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Physiological β-catenin signaling controls self-renewal networks and generation of stem-like cells from nasopharyngeal carcinoma

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Abstract

Background: A few reports suggested that low levels of Wnt signaling might drive cell reprogramming, but these studies could not establish a clear relationship between Wnt signaling and self-renewal networks. There are ongoing debates as to whether and how the Wnt/β-catenin signaling is involved in the control of pluripotency gene networks. Additionally, whether physiological β-catenin signaling generates stem-like cells through interactions with other pathways is as yet unclear. The nasopharyngeal carcinoma HONE1 cells have low expression of β-catenin and wild-type expression of *p53*, which provided a possibility to study regulatory mechanism of stemness networks induced by physiological levels of Wnt signaling in these cells.

Results: Introduction of increased β-catenin signaling, haploid expression of β-catenin under control by its natural regulators in transferred chromosome 3, resulted in activation of Wnt/β-catenin networks and dedifferentiation in HONE1 hybrid cell lines, but not in esophageal carcinoma SLMT1 hybrid cells that had high levels of endogenous β-catenin expression. HONE1 hybrid cells displayed stem cell-like properties, including enhancement of CD24+ and CD44+ populations and generation of spheres that were not observed in parental HONE1 cells. Signaling cascades were detected in HONE1 hybrid cells, including activation of *p53*- and *RB1*-mediated tumor suppressor pathways, up-regulation of *Nanog*-, *Oct4*-, *Sox2*-, and *Klf4*-mediated pluripotency networks, and altered E-cadherin expression in both *in vitro* and *in vivo* assays. qPCR array analyses further revealed interactions of physiological Wnt/β-catenin signaling with other pathways such as epithelial-mesenchymal transition, TGF-β, Activin, BMPR, FGFR2, and LIFR- and IL6ST-mediated cell self-renewal networks. Using β-catenin shRNA inhibitory assays, a dominant role for β-catenin in these cellular network activities was observed. The expression of cell surface markers such as CD9, CD24, CD44, CD90, and CD133 in generated spheres was progressively up-regulated compared to HONE1 hybrid cells. Thirty-four up-regulated components of the Wnt pathway were identified in these spheres.

Conclusions: Wnt/β-catenin signaling regulates self-renewal networks and plays a central role in the control of pluripotency genes, tumor suppressive pathways and expression of cancer stem cell markers. This current study provides a novel platform to investigate the interaction of physiological Wnt/β-catenin signaling with stemness transition networks.

Keywords: Physiological Wnt/β-catenin signaling, Nasopharyngeal carcinoma, Self-renewal network, Chromosome 3 transfer, Stemness transition, Tumor suppressor genes, Cancer stem cell markers

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Background

Multiple groups have shown that different dosage levels of Wnt signaling contribute to distinct cellular activities such as reprogramming, differentiation, tumorigenesis, and epithelial-mesenchymal transition (EMT) events [1-4]. Inappropriate activation of components of this signaling pathway has been observed in some human cancers and differentiating stem cells, in which high levels of Wnt signaling were often detected [1,4-8]. Therefore, Wnt signaling has multiple functions in cell fate determination and is involved in generation of cancer stem cells (CSCs). However, there are unresolved issues for the role of Wnt/β-catenin signaling in the regulation of either self-renewal or differentiation networks in human cells [4,5,7,9,10].

Nasopharyngeal carcinoma (NPC) is a unique cancer, which is particularly prevalent among the southern Chinese, but rare in most other areas around the world [11,12]. Unlike other common tumors, how Wnt signaling influences NPC and crosstalks to other networks to affect cell differentiation and growth, including regulation of CSC markers and EMT events, is unknown. NPC was reported to have an infrequent mutation of tumor suppressor gene (TSG) *p53* and wild-type *RB1* expression [11-14]; they both play critical roles in the control of the reprogramming process, self-renewal, and other cell fate determinations [15-17]. Wnt signaling interacts with p53 signaling [18-20] and usually acts in a dosage-dependent and tissue-specific manner for many cellular processes [1,21-26]. Therefore, it is possible to reveal novel findings by exploring the regulatory mechanism of Wnt signaling in wild-type *p53* expressing tumors such as with NPC HONE1 cells.

We previously established several microcell hybrid cell (MCH) lines derived from HONE1 cells containing a transferred copy of chromosome 3 [11]. Because a physiological or basic level of Wnt signaling acts as a determinant factor in the regulation of stem cells and self-renewing tissues [3,25,27,28] and HONE1 cells have very low endogenous expression of β-catenin, a major mediator of Wnt signaling, we hypothesized that introduction of another copy of the β-catenin gene (*CTNNB1*) via single copy transfer of chromosome 3 may generate physiological levels of β-catenin signaling due to the haploid level of expression of the transferred genes under control by their natural regulators. This approach differs from gene transfection studies that often cause artificial overexpression of the transferred genes [25]. In many cancer cells, stem cell-related genes are often expressed. Exogenous β-catenin signaling may regulate these endogenous signaling networks, thus changing the direction of cellular differentiation. Additionally, *p53*, *RB1*, or other possible TSGs, often serve as negative barriers for the reprogramming and self-renewal processes [15,16].

