

1 **MiR-146a-dependent regulation of CD24/AKT/ β -catenin axis drives stem** 2 **cell phenotype in oral cancer**

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17 **Running title:** MiR-146a regulate CSCs by targeting CD24

18 **Abstract**

19 Cancer stem cells (CSCs) are known to potentiate tumor initiation and maintenance in oral squamous
20 cell carcinoma (OSCC). Increasing evidences suggest that CD44^{high}CD24^{low} population in OSCC
21 exhibit CSC-like characteristics. The role of mi-RNAs in maintenance of oral CSCs has remained
22 unclear. Here we report that CD44^{high}CD24^{low} population within OSCC cell lines and primary HNSCC

23 tumors have an elevated expression of miR-146a. Moreover, over-expression of miR-146a results in
24 enhanced stemness phenotype by augmenting CD44^{high}CD24^{low} population. We demonstrate that miR-
25 146a induces CSC property by stabilizing β -catenin with concomitant loss of E-cadherin and CD24.
26 Interestingly, CD24 has been identified as a novel functional target of miR-146a and ectopic expression
27 of CD24 abrogates miR-146a driven potential CSC phenotype. Mechanistic analysis reveals that higher
28 CD24 levels inhibit AKT phosphorylation leading to β -catenin degradation in non-CSCs. We also
29 validate that the miR-146a/CD24/AKT loop significantly alters tumorigenic ability *in vivo*.
30 Furthermore, we confirmed that β -catenin trans-activates miR-146a, thereby forming a positive
31 feedback loop contributing to stem cell maintenance. Collectively, our study demonstrates that miR-
32 146a regulates CSC-driven properties in OSCC through CD24-AKT- β -catenin axis.

33

34 **Keywords:** miR-146a, β -catenin, AKT, stemness, CD24, OSCC

35

36 **Introduction**

37 Oral Squamous Cell Carcinoma (OSCC) is the most prevalent form of head and neck cancers worldwide
38 with more than 60% individuals diagnosed with advanced tumors¹. It is known that cancer stem-like
39 cells (CSC) leads to aggressive tumor behavior². The oral tumor CSCs are also considered responsible
40 for treatment failures, relapse and development of metastases^{3,4}. Over the past decade, epigenetic re-
41 programming has emerged as a crucial mechanism of regulating cancer stem cell dynamics⁵. Epigenetic
42 regulatory mechanisms include DNA methylation, histone modifications and chromatin remodeling.
43 They show robust effect upon cellular fate and stem cell potential^{6,7}. MiRNAs, a small ncRNAs of 20-
44 22 nucleotides, has recently gained considerable importance in epigenetic modulation of tumor⁸. De-
45 regulation of miRNAs may have critical roles in disease development⁹. They can act either as oncogenes
46 or as tumor-suppressors depending upon the specific genes targeted. In-fact miRNA associated
47 signatures are now considered for cancer specific diagnostic and prognostic purposes¹⁰.

48 MiRNAs not only regulate primary cellular functions like proliferation, differentiation,
49 migration and invasion, but are also directly or indirectly involved in CSC functions¹¹⁻¹⁴. Most of this
50 phenomenon are attributed to altered signaling pathways including cell surface markers, pluripotent
51 transcription factors, chemo-resistance and epithelial-to-mesenchymal transition (EMT) markers¹⁵. Role
52 of miR-34a, miR-145a and miR-200bc family in regulating CD44, Oct4, Sox2, KLF4, Bmi1, Zeb1/2
53 and Notch1 has been well established¹⁶⁻¹⁹. Thus, by precisely regulating the CSC related genes, miRNAs
54 themselves have emerged as inherent modulators of cancer stem cells. MiR-146a is predominantly an
55 onco-miR, which directly targets *IRAK1*, *traf6*, and *numb* genes in OSCC and imparts tumorigenicity
56²⁰. Emerging evidence on miR-146a suggests that it directs the self-renewal process in colorectal cancer
57 stem cells by regulating Snail- β -catenin axis which also contributes to EMT²¹. High nuclear
58 accumulation of β -catenin, along with lowering of E-cadherin is frequently associated with higher tumor
59 grade and poor prognosis in various cancers²². Given the role of Wnt/ β -catenin signaling in CSC
60 maintenance and the miRNAs in regulating wnt pathway, understanding the step-wise regulation of its
61 mediators is crucial²³⁻²⁵.

62 Our recent study has characterized CD44^{high}CD24^{low} cells as the potential CSC population in
63 OSCC³. CD24, a small cell surface protein, was identified as a critical determinant of differentiation in
64 hematopoietic cells and mammary epithelial cells²⁶. Besides having role in adhesion, cadherin switching
65 or migration, CD24 is involved in diverse signaling networks, thus promoting oncogenesis or
66 regression²⁷. Although role of CD44 is well established⁴, the significance of CD24 in determining
67 stemness is less explored, particularly in oral CSCs. In this study, we show that miR-146a imparts
68 CD44^{high}CD24^{low} status to OSCC cells merely by targeting CD24. Changes in β -catenin pools seemed
69 to be an important event in altering the afore-mentioned phenotypes downstream of miR-146a through
70 AKT pathway. We propose that miR-146a/CD24/AKT/ β -catenin axis influences the stemness
71 characteristics of CD44^{high}CD24^{low} population in oral cancer cells.

72

73 **Results**

74 **MiR-146a is over-expressed in CD44^{high}CD24^{low} population of OSCC cell lines and primary**
75 **tumors**

76 To identify the cellular miRNAs regulating CSC phenotype of OSCC cells, we initially screened nine
77 miRNAs that are aberrantly expressed in human cancers with their reported role in cancer stemness and
78 EMT³⁰⁻³². QRT-PCR data showed significant difference in the expression of miR-200b, miR-138, miR-
79 34a, miR-21 and miR-146a between CD44^{high}CD24^{low} and CD44^{low}CD24^{high} population of SCC25 cells
80 (Fig. 1a). Amongst them, we focused on miR-146a in view of its context dependent role in various
81 cancers³³⁻³⁵. MiR-146a is consistently over-expressed in oral CSCs, therefore it was intriguing to explore
82 its possible connection with stemness and the underlying mechanisms. Up-regulation of miR-146a in
83 the CD44^{high}CD24^{low} population of SCC131 and SCC084 cell lines was also confirmed (Fig.1b).
84 Further, miR-146a expression was also increased upon enrichment of isolated CSCs in the sphere
85 forming culture conditions, suggesting its importance in determining oral CSC property (Fig. 1c).
86 Increased miR-146a expression has been reported to predict poor survival of OSCC patients²⁰. Our
87 analyses in TCGA Head and Neck Squamous cell Carcinoma (HNSCC) patient's cohort³⁶, showed
88 increased miR-146a expression in patients with CD44^{high}CD24^{low} signature compared to those with
89 CD44^{low}CD24^{high} profile (Fig. 1d). While there was not much difference in the histological stage of the
90 tumors across the two categories, most of the CD44^{low}CD24^{high} tumors were free of lymph node
91 metastasis (Figure S1a, Supplementary file 1). Moreover, miR-146a expression of the node positive
92 patients was relatively higher than that of the node negative ones, although not statistically significant
93 (Figure S1b). Together, our data suggests high miR-146a expression as a critical determinant of CSC
94 phenotype in oral tumors.

95 **Ectopic expression of miR-146a induces CSC characteristics**

96 We next investigated whether ectopic miR-146a affect CSC markers and found a significant increase
97 in the relative proportion of CD44^{high}CD24^{low} population in SCC131 cells (Fig.2a). Similar results were
98 also obtained with SCC036, SCC084 and SCC25 cells, respectively (Figure S2a). Characteristic sphere
99 forming ability of miR-146a expressing SCC131 and SCC036 cells were also markedly enhanced (Fig.

100 2b). Concomitantly, ectopic expression of miR-146a led to the increased expression of intracellular
101 stem cell markers such as Oct4, Sox2 and C-myc and loss of Involucrin (Fig.2c). Also, we observed a
102 pronounced decrease in CD24 protein levels upon ectopic miR-146a expression in SCC036, SCC131
103 and SCC084 cells (Fig. 2c). However, in SCC131 cells the levels of Oct4 and Involucrin did not show
104 a dose dependent change upon miR-146a over-expression, probably due to high endogenous miR-146a
105 in this cell line (Fig. 2c and Figure S2b). Loss of these markers upon knockdown of miR-146a was also
106 evident in SCC131 and SCC25 cells (Figure S2c). Expression of miR-146a transcripts was validated
107 by qRT-PCR (Figure S2d). Transfection of miR-146a containing mutated seed sequence, however, did
108 not alter the levels of stem-related proteins in a statistically significant manner (Fig. 2d). These results
109 demonstrate that miR-146a contributes to enrichment of CSCs in OSCC through increased expression
110 of stem cell markers.

111 **MiR-146a activates Wnt/ β -catenin pathway and promotes EMT in oral CSCs**

112 The CSC population is sustained by niche signalling, similar to that of normal stem cells. These include
113 Wnt, Notch and Hedgehog pathways that are often involved in maintaining its self-renewal potential³⁷.
114 Accordingly, we observed increased expression of β -catenin and Cleaved Notch1 in CD44^{high}CD24^{low}
115 population of SCC25 and SCC131 cells (Figure S3a, b). While significantly depleted in CD24^{high} cells,
116 β -catenin levels were also higher in CD24^{low} population of SCC084 cells which correlated well with the
117 expression of stemness markers in these cells (Fig.3a). To specifically envisage the role of CD24, we
118 now compared the CD44^{high}CD24^{low} population with the CD44^{high}CD24^{high} cells only. Interestingly we
119 detected nuclear localization of β -catenin in the CD44^{high}CD24^{low} population of SCC131 cells, whereas
120 it remained membrane bound in CD44^{high}CD24^{high} cells (Fig.3b). Transcriptional activity of β -catenin
121 was indicated by the relative wnt reporter activity in the respective cell populations (Figure S3c).
122 Interestingly, upon stable knockdown of β -catenin, not only the stem cell markers were reduced but
123 also a modest increase in CD24 expression was observed (Figure S3d). These results suggest a possible
124 cross talk between CD24 and β -catenin in conferring stemness and EMT to these cells.

