In the present work, *CUL2* transcriptional processing was shown to be cell type-specific. In epithelial cells, *CUL2* transcription produced an mRNA that will be translated to Cul2 protein, which acts as a tumor suppressor, while in mesenchymal cells, the

overexpression of Twist1 promoted the transcription of a CUL2-derived circRNA produced by back-splicing through direct binding of Twist1 to the promoter region of *CUL2*. The CUL2-derived circRNA regulated the translation of Vimentin, thus inhibiting Vimentin-targeting miRNAs, and promoted tumor growth and metastasis (*Figure 1*). Further studies are needed to elucidate the other components that participate in the alternative processing of the CUL2 pre-mRNA that leads to CUL2-derived circRNA production during the EMT process, but the understanding of the Twist1-mediated activation of Vimentin is a significant step in the understanding of EMT and risk stratification in HCC and provides new potential therapeutic targets for HCC.

Acknowledgments

Funding: SAF2017-88606-P from the Ministry of the Economy and Competition (MINECO) co-financed with the European Union FEDER funds and AECC-Catalunya 2017.

Footnote

Provenance and Peer Review: This article was commissioned and reviewed by the Section Editor Chunlin Ou (Cancer Research Institute of Central South University, Changsha, China).

Conflicts of Interest: The author has completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2018.12.01). The author has no conflicts of interest to declare.

Ethical Statement: The author is accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Cite this article as: Navarro A. Twist1 activated circRNA-10720 is a new player in hepatocellular carcinoma metastasis. Transl Cancer Res 2019;8(Suppl 2):S135-S140. doi: 10.21037/tcr.2018.12.01