Delicate control of relevant signaling activities may drive cells into a more de-differentiated status, revealing signaling regulatory mechanisms during the stemness transition process, a series of regulatory relationships that are not fully understood in human cells.
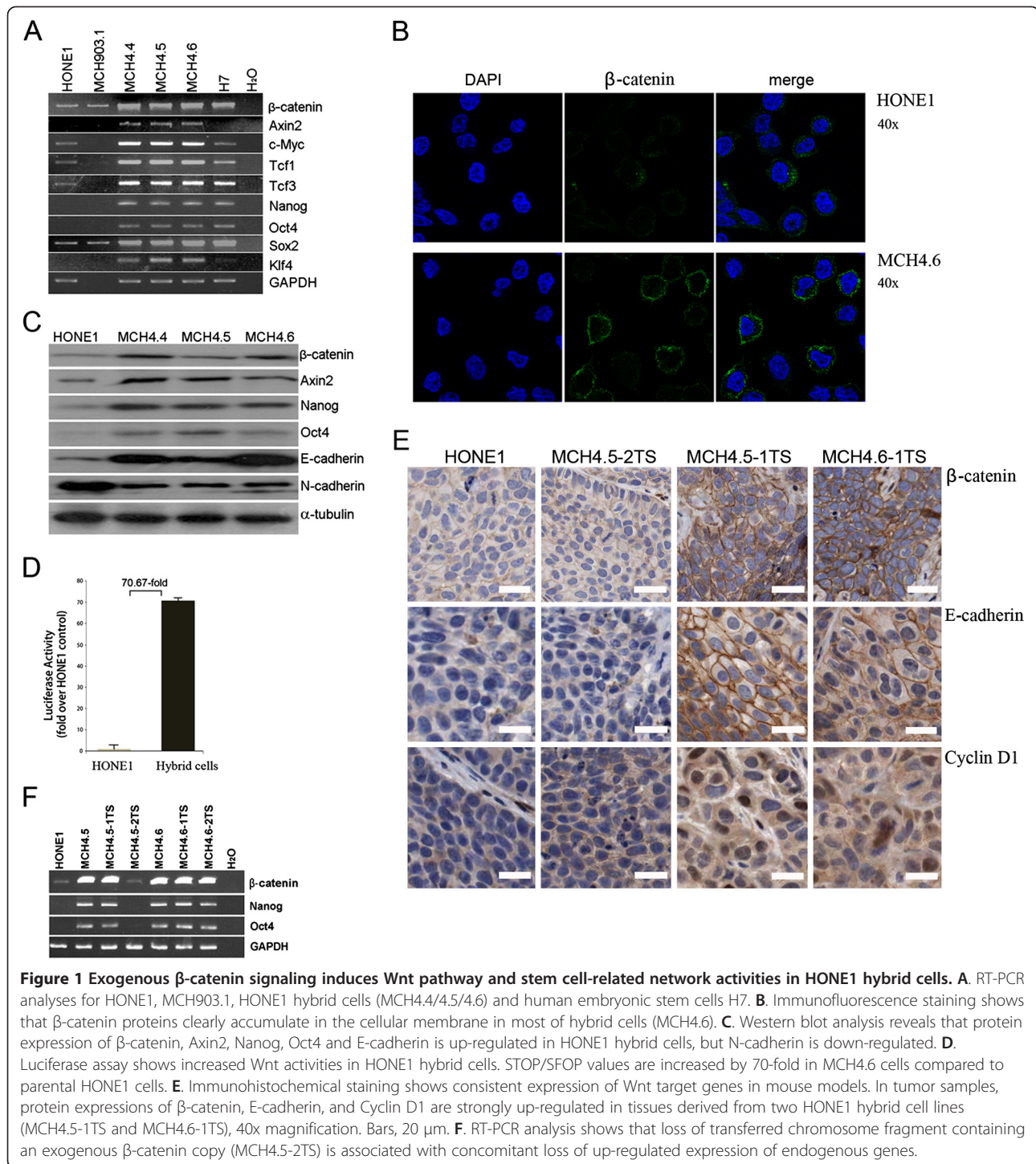
It is important to determine what critical role β-catenin plays in the transferred chromosome by examining the relevant network activities in recipient cells. It is well-accepted now that Wnt/β-catenin signaling interacts with many other signaling networks such as pluripotency, cadherins, EMT, transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), and TSG signaling [1,8,15,16,26,29,30]. If Wnt/β-catenin signaling is activated, these relevant network activities are expected to be detected in treated cells. For example, altered expression of E-cadherin and EMT markers should be found in these cells. Therefore, whether Wnt signaling, initiated at a basic and physiological level, is able to induce other signaling pathways during the progress of stemness transition, or to generate stem-like cells from human cancer cells, such as NPC, is the focus of this study.

Results

Monochromosome 3 transfer confers physiological increases of β-catenin that up-regulates expression of core stem cell genes

We previously established several HONE1 hybrid cell lines that were confirmed to contain an exogenous copy of the intact chromosome 3, following fusion of parental HONE1 and mouse MCH903.1 donor cells [11]. Figure 1A shows that both HONE1 and MCH903.1 cells have similar and low expression levels of the human β-catenin, consistent with their having physiological levels of β-catenin signaling. Human embryonic stem cells, H7 [31], were used as a positive control for mRNA expression of stem cell genes and β-catenin. The up-regulation of β-catenin expression was clearly detected in all three HONE1 hybrid cell lines, as compared to HONE1, and is similar to that detected in H7 cells. Both *c-Myc* and *Axin2* are major targets of the Wnt pathway and *Tcf1* and *Tcf3* are terminal components of the β-catenin signaling pathway in the nucleus. The expression of *Axin2* was detected in HONE1 hybrid cells, but not in H7 cells and parental HONE1 cells. The expression of *Tcf1*, *Tcf3*, and *c-Myc* were obviously up-regulated in these HONE1 hybrid cells, compared with parental HONE1 cells (Figure 1A).

Significant expression of pluripotency genes, *Nanog*, *Oct4*, and *Klf4*, at the RNA level was detected in HONE1 hybrid cells compared to parental HONE1 cells. Both HONE1 and mouse MCH903.1 cells show endogenous expression of human *Sox2* gene, but in HONE1 hybrid cells this gene was up-regulated and had similar expression levels as seen in H7 cells (Figure 1A).