125 The clue that β -catenin level might influence stemness in OSCC cells led us to investigate whether miR-
126 146a regulates β -catenin. Indeed, over-expression of miR-146a lead to the dose dependent increment in
127 β -catenin levels with concordant decrease in E-cadherin (Fig.3c). It is known that miR-146a targets the
128 3'UTR of Numb, a protein that promotes lysosomal degradation of β -catenin and Cleaved Notch1³⁸. We
129 indeed, confirmed reduced levels of Numb upon miR-146a over-expression along with stabilization of
130 Cleaved Notch1 (Figure S3e). Conversely, inhibition of miR-146a activity lead to β -catenin degradation
131 along with the loss of Oct4 as expected (Fig.3d). Notably, we did not observe these changes upon mutant
132 miR-146a over-expression (Figure S3f). To emphasize the contribution of β -catenin in miR-146a
133 induced stemness, we transfected miR-146a in β -catenin shRNA expressing cells and found no change
134 in expression of CSC markers (Figure S3g) as compared to that of non-silencing controls. Strikingly,
135 the ability of anchorage independent growth induced by miR-146a was also dependent on the expression
136 of β -catenin (Figure S3h) suggesting its tumorigenic role in OSCC.

137 To obtain clinical relationships among miR-146a, CD24 and β -catenin, we first checked the correlation
138 between miR-146a and CD24 expression across the NCI-60 cell lines and found it to be significantly
139 negatively correlated (Fig.3e). Moreover, examination of CD44^{high} HNSCC tumors from TCGA dataset
140 revealed a positive correlation between miR-146a expression and β -catenin/CD24 ratio (Fig. 3f).
141 Further, the observed down-regulation of E-cadherin upon miR-146a expression prompted us to address
142 the miR-146a driven EMT phenomenon in OSCC. Indeed, miR-146a was found to be significantly
143 over-expressed in the mesenchymal (MS) cell lines showing higher CD44 and lower CD24 expression³,
144 compared to the epithelial (EP) cell lines of the NCI-60 panel²⁹(Fig.3g). In addition, the miR-146a
145 expression in TCGA tumor samples was negatively correlated with the E-cadherin to Vimentin ratio
146 (Fig.3h). Based on these observations, we propose that miR-146a induced stemness and EMT in OSCC
147 is mediated through lowering of CD24 followed by activation of β -catenin.

148 **MiR-146a targets CD24 in oral CSCs**

149 We considered CD24 as a putative target of miR-146a through which it might impart stemness in OSCC.
150 Although, *in silico* identification of miRNA targets using the prediction software did not reveal CD24

151 as the probable target, we did find matching of miR-146a seed sequence in the CD24 3'UTR in
152 miRANDA (Supplementary file 2). Despite one mismatch, the maximum free energy of miRNA-
153 mRNA binding was favourable enough for hybridization and targeting (Figure S4a). In Fig. 2c, we had
154 already examined that CD24 expression was significantly depleted upon miR-146a transfection in a
155 dose dependent manner in SCC036, SCC131 and SCC084 cells. Alongside, it was up-regulated upon
156 inhibition of miR-146a in SCC131 cells (Fig.4a). We also observed remarkable changes in CD24
157 transcripts in both SCC131 and SCC084 upon modulation of miR-146a (Fig.4b,c). To further confirm
158 that CD24 is a direct target of miR-146a, we co-transfected luciferase reporter vector containing the
159 3'UTR fragment of CD24 gene with either miR-146a expressing vector or anti-miR-146a in SCC131
160 cells. As shown in Fig.4d, e and f, miR-146a over-expression reduced the luciferase activity of CD24
161 3'UTR, while miR-146a inhibitor elevated the same. On the contrary, transfection with mutated mir-
162 146a did not alter the CD24 3'UTR luciferase activity significantly (Fig.4d, Figure S4b). Thus, this data
163 experimentally validates the ability of miR-146a to directly target CD24 gene by binding to its 3'UTR.
164 This justifies the involvement of miR-146a in negative regulation of CD24 expression in oral cancer
165 stem cells.

166 **Mir-146a stabilizes β -catenin by down-regulating CD24**

167 To get a mechanistic insight into the miR-146a mediated β -catenin stabilization, CD24 was over-
168 expressed in miR-146a over-expressing cells. Interestingly, CD24 not only abolished the stemness
169 markers but also the expression of β -catenin and CD44 (Fig.5a and FigureS5a). This indicated that
170 down-regulation of CD24 by miR-146a was instrumental in maintaining the high β -catenin levels and
171 consequently the downstream stemness phenotype. This was further exemplified as CD24 over-
172 expression alone could lead to degradation of β -catenin protein and the associated stem cell markers,
173 while the siRNA mediated knockdown of CD24 showed an opposite effect (Fig.5b,c). Wnt target genes
174 such as *C-myc*, *CD44*, *CCND1* were detected at conspicuous levels upon CD24 knockdown, whereas
175 significantly depleted upon CD24 over-expression in cells expressing miR-146a (Figure S5b, c). These
176 observations suggest an inverse correlation between CD24 and β -catenin signalling in OSCC cells. It
177 was further confirmed by the inhibition of miR-146a-induced β -catenin nuclear mobilization upon

178 ectopic expression of CD24 (Figure S5d). In addition, miR-146a driven increased wnt reporter activity
179 was found to be reduced upon CD24 over-expression (Fig.S5e). To further investigate its downstream
180 effect on stemness phenotype, we performed an *in vitro* sphere-formation assay. We observed a
181 considerably defective spheroid forming ability of miR-146a transfected cells in the presence of CD24
182 (Fig.S5f). Together, these observations suggest the possible contribution of CD24 in regulating Wnt
183 pathway through β -catenin, thereby affecting CSC-like traits.

184 **Involvement of pAKT in CD24 mediated degradation of β -catenin**

185 Next, to elucidate the cause of β -catenin reduction in the presence of CD24, we treated SCC084 and
186 SCC036 cells with MG132, and found β -catenin levels returned to that of un-transfected controls
187 (Fig.5d). This confirms that unlike Numb, CD24 degrades β -catenin in a proteasomally dependent
188 manner. The restored β -catenin also re-established the expression of stem cell marker Oct4 irrespective
189 of CD24 over-expression in both SCC036 and SCC084 cells (Fig.5d). E-cadherin levels, however,
190 remained high in the presence of CD24, irrespective of β -catenin stability (Fig.5d). Notably, CD24 did
191 not affect Numb expression, corroborating the independent participation of CD24 in regulating β -
192 catenin (Figure S6a). To gain mechanistic insights into the CD24 mediated β -catenin degradation, we
193 speculated that CD24 might sequester β -catenin into the membrane bound lipid rafts analogous to that
194 of E-cadherin³⁹. To address this, we checked the CD24 and β -catenin interaction at protein level but did
195 not find them in co-immunoprecipitation (Figure S6b). Alternatively, CD24 may also lead to β -catenin
196 destabilization via AKT-GSK-3 β pathway^{40,41}. Towards this, we did find that CD24 over-expression
197 rescued the miR-146a mediated increase in pAKT (Ser 473) activity (Fig.5e). Here it was appreciably
198 noted that over-expression of CD24 lead to the down-regulation of total AKT protein itself and thereby
199 phospho-AKT (Figure S6c), although it remained stable during MG132 treatment (Figure S6d). On the
200 contrary, knockdown of CD24 in SCC036 increased pAKT and β -catenin levels (Fig.5f). Moreover,
201 pAKT was found to be accumulated in the CD44^{high}CD24^{low} fraction of SCC25 cells thereby indicating
202 its prior involvement in stemness (Figure S6e). In addition, we observed significant depletion of β -
203 catenin upon treatment with pAKT inhibitor (LY294002) which again confirmed its regulation by miR-
204 146a-CD24-pAKT loop (Fig.5g).

205 These cell line related observations was further validated using mouse xenograft model. To check the
206 effect of miR-146a on *in-vivo* tumor formation, SCC084 cells harboring either an empty vector
207 (SCC084/EV) or stably expressing miR-146a (SCC084/miR-146a), were generated and the over-
208 expression of miR-146a with subsequent downregulation of CD24 was confirmed by both qRT-PCR
209 and western blot analysis (Fig. S7a, b and c). SCC084/EV and SCC084/miR-146a cells were then
210 introduced in the right and left flanks of NOD/SCID mice respectively and allowed to form
211 subcutaneous tumor (Fig S7d and e). A significant increase in tumor volume and tumor weight was
212 observed in SCC084 xenografts stably over-expressing miR-146a suggesting enhanced stemness
213 potential of these cells. (Fig 6a and b). Further, to explore the effect of AKT signaling on miR-146a
214 induced tumor, mice with palpable tumors generated from SCC084/EV and SCC084/miR-146a cells
215 were treated with quercetin, a known PI3K/AKT signalling pathway blocker ⁴² (Fig S7d and e). As
216 expected, the tumor formation ability of miR-146a cells was significantly attenuated *in vivo* upon
217 administration of quercetin at regular intervals (Fig 6a and b). While there was no effect of quercetin
218 on miR-146a and CD24 levels, β -catenin over-expression in miR-146a tumors was compromised in
219 presence of quercetin (Fig S7f, g). In order to investigate the effect of CD24 upon the acquired stemness
220 of miR-146a expressing cancer cells *in vivo*, we examined xenograft tumors generated from
221 SCC084/miR-146a cells harboring CD24 expression construct (Fig S7a, b, c, h and i). Notably,
222 compared to the control tumors, volume and weight of CD24-expressing SCC084/miR-146a tumors were
223 not significantly (p-value = 0.7360) altered (Fig 6c and d) suggesting loss of miR-146a driven stemness.
224 Xenograft tumor subjected to qRT-PCR analysis confirms the overexpression of miR-146a and CD24
225 in these cells while β -catenin is down-regulated (Fig. S7j and k). Collectively, these results indicate that
226 CD24/AKT/ β -catenin axis plays an important role in miR-146a regulated CSC-mediated tumor growth
227 *in-vivo*. This was further supported by the soft agar tumorigenesis assays, which clearly showed that
228 CD24 over-expression or AKT inhibition, both rescued the miR-146a induced colony formation in
229 OSCC cells (Fig. S8a and b). The above results strongly prove CD24 as a negative regulator of β -
230 catenin/Wnt signaling pathway through AKT inhibition.

231 **β -catenin transactivates miR-146a expression contributing to positive feedback loop**

232 The upstream regulators of miRNAs have always been involved in feedback regulatory mechanisms
233 and are not much investigated. Analysis of miR-146a promoter has revealed the binding sites for NF-
234 κ B, TCF4/ β -catenin and STAT3, suggesting possible transcriptional modulation^{21,43,44}. β -catenin
235 enhances stemness features by driving the intracellular levels of c-myc and other yamanaka factors
236 (Fig.7a). This apparently contributes to the enhanced tumorigenic properties in response to high miR-
237 146a levels. Interestingly, we found that β -catenin also promotes the expression of miR-146a, which
238 might augment the stemness acquiring ability of the cancer cells (Fig.7a). However, expression of miR-
239 146a was significantly reduced in the presence of both dnTCF4 and Numb which inhibits β -catenin
240 binding to the promoter and degrades it respectively (Fig.7b). Change in miR-146a promoter activity
241 under similar conditions suggests that β -catenin is involved in trans-activation of miR-146a promoter
242 (Figure S9a). We hypothesize that β -catenin mediated induction of miR-146a contributes to β -catenin
243 mediated CD24 reduction (Fig.7a). ChIP-qPCR assay confirmed that β -catenin binds to miR-146a
244 promoter *in vivo* (Fig.7c). Further, the recruitment of β -catenin was found to be significantly enhanced
245 in absence of CD24 (Fig.7c). Moreover, miR-146a promoter activity was significantly increased in
246 presence of miR-146a, while reduced upon ectopic expression of CD24 (Fig. 7d). However, the
247 constructs with either mutated or deleted TCF4 binding sites showed no significant difference in
248 promoter activity (Figure S9b). This may be due to the alternating levels of β -catenin which shoots up
249 in miR-146a over-expression condition and gets depleted in presence of CD24. Transient ChIP assays
250 with the same luciferase constructs also confirmed that β -catenin indeed binds to miR-146a promoter,
251 which is impeded upon CD24 over-expression (Fig. 7e). These data positively confirm the feedback
252 activation loop by β -catenin that further trans-activates miR-146a expression to shift the equilibrium
253 towards CSC maintenance.

254 Discussion

255 Oral cancer progression has been largely attributed to both genetic and epigenetic heterogeneity.
256 Tumorigenic cells can arise from the non-tumorigenic cancer cells owing to spontaneous conversion to
257 a stem-like state⁴⁵. The origin and plasticity of such cells, called cancer stem cells (CSCs), have always

258 been a matter of debate. Nevertheless, CSCs are known to be responsible for chemo-resistance, tumor
259 recurrence and metastasis. Detail molecular characterization of CSCs is therefore of paramount
260 importance for eliminating them from its roots. CD24 has been routinely used with CD44 for the
261 prospective isolation of CSCs in colorectal, prostate and breast cancers ⁴⁶. Given the critical role of
262 cellular miRNAs in regulating CSC characteristics, current anticancer therapies have provided an
263 important avenue towards exploiting them for effective cellular targeting⁴⁷. Targeting of CD44 by
264 miRNAs in NSCLC, prostate and ovarian cancer has been previously demonstrated to attenuate
265 stemness^{17,48,49}. However, miRNA mediated regulation of CD24 remains to be determined.

266 Consistent with its oncogenic functions, miR-146a promotes symmetric division of colorectal
267 CSCs, thereby promoting stemness ²¹. The miRNA is also involved in development of melanoma by
268 activating Notch1 signaling leading to drug resistance ⁵⁰. However, little is known about its role in
269 regulating expression of CSC-related CD markers in oral cancer. In the present study, we detected
270 significantly higher expression of miR-146a in CD44^{high}CD24^{low} population of OSCC cell lines as well
271 as in tumor specimens. We therefore speculated whether miR-146a expression maintains CSC traits or
272 miR-146a accumulation is a consequence of induced stemness. Notably, ectopic expression of miR-
273 146a induce CSC-phenotype i.e. CD44^{high}CD24^{low} population together with increased β -catenin activity
274 in OSCC cell lines. CD44 is a well-known transcriptional target of β -catenin along with C-myc and
275 CCND1²¹, which clearly indicates a molecular link with miR-146a induced CD44 expression. However
276 the effect of miR-146a upon CD24 expression under these conditions was particularly intriguing.
277 Hence, we examined whether CD24 is a direct target of miR-146a and experimentally confirmed that
278 miR-146a binds to the 3'UTR of CD24 thereby repressing it post-transcriptionally. Loss of E-cadherin
279 upon miR-146a over-expression and positive correlation with the mesenchymal marker vimentin was
280 also evident. Hence, in addition to its novel role in acquiring stemness, our results re-confirmed miR-
281 146a as a key regulator of EMT⁵¹.

282 Wnt/ β -catenin has shown great potential for CSC-targeting in cancer³⁷. Our study shows that
283 CSC characteristics in OSCC is attributed to the elevated β -catenin along with depleted CD24. The
284 anticipation that CD24 leads to proteasomal degradation of β -catenin was found to be true and

285 apparently it also abolished the β -catenin mediated stemness. This is a novel functional interaction
286 through which miR-146a regulates β -catenin in oral cancer cells. Our study, thus points towards the
287 tumor-suppressor functions of CD24, supporting our previous observation of reduced CD24 expression
288 in oral tumors compared to the normal tissue (our own data)³. Although growth inhibition was achieved
289 by knocking down CD24 in colorectal and pancreatic cancer⁵², no such effects were observed in oral
290 cancer. Perhaps the variable cell-type specific distribution pattern underlies the paradoxical role of
291 CD24 in oral cancer⁵³. Activated PI3K-AKT pathway is one of the primary events in carcinogenesis⁵⁴.
292 Its contribution to stem cell self-renewal and proliferation (both normal and cancer) has also been
293 extensively studied⁵⁵. Receptor Tyrosine Kinase (RTKs) mediated growth signals (through EGF, IL-6,
294 TGF- β etc.) impinges upon AKT through activation of the PI3K kinase⁵⁶. Stability of phospho-AKT
295 and other kinases play key role in maintaining cancer stem cells of chronic myeloid leukemia (CML),
296 NSCLC, breast, prostate and colorectal cancer⁵⁵. Further, signaling pathways like WNT are often linked
297 with AKT activation that eventually contribute to expression of stem cells-related factors, chemo-
298 resistance genes, and CSC markers^{54,57}. Here we show that CD24, the cell surface CSC marker lie
299 upstream of AKT protein, similar to that of TWIST and FOXO transcription factors which is also known
300 to inhibit CD24^{40,58-60}. However, the precise mechanism by which expression/stability of AKT protein
301 is regulated by CD24 is unknown. CD24 has been shown to possibly modulate phospho-AKT levels⁶¹,
302 which might affect its downstream targets such as GSK-3 β ⁴¹. Activated GSK-3 β mediates
303 phosphorylation and ubiquitination of β -catenin, thereby leading to its degradation⁴¹. Therefore, it was
304 incumbent on us to ask whether CD24 induce AKT and subsequently affect β -catenin stability in miR-
305 146a induced oral CSCs. Indeed, MG132 treatment was found to re-stabilize β -catenin by relieving
306 pAKT inhibition in cells over-expressing CD24. Moreover, direct AKT inhibition in the miR-146a
307 transfected cells depleted β -catenin, irrespective of CD24 level suggesting AKT is downstream of
308 CD24. Thus, we logically elucidated the molecular mechanism underlying CD24 mediated β -catenin
309 degradation in oral cancer cells. We have specifically shown that CD24 over-expression decrease levels
310 of phospho-AKT leading to β -catenin instability. The role of miR-146a/CD24/AKT/ β -catenin axis in

311 maintaining the oral cancer stem cell populations is thus mechanistically evident. Studies from in-vivo
312 tumor model system also confirms that these molecular mechanisms directly affect tumorigenesis.

313 Further, the recruitment of β -catenin onto miR-146a promoter was found to be negatively
314 regulated by CD24 which might contribute to the fine tuning of stemness. These results clearly establish
315 a cross-regulatory network between miR-146a and β -catenin, governed by a stem-related marker, CD24
316 in OSCC cells. Our study thus provides strong evidences which suggest that miR-146a promotes CSC
317 characteristics of oral cancer cells by down-regulating CD24. Repression of CD24 leads to AKT
318 stabilization followed by activation of Wnt/ β -catenin signaling. Based on our observation, we propose
319 a model wherein, AKT activity is an important determinant of miR-146a dependent β -catenin signaling
320 (Fig.8). It should be noted, however, that β -catenin mediated CSC induction might be due to the
321 induction of miR-146a expression or vice-versa. Taken together, the present study highlights a novel
322 mechanism of miR-146a mediated self-renewal capacity of Oral CSCs that may have a prognostic or
323 therapeutic value in oral cancer.

324 **Materials and methods**

325 **Cell culture and transfection**

326 Various human Oral Squamous Cell Carcinoma (OSCC) cell lines SCC131, SCC084 and SCC036 were
327 obtained from Dr. Sussane Gollin, University of Pittsburgh. These cells were maintained in 5% CO₂ at
328 37°C in DMEM medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (Life
329 Technologies, Thermo Fisher Scientific Inc., MA, USA). The ATCC (American Type Culture
330 Collection) oral cancer cell line, SCC25 was cultured in complete DMEM-F12 medium and 400ng/ml
331 Hydrocortisone (Sigma Aldrich) under similar conditions. For transfection, Lipofectamine™ 2000
332 (Invitrogen) was used in serum free medium. Transfected cells were harvested after 48 hrs or 72 hrs for
333 over-expression or knockdown studies respectively.

334 **Plasmid constructs, miRNA inhibitors and siRNAs**

335 We obtained mir-146a and mir-146a SDM expressing pU61 construct from Dr. Nitai. P. Bhattacharjee
336 (SINP, Kolkata). The TOP-flash/FOP-flash reporters, dnTCF4, Numb, and pcDNA3.1 empty vector
337 along with miR-146a promoter LucA, LucB and mLucA Luciferase constructs were kind gifts from
338 Muh-Hwa Yang (Taiwan). Human CTNNB1 expression plasmid deposited by Eric Fearon was
339 purchased from Addgene (#16828). CD24 cDNA cloned into the pCDNA3.1 vector and the full-length
340 3'-UTR of CD24 cloned into the HindIII site of pMIR (Ambion) were obtained from Heike Allgayer
341 (University of Heidelberg, Germany). Anti-miR-146a (AM-34a) (ID: AM11030) were obtained from
342 Ambion, CD24 siRNA (a pool of 3 target specific siRNAs) and Scramble siRNA from Santa Cruz.
343 CTNNB1 shRNA constructs (Addgene # 18803) were provided by Dr. Mrinal Kanti Ghosh, IICB,
344 Kolkata. MiR-146a over-expression cassette was sub-cloned from pU61 into the pLKO.1 TRC vector
345 (Addgene plasmid # 10878). Packaging plasmids psPAX2 (Addgene plasmid # 12260) and pMD2.G
346 (Didier Trono, Addgene plasmid# 12259) was used to generate the miR-146a over-expression lentiviral
347 particles and target cells were infected following the manufacturer's protocol. Stable transduced cells
348 were selected by puromycin (Gibco) and over-expression efficiencies were verified by qRT-PCR and
349 western blotting. CD24 was co-transfected and clones were selected by G418.