Figure 1 Exogenous β-catenin signaling induces Wnt pathway and stem cell-related network activities in HONE1 hybrid cells. A. RT-PCR analyses for HONE1, MCH903.1, HONE1 hybrid cells (MCH4.4/4.5/4.6) and human embryonic stem cells H7. **B**. Immunofluorescence staining shows that β-catenin proteins clearly accumulate in the cellular membrane in most of hybrid cells (MCH4.6). **C**. Western blot analysis reveals that protein expression of β-catenin, Axin2, Nanog, Oct4 and E-cadherin is up-regulated in HONE1 hybrid cells, but N-cadherin is down-regulated. **D**. Luciferase assay shows increased Wnt activities in HONE1 hybrid cells. STOP/SFOP values are increased by 70-fold in MCH4.6 cells compared to parental HONE1 cells. **E**. Immunohistochemical staining shows consistent expression of Wnt target genes in mouse models. In tumor samples, protein expressions of β-catenin, E-cadherin, and Cyclin D1 are strongly up-regulated in tissues derived from two HONE1 hybrid cell lines (MCH4.5-1TS and MCH4.6-1TS), 40x magnification. Bars, 20 μm. **F**. RT-PCR analysis shows that loss of transferred chromosome fragment containing an exogenous β-catenin copy (MCH4.5-2TS) is associated with concomitant loss of up-regulated expression of endogenous genes.

The increased level of β-catenin protein accumulating around membranes was clearly detected in the majority of hybrid cells compared to parental HONE1 cells by immunofluorescence staining (Figure 1B). As expected, up-regulated protein levels of β-catenin, Axin2, Nanog, and Oct4 were detected by Western blot analyses (Figure 1C). Compared with parental HONE1 cells,

E-cadherin was up-regulated and N-cadherin was down-regulated in the HONE1 hybrid cells.

Enhanced Wnt/TCF/LEF network activity is maintained *in vivo*

A luciferase assay was performed using a Wnt/TCF reporter plasmid that carries a large array of Wnt response

elements and is, therefore, a sensitive reporter of Wnt signals. As shown in Figure 1D, transient transfection of the superTOPGAL reporter detected an increased level of Wnt signaling by approximately 70-fold in HONE1 hybrid cells.

Immunohistochemical staining of the xenograft tumors demonstrated that β-catenin, E-cadherin, and Cyclin D1, the target of Wnt signaling, were strongly up-regulated in tumor segregants (TSs) derived from HONE1 hybrids, as compared with control tumors from the parental HONE1 and MCH4.5-2TS cells. Both β-catenin and E-cadherin proteins clearly accumulated at the membrane (Figure 1E), consistent with the pattern shown in Figure 1B cultured cells.

It has long been known that some regions of transferred chromosomes in TSs are selectively lost during tumor growth and the expression of relevant exogenous genes reverts back to levels similar to parental HONE1 cells [11,12,32-34]. Xenograft tumors derived from HONE1 hybrid cells were, therefore, analyzed for β-catenin expression (Figure 1F). For three of the HONE1 hybrid tumor sets (MCH4.5-1TS/4.6-1TS/4.6-2TS), increased β-catenin levels were evident as well as increased levels of endogenous stem cell genes Nanog and Oct4, compared to corresponding hybrid cells. A fourth hybrid cell line tumor, MCH4.5-2TS (see Figure 1E), did not show up-regulated expression of exogenous β-catenin and also did not have increased expression of Nanog and Oct4.

Physiological expression of exogenous β-catenin is essential for the up-regulation of the endogenous core stem cell network

To further confirm whether expression of exogenous β-catenin is a major determinant in the regulation of the core stem cell network in HONE1 hybrid cells, we performed inhibitory assays using short hairpin RNAs (shRNAs) that silence β-catenin expression in infected cells. A scrambled shRNA was used as a negative control in all experiments. HONE1 hybrid cells with infected scramble shRNA have up-regulated expression of β-catenin compared to parental HONE1 cell (Top panel, Figure 2A). HONE1 hybrid cells with infected β-catenin shRNA have reduced expression of β-catenin and representative results from various passages of cell populations after infection of shRNA plasmids are shown in the bottom panel, Figure 2A. β-catenin expression was reduced by approximately 54%, 97%, 70%, and 79% in MCH4.5 and MCH4.6 cell lines at different passage levels after infection. In these β-catenin shRNA-infected cells, expression of Nanog, Oct4, Sox2, and Klf4 was inhibited as observed by qPCR analyses (Figure 2B).

Figure 2C shows that expression of β-catenin, E-cadherin, Sox2, Zeb1, and Fn1 proteins were clearly

decreased in both β-catenin shRNA-treated cell lines. The inhibited expression of Oct4 was seen in MCH4.6 (4), and inhibition of Klf4 was detected in MCH4.5(5), but no obvious expression change was detected for N-cadherin and Nanog. These results suggest that β-catenin signaling in HONE1 hybrid cells is involved in both stemness and EMT networks.

HONE1 hybrid cells are poorly differentiated and exhibit stem cell-like properties

Parental HONE1 cells grow as monolayers that do not form spheres under ordinary culture conditions, evidenced by our long-term culture of these cells [11]. In contrast, after 45 to 90 days in standard culture, 158 spheres from independent experiments were observed in the HONE1 hybrid cultures (Figure 3A). The size of all spheres was larger than 20 μM in diameters, which was not detected in parental HONE1 and HONE1 hybrid cell lines with infected β-catenin shRNA. These results suggest that sphere-forming cells had lost contact inhibition and had undergone a dedifferentiation process.

Since CSC markers, CD24 and CD44, are commonly expressed in many stem-like cells, we investigated expression changes of these two markers by FACS analysis. CD24+, CD44+, and CD24+/CD44+ populations were markedly increased from 23% to 53.2%, 35.7% to 77.9%, and 42.7% to 77.2%, respectively, in HONE1 hybrid cells compared to parental HONE1 cells (Figure 3B).

Physiological Wnt levels trigger multiple signaling activities during the regulation of stem cell gene networks

The qPCR array results confirmed that pluripotency genes, Sox2, Klf4, Oct4, and Nanog, are clearly up-regulated in HONE1 hybrid cells, compared with parental HONE1 cells. In addition, many other relevant genes such as HOXA9, RB1, ZIC1, WRN, TDGF1, LIN28B, and WT1 are considerably up-regulated in these HONE1 hybrid cells (Figure 4A), supporting the stem cell-like properties of these cells and activation of known genes related to self-renewal networks.