350 **Quantitative Real Time PCR**

351 TRIzol (Invitrogen, Thermo Fisher Scientific Inc., MA USA) method was used for isolation of total
352 RNA as per manufacturer's instructions. 250 ng of RNA were converted to cDNAs using stem-loop
353 primers specific for reverse transcription of individual miRNAs²⁸. MiRNA cDNAs were amplified with
354 forward primers specific for individual miRNAs and a URP, with U6 snRNA as an endogenous
355 reference control. For mRNA expression changes, protocol was similar to that described previously³.
356 SYBR Green master mix (Roche, USA) was used to carry out qRT-PCR in the 7500 Fast Real-Time
357 PCR instrument (Applied Biosystems, USA). Fold change values ($2^{-\Delta\Delta CT}$) were calculated from average
358 of three independent experiments. Primer sequences of genes, miRNA forward and loop primers are
359 listed in Supplementary File 3.

360 **Analysis of TCGA and NCI-60 datasets**

361 RNA and miRNA-seq data were acquired for a total of 292 HNSCC tumor specimens from TCGA (The
362 Cancer Genome Atlas) data portal (<https://tcga-data.nci.nih.gov/tcga/>). We first grouped top 25% of
363 CD44 high and low expressing tumors and then further sub-grouped 25% of these tumors based on
364 CD24 expression (Supplementary File 1). These were designated as CD44^{high}CD24^{low} and
365 CD44^{low}CD24^{high}, wherein we checked the differential expression of miR-146a and calculated statistical
366 significance using R Limma Package. Node status of these patients was also correlated with miR-146a
367 expression using GraphPad Prism5 software. NCI-60 miRNA expression dataset (GEO accession
368 number GSE26375) was analyzed to compare the miR-146a expression between the epithelial and
369 mesenchymal groups as classified earlier²⁹ using Mann Whitney's u test.

370 **Flow cytometry**

371 Briefly, 48 hours post transfection of miR-146a, approximately 1×10^6 cells were harvested in 1X PBS
372 consisting of 1% FBS and 0.02% sodium azide. CD44-PE and CD24-FITC (BD Pharmingen)
373 conjugated antibodies were used for double staining. Cells were then washed and subjected to flow
374 cytometry on the BD LSRFortessa and analysis was done using BD FACSDiva 6.2 software. Debris
375 and clumps were excluded and the size gate was decided using forward and side scatter analysis of
376 unstained cells. Isotype controls were included for the non-specific staining.

377 **Sphere forming assay**

378 MiR-146a transfected cells were cultured overnight. Next day, cells were trypsinised and a single cell
379 suspension was ensured. Low attachment 6-well plate were used for re-seeding the cells at a density
380 of 5000 cells/ml in DMEM-F-12 serum free media containing 1% B27 supplement, 20 ng/ml of EGF
381 and 20 ng/ml of bFGF (Invitrogen). 500 μ l of media was added every 2-3 days. Photographs of the
382 spheres were taken under inverted microscope (Leica TCS SP8; Germany) with 20X magnification at
383 7-14 days. All experiments were done in biological triplicates.

384 **Immuno-fluorescence**

385 Sorted populations of SCC131 were grown on cover-slips overnight and then fixed with chilled aceto-
386 methanol (1:1). 0.03% Saponin (Calbiochem, Germany) was used for permeabilization followed by
387 blocking with 3% BSA. Rabbit monoclonal antibody against β -catenin and mouse monoclonal antibody
388 against CD24, CD44 (Cell signaling technology) were added at a dilution of 1:200 and incubated
389 overnight. It was then probed with anti-rabbit-FITC and anti-mouse Alexa-Flour 633nm conjugated
390 secondary antibody (molecular probes) and counter stained with DAPI (Invitrogen) for nuclear staining.
391 Images were taken under a confocal microscope (Andor Spinning Disc Confocal Microscope, Andor
392 Technology, Belfast, Ireland) at 60X magnification.

393 **Western blotting**

394 Cell lysates were prepared after 48 hrs of transfection in NP-40 lysis buffer (Invitrogen) and protease
395 inhibitor cocktail (1X) was added for enhanced protein stability. Bradford reagent (Sigma) was used to
396 determine concentrations and equivalent amounts of denatured protein samples were subjected to SDS-
397 PAGE (8%-10%), separated by size and transferred on to PVDF membrane (Millipore, Billerica, USA).
398 Antibodies used for immuno-blotting were polyclonal β -catenin, E-cadherin, CD44 and CD24,
399 Involucrin (Santa Cruz Biotechnology, CA, USA), polyclonal Oct4 and Sox2 (Abcam), polyclonal C-
400 myc, Akt and phospho-Akt (Cell Signaling Technology, USA). Bands were obtained using ECL
401 substrate (Thermo Scientific, USA) from HRP-conjugated secondary antibody (Sigma). Proteasome
402 Inhibitor MG132 (Calbiochem) and Akt inhibitor LY294002 (Cell signaling Technology, USA) were
403 both used at a concentration of 50 μ M. Transfected cells were treated for 4 hours before harvesting.
404 Band intensities of each protein were analyzed by ImageJ to obtain densitometric values for their
405 quantification. These were normalized to β -actin for individual experimental sets and fold change
406 calculated. All the histograms were expressed as means \pm S.D. of three different experiments and p
407 values computed in GraphPad Prism 5 (Student's two tailed t test).

408 ***In vivo* tumor xenograft experiments**

409 Animal experiments were performed following guidelines of the institutional animal ethics committee
410 of National Centre for Cell Science, Pune. All the animals were issued under the project

411 IAEC/2012/B183. To investigate the effect of miR-146a overexpression on Oral squamous cell
412 carcinoma (OSCC) growth *in vivo*, 3×10^6 empty vector- and microRNA overexpression construct-
413 containing SCC084 cells were injected subcutaneously into the dorsal flanks of eight NOD/SCID male
414 mice (18 weeks old) on left and right side respectively. When palpable tumors could be seen the mice
415 were segregated into groups of four each. Mice in one of the groups were injected with 25 mg/kg of
416 body weight of Quercetin (Sigma) on every alternate day for a period of 15 days. The experiment was
417 terminated when the average miR-146a over-expressing SCC084 tumor volumes in the group which
418 received no quercetin reached about 1200 mm^3 . At the termination of the experiment, the animals were
419 sacrificed by CO_2 asphyxiation and the tumors were collected for further analysis. Tumor diameters
420 were measured each time the Quercetin was injected and at the termination of the experiment using
421 digital Vernier Caliper. Excised tumor tissues were weighed and then stored in RNAlater solution
422 (ThermoFisher Scientific) in -20°C freezer. Tumor volumes were determined using the following
423 formula: $\pi/6[(d1 \times d2)^{3/2}]$; where d1 and d2 are two different diameters of a tumor. In another experiment,
424 to investigate effect of simultaneous overexpression of miR-146a and CD24 on OSCC growth *in vivo*,
425 3×10^6 empty vector- and miR-146a and CD24 overexpression constructs-containing OSCC-084 cells
426 were injected subcutaneously into the dorsal flanks of four NOD/SCID male mice (15 weeks old) on
427 left and right side respectively. Tumors volumes were measured when palpable growth could be
428 observed. The experiment was terminated when tumor volumes reached 1300 mm^3 . The animals were
429 euthanized by CO_2 asphyxiation and the tumors were collected. Tumor tissues were processed as
430 described previously for the other experiment.

431 **Reporter assays**

432 Cells seeded in 24 well plates were co-transfected with miR-146a OE plasmid and either CD24 3'UTR
433 or miR-146a promoter luciferase construct using LipofectamineTM 2000 (Invitrogen). The TOP-Flash
434 and FOP-Flash reporters were also used under similar conditions. Promega dual luciferase assay system
435 was performed according to the manufacturer's protocol. After 48 hr of transfection, medium was
436 washed off with 1x PBS and cells were lysed with Passive Lysis Buffer (Promega) and luminescence
437 was measured in Promega Glomax 20/20 luminometer. The luminescence values were transfection

438 normalized with the internal control pRL-TK (50 ng, Renilla Luciferase; Promega). Experiments were
439 performed with three biological replicates.

440 **Chromatin immunoprecipitation**

441 Cells seeded in 10cm dishes were transfected. After 48hrs, 1X formaldehyde solution was added for
442 DNA-protein crosslinking. Cells were lysed in SDS lysis buffer followed by sonication in Bioruptor
443 (Diagenode) to obtain 200-1000 bp chromatin fragments. ChIP dilution buffer was used to dilute the
444 sheared chromatin followed by preclearing with Protein G Agarose beads (Sigma) for 30min. After
445 preclearing, 20% of the lysate was kept aside as the input and the remaining was divided equally for IP
446 and IgG. Immunoprecipitation was carried out using 5 μ g of β -catenin (Santa Cruz) and normal IgG
447 control (Sigma) and incubated overnight. The following day, Protein G Agarose beads were added to
448 collect the Antibody/Antigen/Chromatin complex. The complex was washed briefly with cold low salt
449 immune complex wash followed by high salt immune complex buffer, lithium chloride immune
450 complex buffer and Tris-EDTA buffer. It was then reverse-crosslinked and the DNA purified using
451 Phenol/Chloroform extraction method. PCR amplification of the immunoprecipitated DNA was carried
452 out using primers listed in Supplementary File 1. Composition of the ChIP buffers are provided in
453 Supplementary Information.

454 **Statistical analyses**

455 Three different experiments were subjected to an independent two-tailed Student's *t* test to measure the
456 significance value of results under varying biological parameters. R package was used to generate the
457 correlation graphs and calculate p values. * indicates $P \leq 0.05$ and ** indicates $P \leq 0.01$.

458

459 **Supplementary information**

460 Supplementary Figure and Figure Legends (S1, S2, S3, S4, S5, S6, S7, S8, S9)

461 Supplementary methods

462 **Supplementary File 1:** Analysis of TCGA data for Head and Neck Squamous Cell Carcinoma
463 patients (Excel File)

464 **Supplementary File 2:** Snapshots of miRANDA analysis for miR-146a-5p seed sequence and CD24
465 3'UTR matching showing their free energy of binding (pdf)

466 **Supplementary File 3:** List of Primers for the qRT-PCR (Excel File)

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480 **Author contributions**

481 SG and SR conceived and designed the study. Some experiments were designed by DG and SD.
482 Experiments, data collection and statistical analyses were performed by SG, DG, SD and PD. Some
483 experiments were performed by RB and MG. The manuscript was written and edited by SG, DG, SD,
484 GC and SR. All authors read and approved the final manuscript.

485 **Conflict of interest**

486 The authors declare that they have no conflict of interest.

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656

657 **FIGURE LEGENDS**

658

659 **Fig. 1.** Over-expression of miR-146a in CD44^{high}CD24^{low} cells of Oral Squamous Cell Carcinoma. (a)
660 Total RNA extracted from the stem (CD44^{high}CD24^{low}) and the non-stem (CD44^{low}CD24^{high}) sub-
661 populations of SCC25 cells were reverse-transcribed using stem-loop primers specific for subsequent
662 Real time PCR analysis of various miRNAs using respective forward primers and Universal reverse
663 primer (Additional File 1). (b) Expression of miR-146a was re-analyzed in UPCI: SCC131 and UPCI:
664 SCC084 by qRT-PCR. (c) Quantification of miR-146a transcripts in the spheres enriched from
665 CD44^{high}CD24^{low} cells of SCC131 compared to that grown under differentiating adherent conditions.
666 Data is representative of 3 independent experiments and the bar graph is shown with mean ± SD (right).
667 U6snRNA was used to normalize relative expression values. Students t test used to calculate p-value (*
668 P<0.05, **P<0.01, *** P<0.001). (d) Box-Scatter plot showing the differential expression of miR-146a
669 in the CD44^{high}CD24^{low} and CD44^{low}CD24^{high} subgroup of HNSCC tumors obtained from TCGA. (p-
670 value was calculated in R package to show statistical significance).

671

672 **Fig. 2.** Cancer stem cell characteristics induced by miR-146a in OSCC cell lines. (a) MiR-146a
673 transfected SCC131 cells were analyzed by flow cytometry and mean fluorescence values of CD44
674 (PE) and CD24 (FITC) are shown. (b) Equal number of vector and miR-146a transfected UPCI:
675 SCC131 and UPCI: SCC036 cells were seeded in ultralow-attachment 6-well plate at clonal density.
676 Sphere forming structures was captured at five random fields at 20X magnification using phase contrast
677 microscope (Leica CTR4000) with scale bar equal to 50 μ m. (c) Representative images of western blots
678 showing dose dependent increment of Sox2, C-myc, Oct-4, Involucrin and CD44, CD24 in the UPCI:
679 SCC036, UPCI: SCC131 and UPCI: SCC084 upon ectopic expression of miR-146a. β -actin bands was
680 used to normalize the data. Band intensities of each protein has been quantified from three biological
681 replicates and the average \pm sd is plotted in GraphPad prism 5 showing statistical significance in the
682 respective graphs (below)(* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)(d) Similar western blots upon
683 transfection of miR-146a with mutated seed sequences in UPCI:SCC131 and its graphical
684 representation (below).

685

686 **Fig. 3.** MiR-146a induced β -catenin/Wnt Signaling in CD44^{high}CD24^{low} population. (a) Isolation of
687 CD24^{low} and CD24^{high} cells from SCC084, as shown by qRT-PCR of CD24. Western blot images of β -
688 catenin, Oct-4, E-cadherin and Involucrin in the respective populations along with its quantitative plot
689 indicating the statistical data. (b) Representative confocal immunofluorescence images (60X
690 magnification) of CD44^{high}CD24^{high} and CD44^{high}CD24^{low} subpopulation of SCC131 showing β -catenin
691 (green) and CD44 (red) counterstained with DAPI (blue) (scale bar equal to 10 μ m) (c) Each of the
692 SCC cell lines were subjected to western blot analysis of β -catenin and E-cadherin upon increasing
693 doses of miR-146a. (d) β -catenin and Oct-4 immunoblotting upon increasing doses of anti-miR-146a.
694 Data normalized with β -actin. All the data has been graphically represented beneath the respective
695 figures (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) (e) Association of miR-146a expression with CD24 in the
696 NCI-60 cell lines (n=59) and (f) with β -catenin/CD24 ratio in the CD44^{high} tumors of the TCGA HNSCC
697 patients (n=146). Statistical significance was determined by Pearson correlation test. Pearson
698 correlation coefficient is shown in each plot. (g) Box plots showing miR-146a expression in NCI-60

699 cell lines classified as epithelial (EP) and mesenchymal (MS) subgroups. p value has been calculated
700 using mann-whitney's u-test. **(h)** Correlation (Pearson) of miR-146a with CDH1/VIM expression ratio
701 based on the RNA-Seq data from 292 TCGA HNSCC specimens

702

703 **Fig. 4.** MiR-146a targets CD24 post-transcriptionally. **(a)** Immunoblotting of CD24 in SCC131 cells
704 subjected to miR-146a knockdown showing significant up-regulation upon normalization with β -actin.
705 **(b)** qRT-PCR to detect CD24 transcripts in the anti-miR-146a treated or **(c)** miR-146a over-expressed
706 SCC131 and SCC084 cells. 18srRNA served as an endogenous control and p-values calculated by
707 Student's t-test. Schematic representation of the CD24 3'UTR luciferase constructs. **(d)** Luciferase
708 activity of the CD24 3'UTR reporter gene in SCC084 cells with increasing expression of miR-146a or
709 miR-SDM (mutant miR-146a). **(e)** CD24 3'UTR reporter activity upon miR-146a over-expression and
710 **(f)** miR-146a knockdown in SCC131 cells. Data in **(d)**-**(f)** are mean \pm SD, normalized with pRL-TK
711 vector and statistical significance was measured by paired Student's t test (two-tailed).

712

713 **Fig. 5.** MiR-146a promotes stemness by down-regulating CD24 and leads to AKT mediated
714 stabilization of β -catenin. **(a)** Immunoblot analysis of SCC131 transfected with either a vector control,
715 miR-146a with or without CD24 showing rescued expression of β -catenin and E-cadherin as well as
716 Involucrin Oct-4, Sox2, C-myc. **(b)** Immunoblotting of the same proteins with increasing dose of CD24
717 expression and **(c)** upon knockdown of CD24 in SCC131 cells. β -actin is loaded as an endogenous
718 control. Histograms show fold changes in the densitometric values of band intensity and shown as
719 means \pm S.D. of 3 individual experiments. **(d)** Western blot analysis revealed degradation of β -catenin
720 upon CD24 over-expression revived with MG132 treatment in SCC084, SCC036. **(e)** Phospho-AKT
721 levels upon miR-146a alone or in combination with CD24. Total AKT is also shown. **(f)** Phospho-AKT
722 and Total AKT levels upon siRNA mediated down-regulation of CD24 in SCC036 cells. **(g)** Effect of
723 pAKT inhibitor upon β -catenin levels in SCC084 cells. Histograms showing fold change in the

724 densitometric values of band intensity is represented as avg \pm S.D. of n different experiments (n=3) (*
725 P<0.05, **P<0.01).

726

727 **Fig. 6.** MiR-146a promotes *in vivo* tumor growth which is rescued upon CD24/AKT modulation (a)
728 Bar graphs showing relative weight (mg) of the tumors xenograft tumors generated from control or
729 miR-146a expressing SCC084 treated without or with Quercetin. Data represent mean \pm SEM (n=4).
730 P-values were computed using two-tailed Student's t-test. (b) Line graph showing relative growth rate
731 of tumors in response to Quercetin in SCC084 cells harboring either control vector or stably expressing
732 miR-146a. Once tumors reached a palpable size, one set of mice were injected with Quercetin (10
733 mg/kg) intraperitoneally and after 10 successive treatment, change in tumor volume was measured at
734 regular interval up to 20 days. Data represent mean \pm SEM (n = 4) and *p*-values shown using Student's
735 t test. (c) Bar graphs showing relative weight (mg) of the tumors described in xenograft tumors
736 generated from SCC084 harboring either control vector or stably expressing miR146a and CD24. Data
737 represent mean \pm SEM (n=4). P-values were assessed using 2-tailed Student's t-test (d) Line graph
738 showing relative growth rate of tumors described in (d). Data represent mean \pm SEM (n = 4). For all the
739 experiments, *p*-values were calculated in graph-pad prism5 using two-tailed Student's t-test algorithm.
740

741 **Fig.7.** β -catenin transactivates miR-146a mediating a positive feedback loop. (a) Increase in stemness
742 markers upon β -catenin over-expression as revealed by western blot and the densitometric analysis of
743 its band intensities (right below). Data was normalized with corresponding β -actin. Concomitant miR-
744 146a expression in β -catenin transfected SCC131 as quantified by qPCR. (b) Increase in miR-146a
745 transcripts upon β -catenin over-expression is dose dependently inhibited in the presence of either
746 dnTCF4 or Numb. (c) Chromatin Immunoprecipitation assays in SCC084 cells transfected with either
747 scramble siRNA or CD24 siRNA showing recruitment of β -catenin upon endogenous miR-146a
748 promoter. Schematic of miR-146a promoter locus and reporter constructs namely LucA: wild-type;
749 mLucA: TCF-4 binding site (TBS) mutation; LucB: TBS deletion[21]. (d) Relative luciferase activity

750 of LucA in SCC084 cells under various transfections as indicated. Data represent average of n=3
751 individual experiments (along with technical replicates). (e) Transient ChIP assay with same constructs
752 as shown. The percentage enrichment of amplified product was normalized to input and graphically
753 presented. Data represent mean \pm sd and n=3 different experiments (along with technical replicates* P <
754 0.05, ** P < 0.01). We used Student's t -test calculate p -value.

755

756 **Fig.8.** Model –schematic representation of transient inter-conversions between stem and non-stem oral
757 cancer cells. Cancer stem cell induction may be triggered with over-expression of miR-146a that targets
758 CD24 and leads to β -catenin protein stabilization via AKT activation. Wnt signalling intermediates
759 promote stem-like de-differentiated state in the tumor cells. Accumulation of β -catenin might further
760 drive miR-146a expression and amplify the stemness characteristics. Stochastic intra-cellular signals
761 may induce differentiation, probably by aberrant activation of CD24. This leads to decline in pAKT
762 levels and hence proteasomal degradation of β -catenin with subsequent loss of miR-146a.