The exogenous copy of β-catenin in HONE1 hybrid cells induced signaling cascades involved in multiple pathways such as Wnt, TGF-β superfamily containing BMP and Activin receptors, pluripotency maintenance, and FGF signaling (Figure 4B). For Wnt signaling, enhanced expression of the non-canonical NFAT family and Wnt receptors, FZD3, FZD5, and FZD6, was detected in these cells. However, the Stem Cell Signaling Array did not detect significant changes of signals from Hedgehog and Notch pathways (Additional file 1: Table S1).
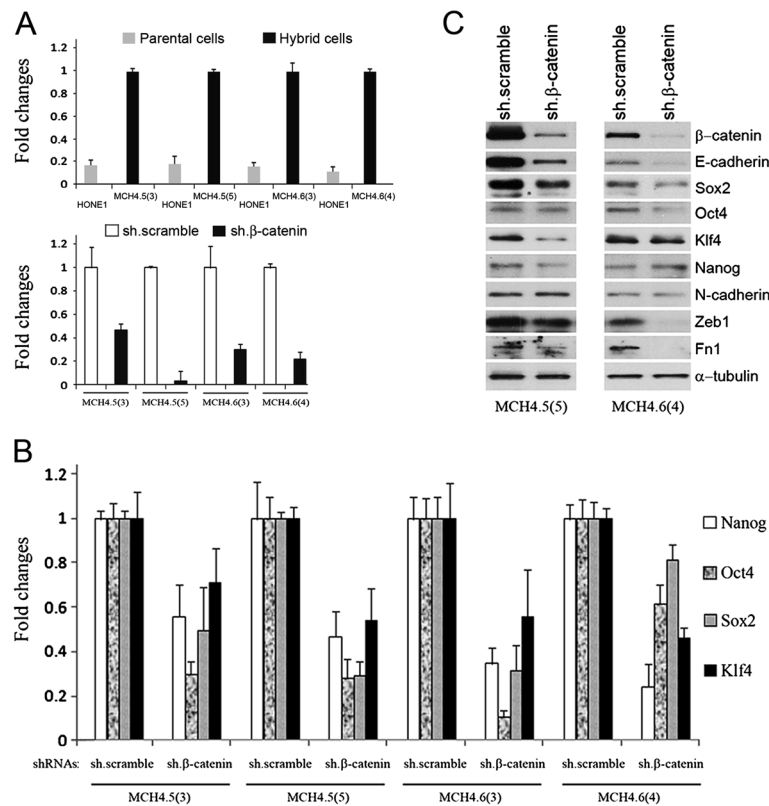
Figure 2 shRNA knockdown of β-catenin is associated with the regulation of Wnt pathway, EMT, and pluripotency networks. A. The top panel shows that HONE1 hybrid cells have an up-regulated expression of β-catenin by qPCR analyses, compared to the parental HONE1 cells. MCHs 4.5 and 4.6 are infected by scramble (empty bars) and β-catenin shRNAs (black bars). The number in parenthesis refers to the growth passages of cells after infection. Fold-changes reflect inhibitory expression of β-catenin in these cells compared to corresponding scramble negative controls. β-catenin expression is reduced in all four cell lines, as shown in the bottom panel. **B**. qPCR analyses show inhibited expression of *Nanog, Oct4, Sox2,* and *Klf4* in β-catenin shRNA infected cells. **C**. Western blot assay confirms that β-catenin shRNAs inhibit expression of β-catenin, E-cadherin, core stem cell factors (Oct4, Klf4, and Sox2), and EMT factors (Zeb1 and Fn1) in the treated HONE1 hybrid cells.

BIO activation of Wnt signaling generates similar signaling activities as induced by chromosome 3 transfer in HONE1 cells

The GSK-3-specific inhibitor, Bromoindirubin-3'-oxime (BIO), activates Wnt signaling in stem cells and somatic cells [5,10,27,35] and this small molecule was, therefore, used to treat parental HONE1 cells to determine whether it had an effect on core stem gene signaling networks, similar to that seen in the HONE1 hybrid cells. As shown in Figure 4C, 5 μM BIO treatment induced the highest mRNA expression of β-catenin and *Axin2*. BIO treatment in HONE1 cells up-regulated mRNA expression of endogenous stem cell genes, *Nanog* and *Oct4*, and EMT markers and regulators such as *Twist, Snail, Cyclin D1, Slug, Fn1,* and *E-cadherin*. BIO treatment also caused an up-regulation of N-cadherin in HONE1 cells, suggesting that BIO treatment does not fully mimic chromosome 3 transfer effects of inducing physiological signaling in HONE1 cells. Bio-induced β-catenin accumulation was mainly detected at the membrane in treated cells (Figure 4D).

To verify the critical role that physiological signaling levels exert, BIO treatment was used to generate high, non-physiological levels of β-catenin protein expression in cells. Western blot analyses indicated that β-catenin, Axin2, and E-cadherin were strongly up-regulated in BIO-treated HONE1 cells. These results were also confirmed in MCH4.5-2TS cells that lost a transferred copy of *β-catenin* gene. Importantly, endogenous protein levels of Nanog and Oct4 were not up-regulated in β-catenin over-expressing HONE1 and MCH4.5-2TS cells (Figure 5A).