763

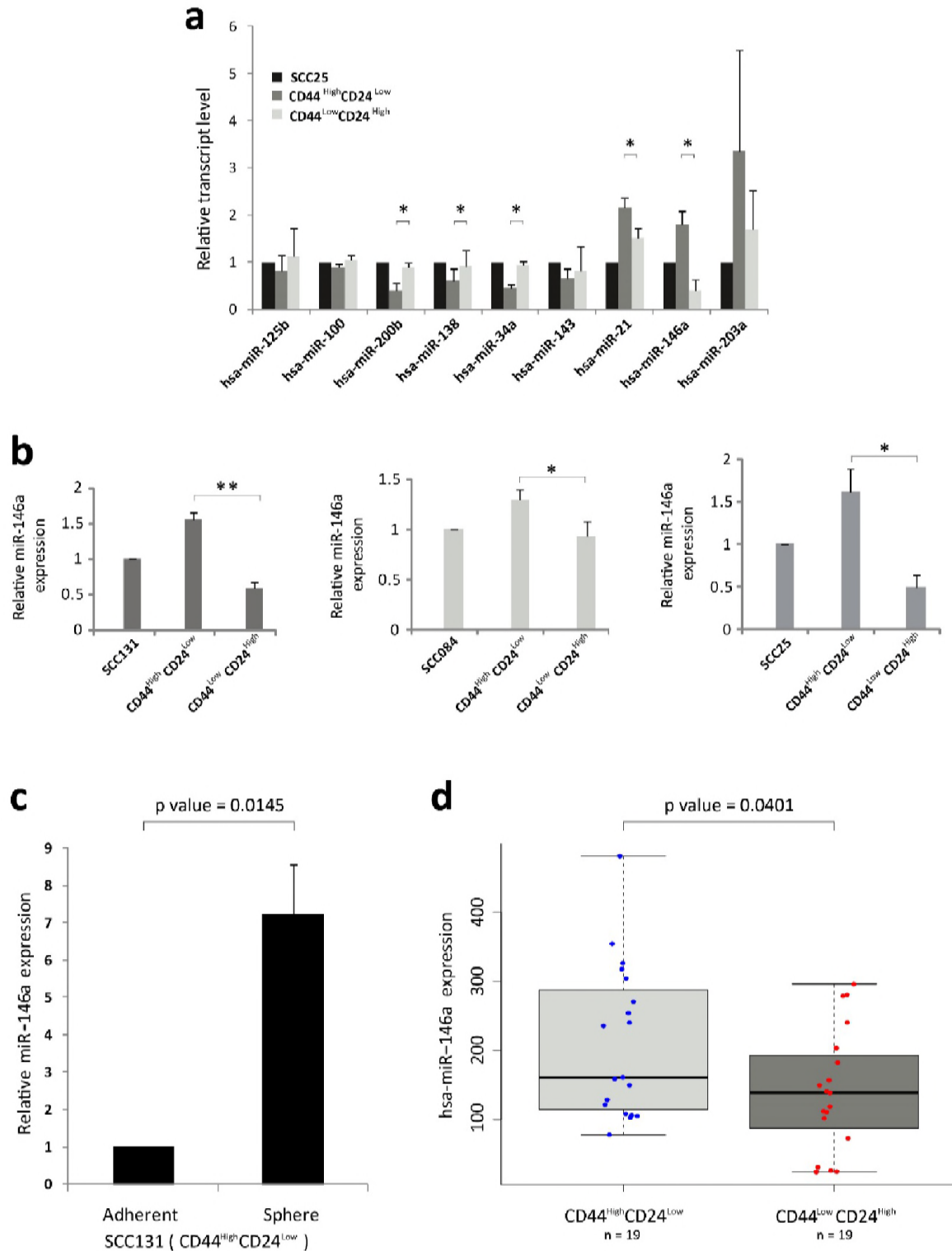
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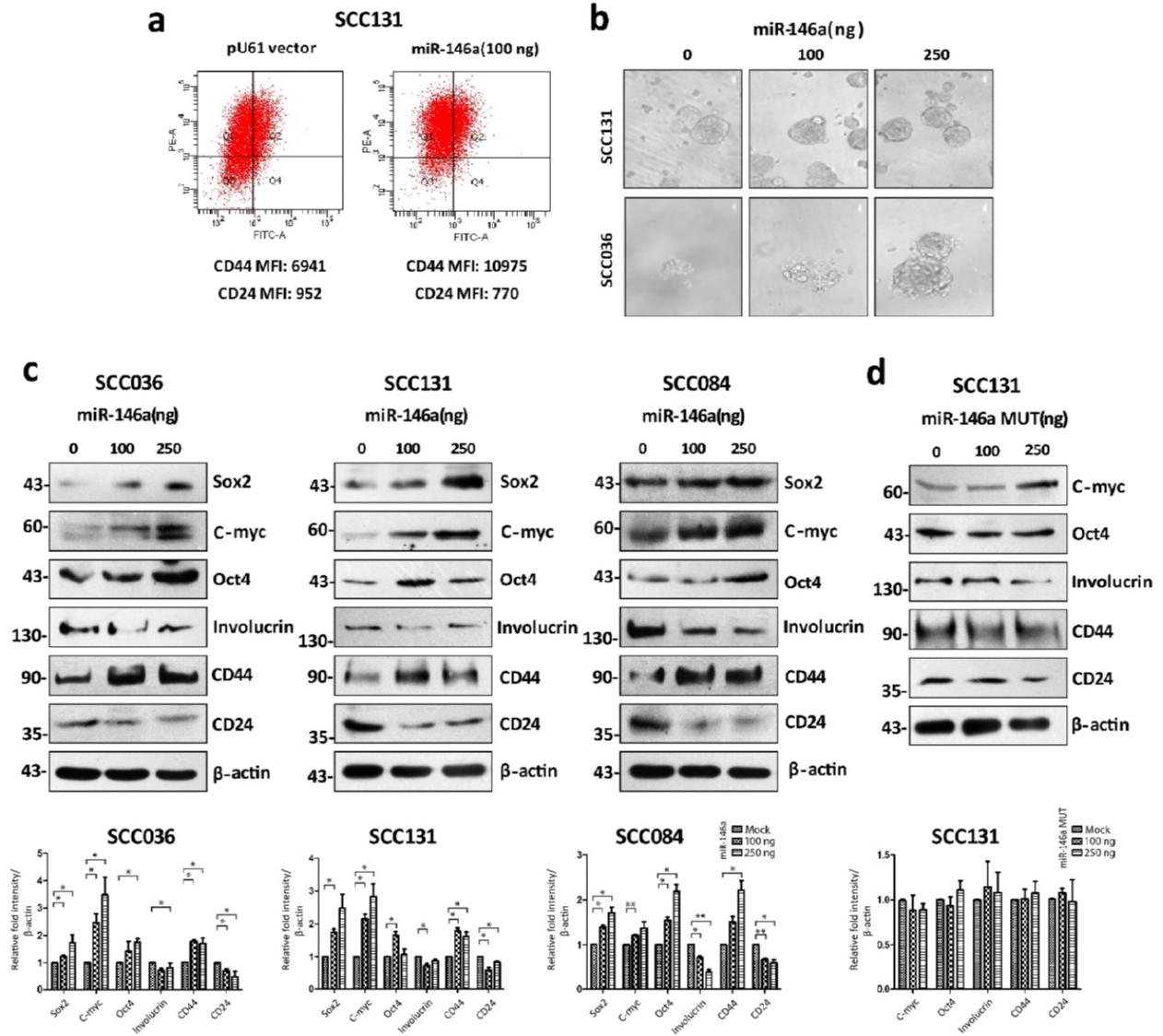
768 **Figure 1**



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771 **Figure 2**



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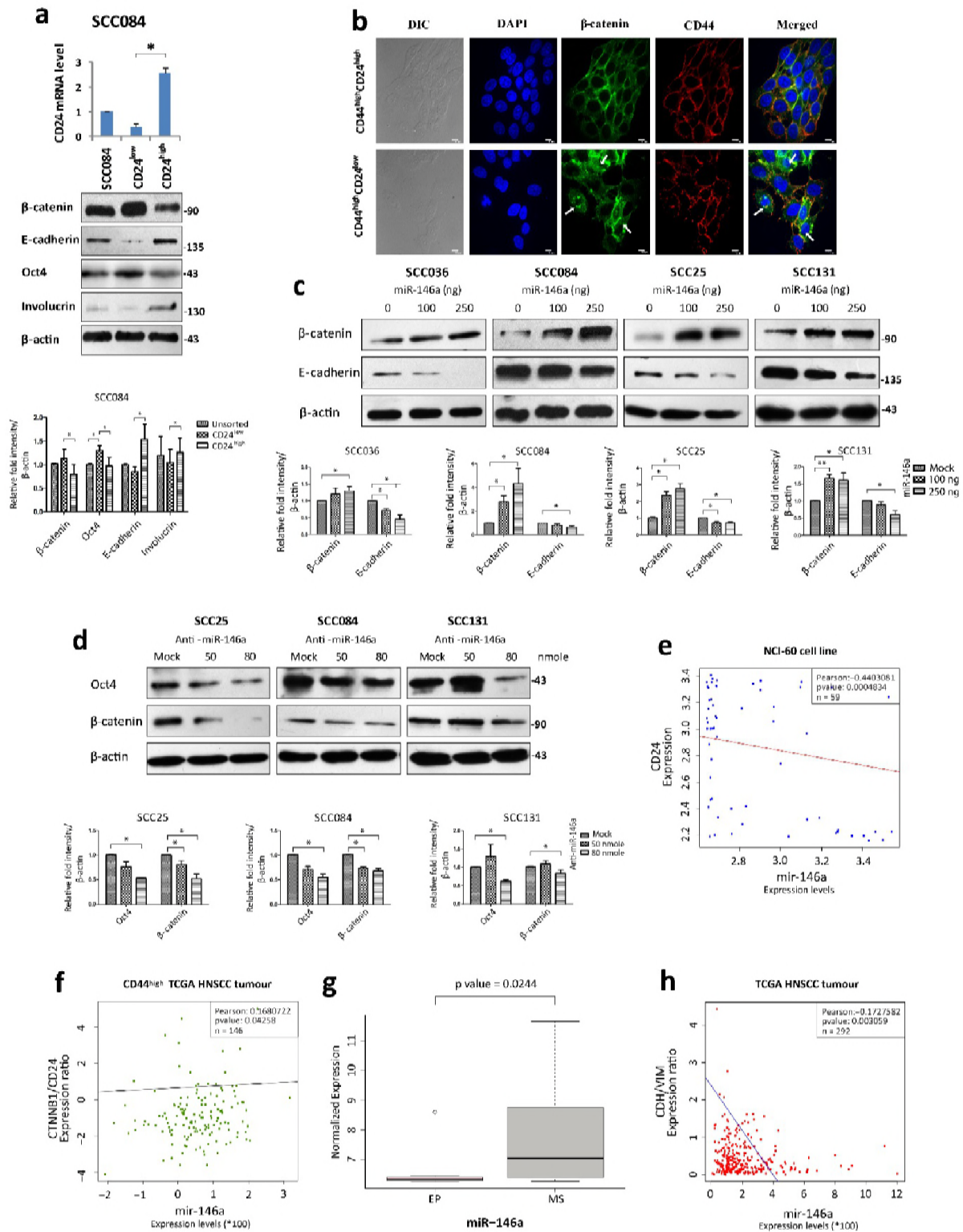
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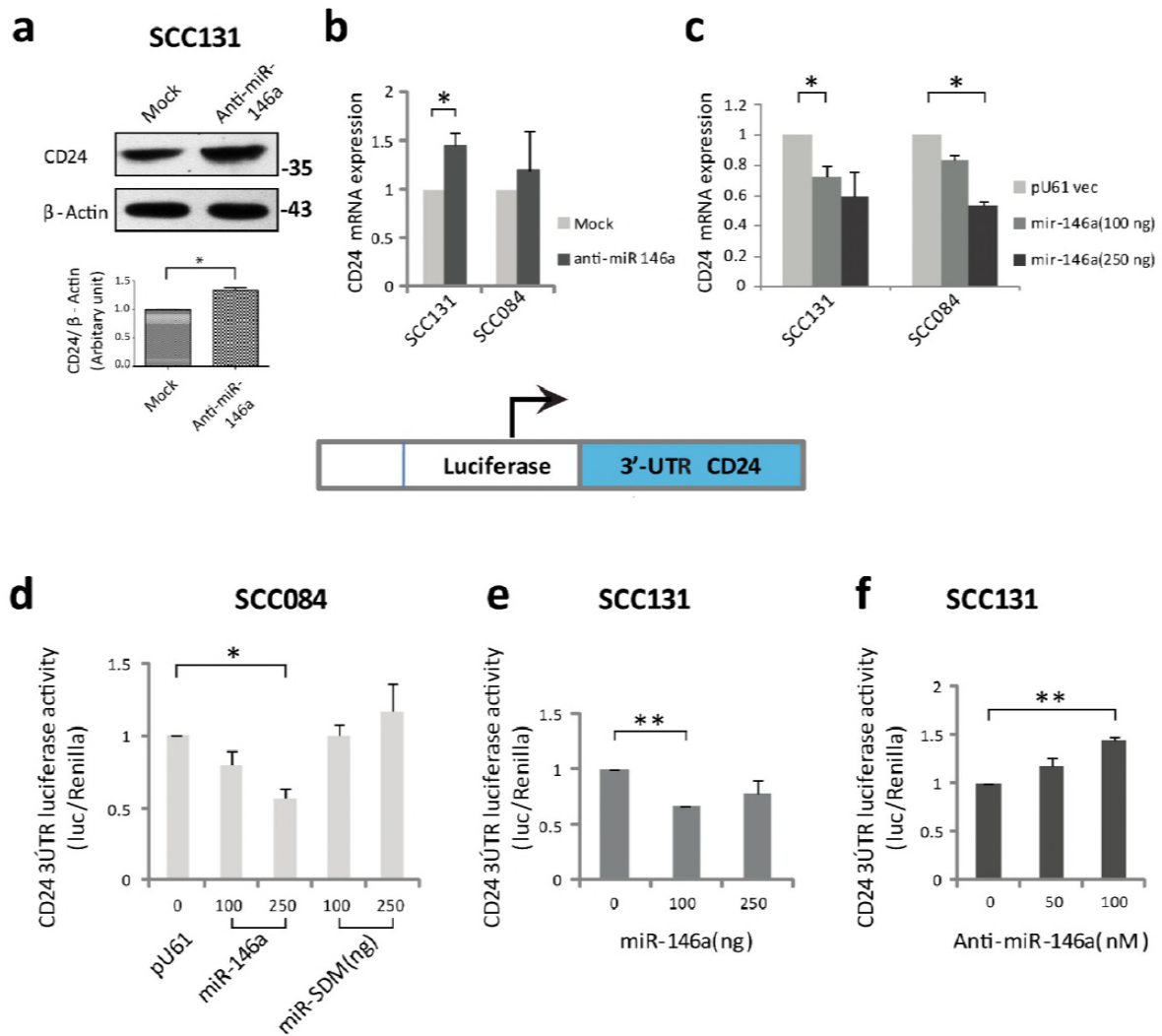
778 **Figure 3**



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781 **Figure 4**



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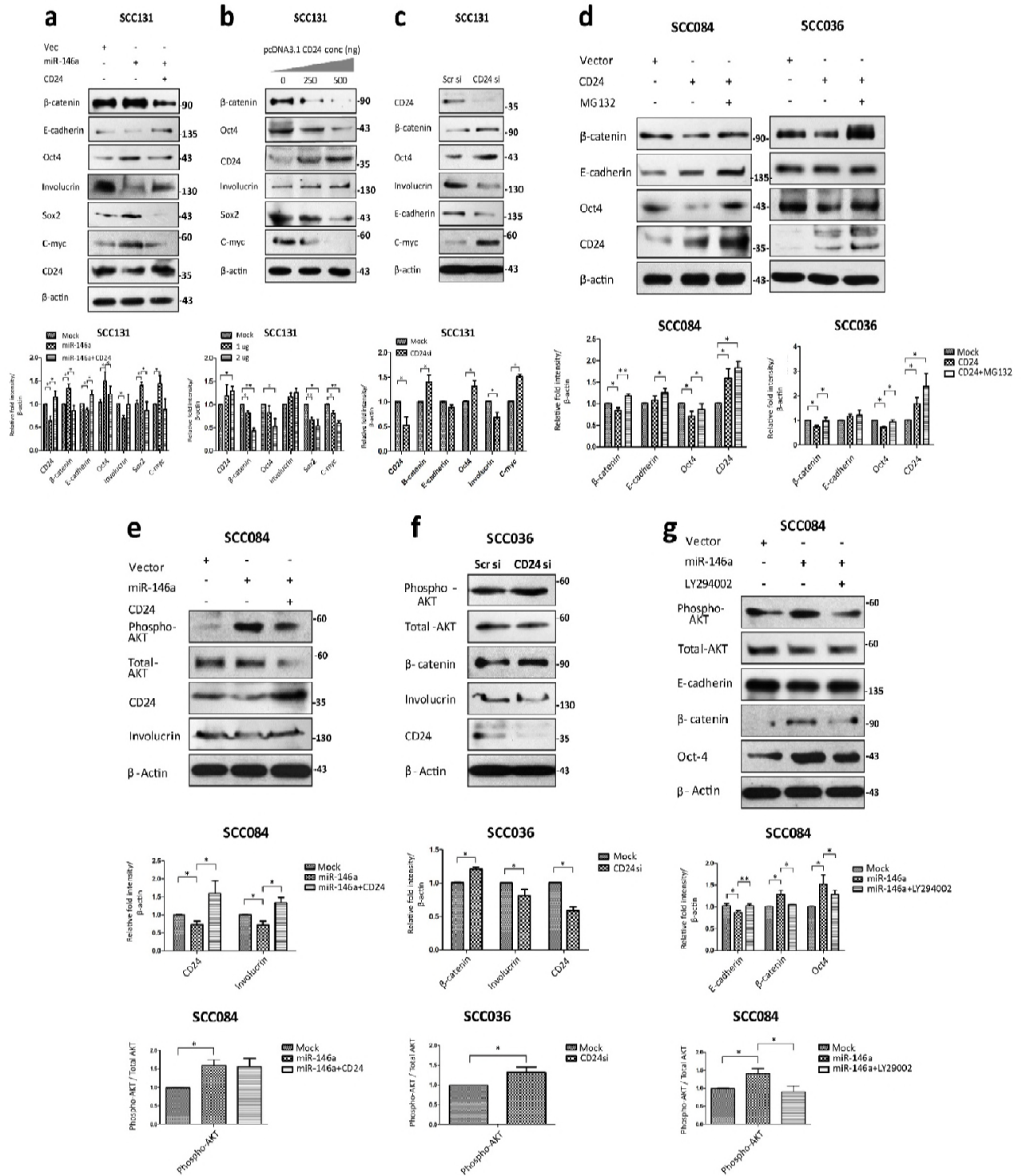
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789 **Figure 5**