Chromosome 3 transfer does not affect cells with high levels of endogenous β-catenin expression

To determine whether physiological β-catenin signaling might regulate other human cells, esophageal carcinoma SLMT1 cells were examined [36]. The endogenous levels of β-catenin expression in SLMT1 were higher than that of HONE1 cells, likely due to an alternative transcript of β-catenin gene in exons 3/ 5 in SLMT1 cell observed by RT-PCR and sequencing analyses (data not shown). The expressions of both β-
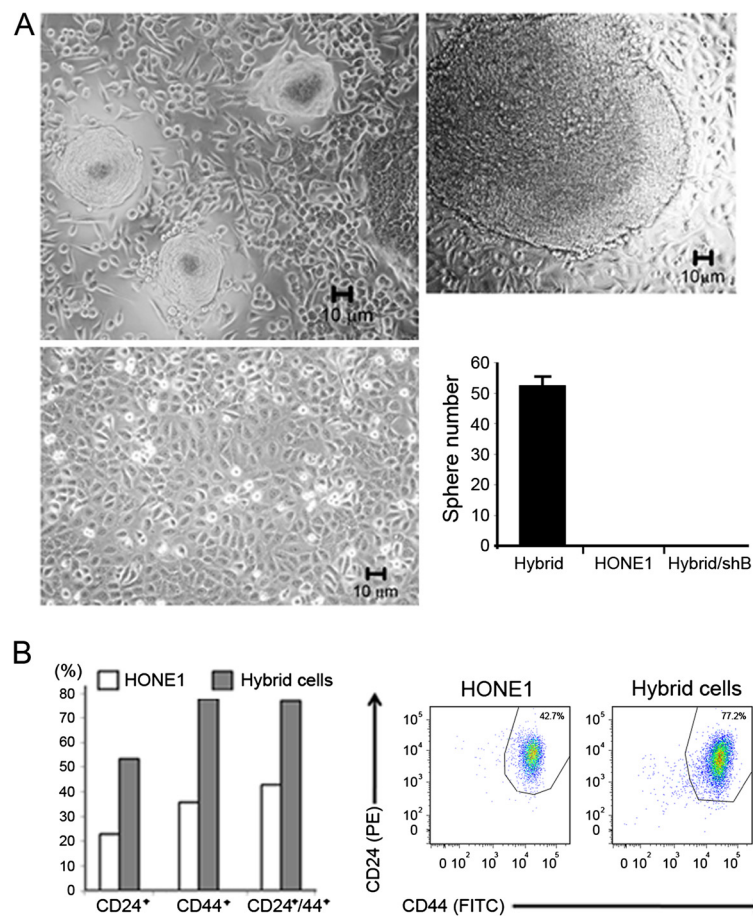
Figure 3 HONE1 hybrid cells have properties of stem-like cells. A. Phase-contrast images of spheres that could be grown on the surface of monolayer epithelial cells or bottom of flask (upper right and upper left). The bottom picture shows image of parental HONE1 cell growth. The bar graph indicates that parental HONE1 cells and HONE1 hybrid cells with infected β-catenin shRNA (Hybrid/shB) do not form spheres in the same culture conditions. **B**. FACS analyses (bar chart, Left) show that HONE1 hybrid cells (solid bars) contain more CD24+, CD44+, CD24+/CD44+ populations than those of HONE1 cells (empty bars). The figure (right) shows that hybrid cells have increased numbers of double positive CD24+/CD44+ populations.

catenin and c-Myc mRNAs remain unchanged in SLMT1 hybrid cells, MCH3.11/3.22 [36], with an introduced copy of chromosome 3, compared with their parental SLMT1 cells (Figure 5B). Nanog and Oct4 protein expression was also unaltered in SLMT1-derived hybrid cells (Figure 5C).

Tumor suppressor gene-mediated pathways are activated in HONE1 hybrid cells

Because the tumor suppressor *p53* circuit communicates with the Wnt pathway and serves as a major target in stem cells [15,18], the expression of *p53* in HONE1 hybrid cells was examined at both the RNA and protein levels. Interestingly, *p53* was up-regulated and triggered by Wnt signaling in all these hybrid cells by both RT-PCR (Figure 6A) and Western blot analyses (Figure 6B). As described previously, other tumor suppressors, such as *RB1* and *WT1* (Figure 4A),

were also activated in these cells, consistent with multiple tumor suppression pathways being activated following introduction of physiological Wnt/β-catenin signaling in HONE1 cells.

Since tumor suppression pathways were activated in HONE1 hybrid cells due to the up-regulation of Wnt/β-catenin signaling, it may affect growth ability of cells. To test these possibilities, MTT and colony formation assays in matrigel were performed with both stable scramble and β-catenin shRNA-infected HONE1 hybrid cells. The results indicated that tumor cell growth was inhibited by the knockdown of Wnt/β-catenin signaling in these cells (Figures 6B and 6C), reflecting that activation of tumor suppressive signaling has limited influence to control cell growth in HONE1 hybrid cells. However, Wnt/β-catenin signaling may stimulate cell growth during stemness transition process.
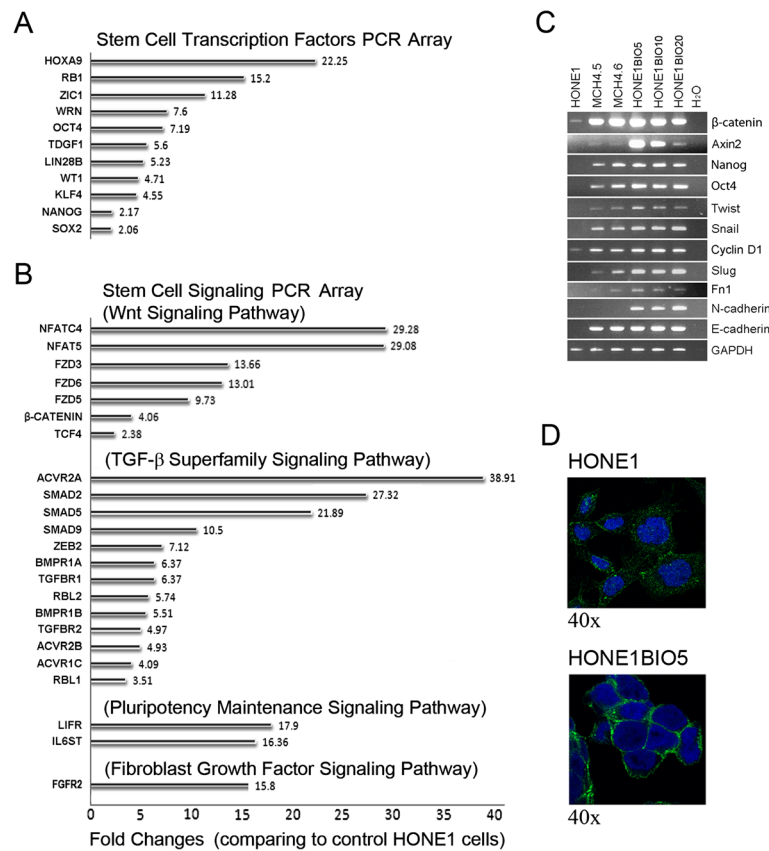
Figure 4 Physiological β-catenin signaling triggers multiple signaling pathways in HONE1 hybrid cells, and BIO-induced Wnt signaling activities in HONE1 cells. A. Stem Cell Transcription Factors Array shows major transcription factors that are up-regulated in HONE1 hybrid cells. **B**. The bottom panel demonstrates Stem Cell Signaling Array, showing that four signaling pathways are activated in HONE1 hybrid cells. **C**. RT-PCR analyses show that both β-catenin and *Axin2* are strongly expressed in BIO-treated cells. Except for *N-cadherin* that shows reduced levels in HONE1 hybrid cells (also see Figure 1C, expression for all tested genes, β-catenin, *Axin2, Nanog, Oct4, Twist, Snail, Cyclin D1, Slug, Fn1,* and *E-cadherin,* is up-regulated in both HONE1 hybrid (MCH4.5 and MCH4.6) and BIO-treated cells compared to mock-treated HONE1 cells. **D**. Immunofluorescence staining shows that expressed proteins in BIO-treated HONE1 cells (5 μM) are mainly accumulated in the membrane (40x magnification) as compared to parental HONE1 cells. This is also seen in HONE1 hybrid cells (Figures 1B and 1E).

Wnt signaling and stemness network activities are progressively up-regulated in sphere-forming cells

In the dedifferentiation progression of HONE1 to hybrid to sphere cells, RNA expression of four core stem cell genes, *Nanog, Oct4, Sox2,* and *Klf4*, was continuously up-regulated in spheres compared to derived hybrid cells (data not shown). Furthermore, some genes that encode CSC surface markers (Table S2) were either expressed or up-regulated by RT-PCR analysis in spheres, including CD9, CD24, CD44, CD90, and CD133. Notably, expression of genes that encode CD24 and CD133 was detected in sphere-forming cells derived from pooled large and small spheres from different experiments (Figure 7A).

Consistent with findings obtained from Stem Cell Signaling Array analysis (Figure 4A), many Wnt pathway components were activated, including both those in canonical and non-canonical signaling. In spheres, *LEF1*

was further increased 23-fold, compared to hybrid cells. Other markedly up-regulated genes are *DKK1* (23.5-fold), *FZD6* (29.7-fold), *Wnt11* (34.9-fold), and *FBXW2* (36.8-fold). Additionally, expression of some components of Wnt signaling, such as *GSK3A* (–9.9-fold) and the *TLE* repressor *AES* (–9.6-fold), were clearly inhibited in these spheres, as seen in Figures 7B and 7C.

Discussion

Wnt signaling plays a substantial role in the control of development of several types of tissues through a dosage-dependent fashion. These regulated cells include crypt progenitor [28,37], hair follicle [38], and hematopoietic stem cells [39]. All these observations suggest that Wnt signaling is a dominant force in the control of proliferation of progenitor or stem cells. Our data further suggest that basic, moderate, physiological levels of Wnt signaling are sufficient for these
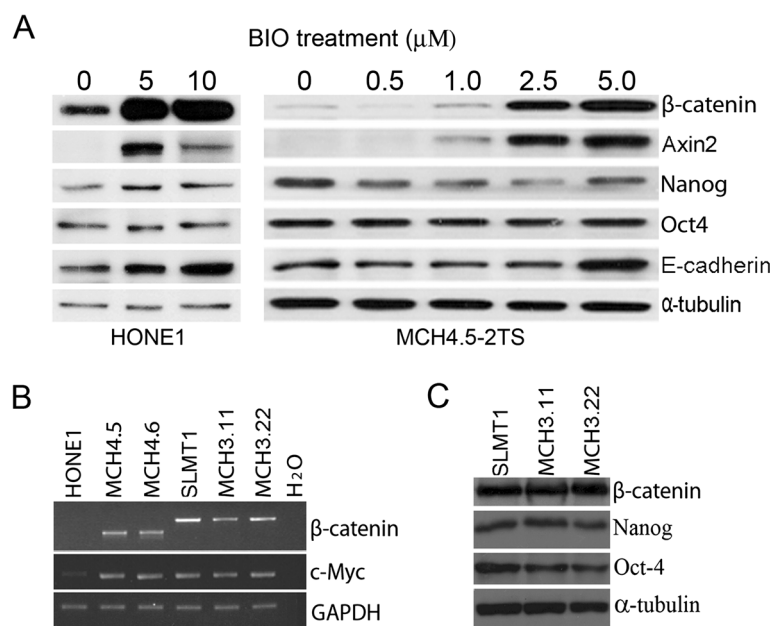
Figure 5 The high levels of β-catenin signaling do not up-regulate Nanog and Oct4 expression in both HONE1 and SLMT1 cells. A.
Western blotting shows that both concentrations of 5 μM and 10 μM BIO treatment induce strong over-expression of β-catenin in treated cells,
but 10 μM BIO treatment causes strong cytotoxic effects in cells and possible reduced expression of Axin2 in HONE1 cells. Compared to mock-
treated HONE1 and MCH4.5-2TS cells, no obvious up-regulation of both Nanog and Oct4 expression is detected in β-catenin over-expressing
cells, but E-cadherin is clearly up-regulated in both BIO-treated cell lines. **B**. SLMT1 cells have high levels of expression of β-catenin and do not
respond to exogenous β-catenin signaling. RT-PCR analysis shows that HONE1 hybrid cells have an up-regulated expression of β-catenin and *c-
Myc* compared to their parental HONE1 cells, but no up-regulated change of β-catenin (exons 3/5) and *c-Myc* is detected in SLMT1 and its
hybrids, MCHs 3.11 and 3.22. **C**. Western blotting analyses do not detect changes of proteins β-catenin, Nanog, and Oct4 in SLMT1 hybrid cells,
compared with their parental SLMT1 cells.