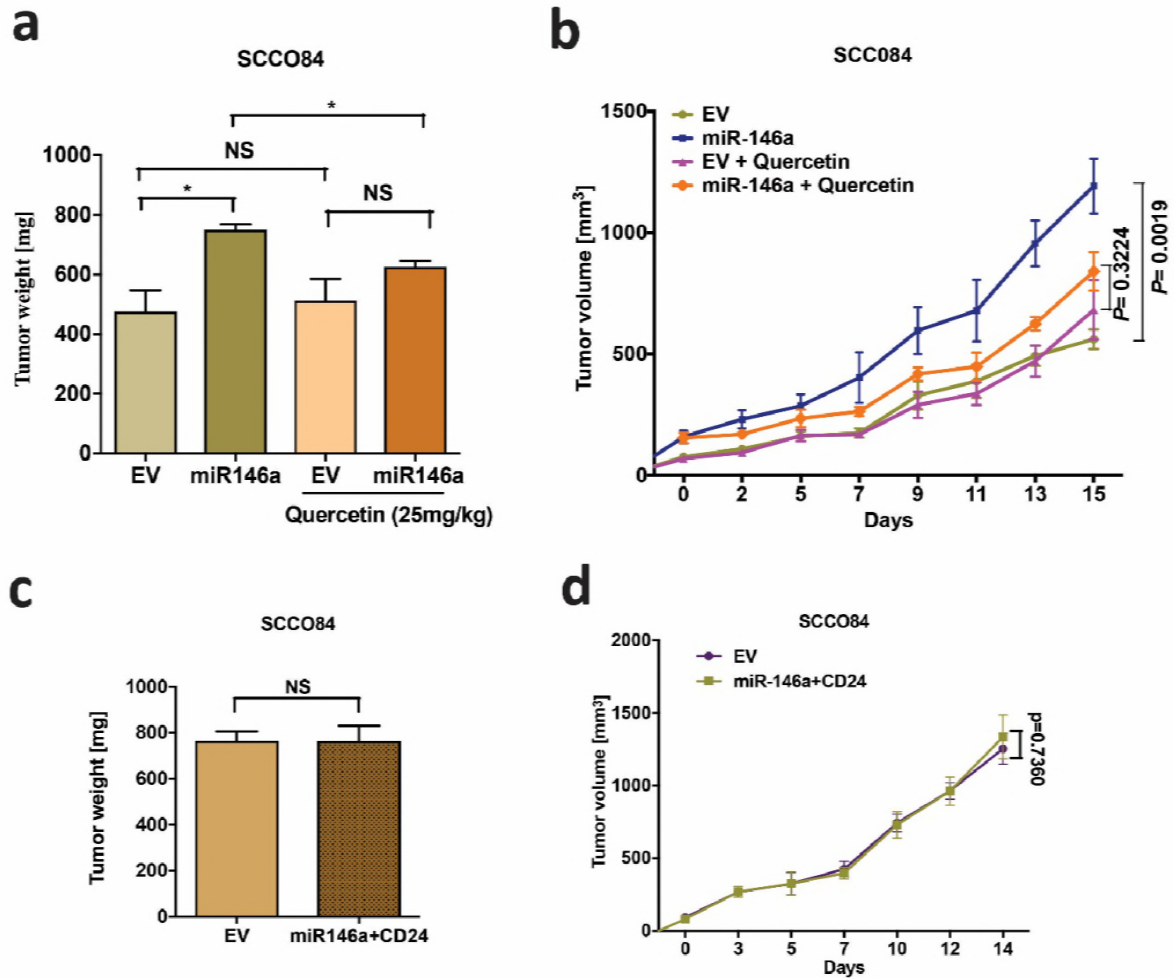


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793 **Figure 6**



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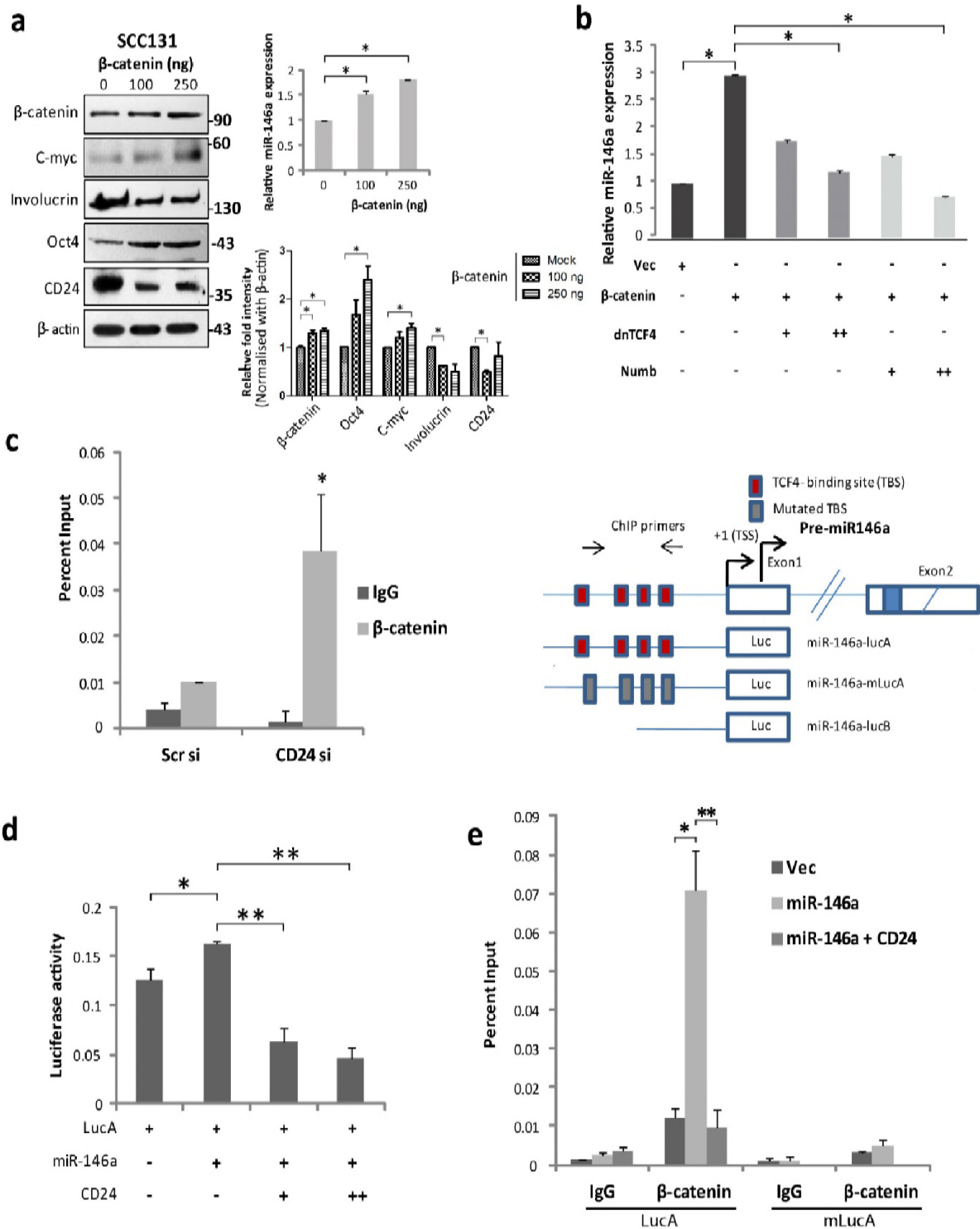
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801 **Figure 7**

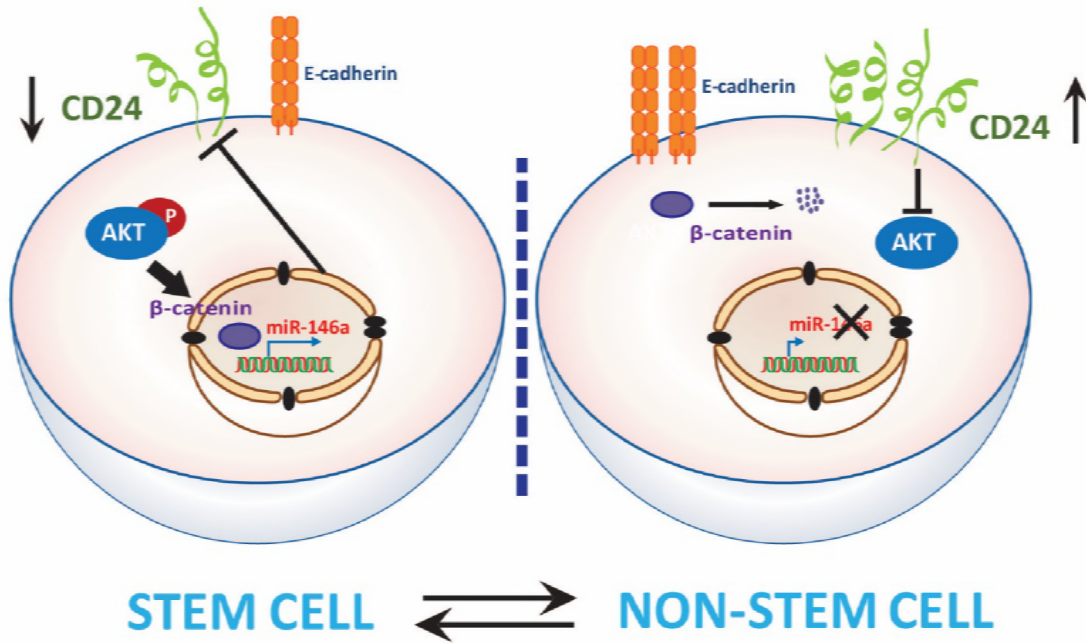


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803

804 **Figure 8**

Proposed Model



Node Postive group

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TCGA-C9-A47Z	39.004298
TCGA-F7-A624	#N/A
TCGA-IQ-A61K	#N/A
TCGA-BA-A6DF	#N/A
TCGA-IQ-A61G	#N/A
TCGA-IQ-A61J	#N/A
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TCGA-BA-A6DA	#N/A
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TCGA-CV-5440	105.071181
TCGA-CV-5436	183.723601
TCGA-CV-5431	423.784951
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TCGA-DQ-7594	165.354955
TCGA-CV-7236	238.921865
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TCGA-CN-6996	258.186615
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Node Negative group

bcr_patient_barcode	hsa-miR-146a
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TCGA-CN-6021	142.610915
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TCGA-CV-7406	103.415984
TCGA-IQ-7631	9.426839
TCGA-CV-6955	298.004749
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TCGA-CV-7090	270.239157
TCGA-CV-6003	114.365252
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TCGA-CV-6441	159.532248
TCGA-BA-A6DE	#N/A
TCGA-F7-8489	220.312975
TCGA-CR-6487	327.350047
TCGA-CV-7440	169.713168
TCGA-CV-7437	240.395329
TCGA-CV-7434	339.201541
TCGA-CV-6935	110.994004
TCGA-CV-6948	88.064455
TCGA-CQ-5331	351.738909
TCGA-CN-4723	310.504995
TCGA-CN-4725	89.807373
TCGA-CN-4727	242.466603
TCGA-CQ-6225	57.80891
TCGA-CQ-6223	166.688126
TCGA-CQ-6218	161.104923
TCGA-D6-6825	247.021917
TCGA-CQ-6224	69.350806
TCGA-BA-5152	631.609232
TCGA-DQ-5625	106.458022
TCGA-D6-6517	220.940955
TCGA-D6-6515	317.356789
TCGA-CN-4733	289.702104
TCGA-CN-4738	254.287346
TCGA-CN-4739	434.852353
TCGA-D6-A4ZB	186.485982
TCGA-CR-7364	116.255161
TCGA-CV-7183	87.069539

TCGA-CV-7177	112.674656
TCGA-CV-7180	182.869117
TCGA-H7-7774	295.608723
TCGA-CQ-7068	181.791608
TCGA-CQ-6221	480.55317
TCGA-CQ-7065	23.8474
TCGA-CV-5973	24.400472
TCGA-CQ-7069	27.764594
TCGA-HD-7831	95.771431
TCGA-CR-7369	143.038792
TCGA-CR-7368	213.989357
TCGA-CN-4734	#N/A
TCGA-CN-4737	79.848209
TCGA-CN-4736	326.181646
TCGA-BB-4228	110.911183
TCGA-CN-4741	415.204395
TCGA-CN-4740	223.561348
TCGA-BB-4224	53.407918
TCGA-BA-5151	188.891666
TCGA-HD-7917	101.231353
TCGA-CQ-7067	86.701001
TCGA-BB-7863	83.382842
TCGA-P3-A5QA	#N/A
TCGA-P3-A5Q6	#N/A

ChIP Primers

Endogeneous miR-146a promoter

146A_P2_F: CCCAAGTAGCTGGGACTACA
146A_P2_R: TAGGCGAGATGTGGTGACTC

Luciferase miR-146a promoter

TBS-chip_F CTTGAATGTTCACATTTCCAGAG
TBS-chip_R GGCAGAAAGCTCCCTTGTTTC