cellular processes [3]. To investigate the hypothesized
role of Wnt signaling in the regulation of stemness
networks in human cells, HONE1, SLMT1, and their
hybrid cell lines were used. As expected, strong activa-
tion of endogenous tumor suppressors *p53*, *RB1*, and
WT1 was detected in HONE1 hybrid cells, indicating
that genomic integrity controlled by multiple tumor
suppressor pathways within the parental HONE1
cells may be a key factor for maintaining a balanced
environment to keep a low level of β-catenin expres-
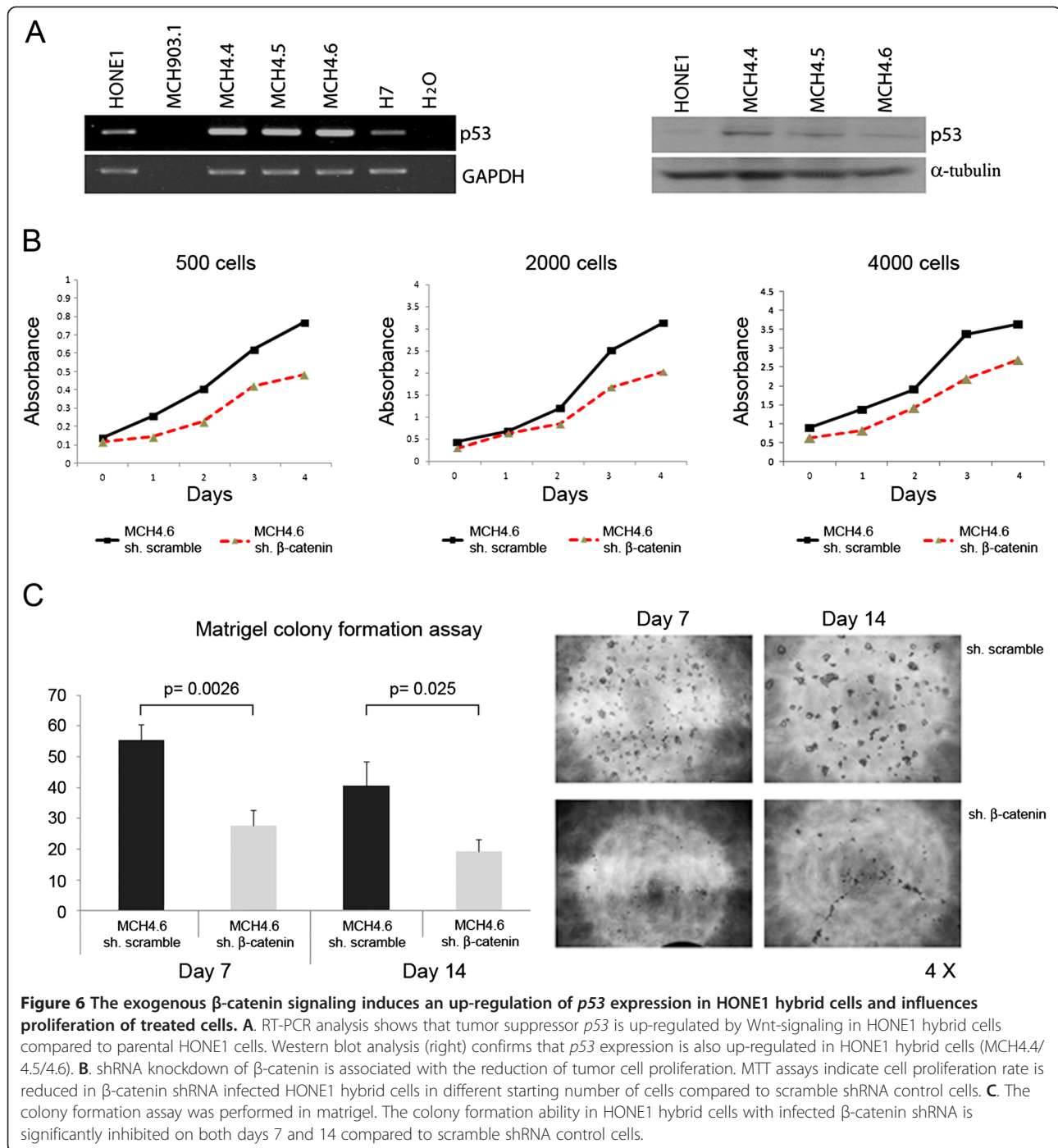sion [18-20].

Consistent with findings of both components of Wnt
pathways and the core stem cell network being
expressed or up-regulated in HONE1 hybrid cells, we
found that the Wnt signal was clearly increased in
HONE1 hybrid cells, as compared with untreated
HONE1 cells. These *in vitro* observations were further
confirmed *in vivo* with animal studies, indicating that
there was consistent, up-regulated Wnt/β-catenin sig-
naling in HONE1 hybrid cells.

To exclude the possibility that genes other than
β-catenin on transferred chromosome 3 induced Wnt
signaling in HONE1 hybrid cells, we specifically si-
lenced β-catenin expression using β-catenin shRNA
in these hybrid cells. Following the inhibition of β-

catenin, expression of core stem cell genes and EMT
markers was also decreased in the treated hybrid cells.
These observations strongly indicate that β-catenin
signaling, introduced by chromosome 3 transfer, is a
dominant force in the regulation of both stemness and
EMT networks in HONE1 cells, an expression pattern
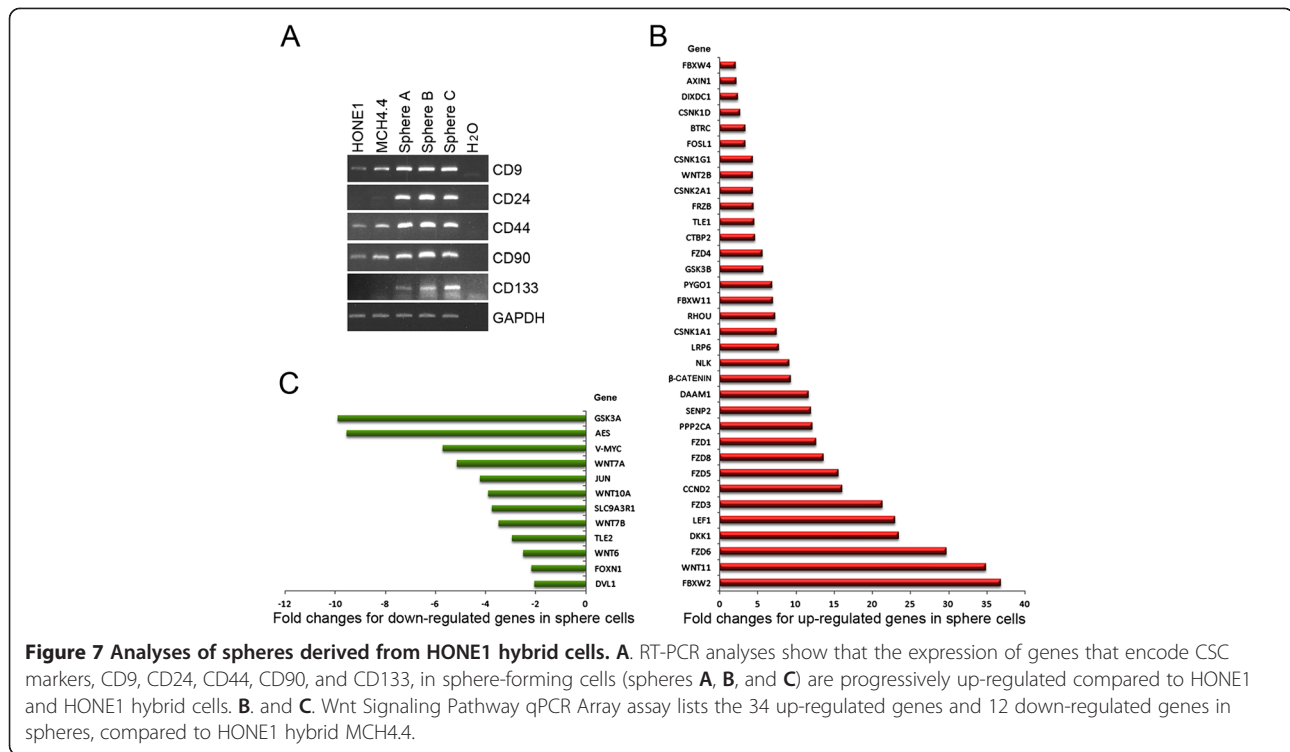seen in other tissues [28,30,37-39].

Both chromosome 3 transfer and BIO treatment in
HONE1 cells regulated EMT genes or its regulators.
However, BIO only had similar, not identical biological
effects as the chromosome 3 transfer, as evident in the
control of cadherin switching. The Wnt signal control-
ling these EMT and cadherin networks was evidenced by
the fact that β-catenin protein accumulated at the mem-
branes, as seen in BIO-treated cells, hybrids with trans-
ferred chromosome 3, and recent stemness studies [40].
This may be another regulatory process for entry control
of free β-catenin into the nucleus, but the detailed
mechanism of nuclear localization of β-catenin and the
critical role E-cadherin plays in these processes are still
not fully understood [8,9,21,29,41,42].

The qPCR Array results confirmed that physiological
β-catenin signaling triggered signals through additional
pathways including pluripotency maintenance, FGF, and
TGF-β superfamily signaling in HONE1 hybrid cells. For

Figure 6 The exogenous β-catenin signaling induces an up-regulation of *p53* expression in HONE1 hybrid cells and influences proliferation of treated cells. A. RT-PCR analysis shows that tumor suppressor *p53* is up-regulated by Wnt-signaling in HONE1 hybrid cells compared to parental HONE1 cells. Western blot analysis (right) confirms that *p53* expression is also up-regulated in HONE1 hybrid cells (MCH4.4/4.5/4.6). **B**. shRNA knockdown of β-catenin is associated with the reduction of tumor cell proliferation. MTT assays indicate cell proliferation rate is reduced in β-catenin shRNA infected HONE1 hybrid cells in different starting number of cells compared to scramble shRNA control cells. **C**. The colony formation assay was performed in matrigel. The colony formation ability in HONE1 hybrid cells with infected β-catenin shRNA is significantly inhibited on both days 7 and 14 compared to scramble shRNA control cells.

example, Smad2/5/9 and TGF-β receptors were clearly activated, which confirms previous reports that the TGF-β signaling pathway is associated with the pluripotency gene network and the EMT process [30,41]. Many embryonic development genes such as *Activin* receptor, *ACVR2A*, were activated following the introduction of physiological β-catenin signaling in treated cells. This suggests that there are additional signals such as *Activin* that develop during culture and may serve to drive the reprogramming or cell

self-renewal processes induced by Wnt signaling. It is also notable that the LIFR- and IL6ST-mediated pluripotency maintenance pathways, as well as *BMP* receptors and *Smad* families, were activated in HONE1 hybrid cells. Since these signals have well-known links to self-renewal programs, their increased expression provides additional evidence that physiological Wnt/β-catenin signaling is involved in the regulation of the cell self-renewal networks [4,5,8,17,43].

Figure 7 Analyses of spheres derived from HONE1 hybrid cells. A. RT-PCR analyses show that the expression of genes that encode CSC markers, CD9, CD24, CD44, CD90, and CD133, in sphere-forming cells (spheres **A**, **B**, and **C**) are progressively up-regulated compared to HONE1 and HONE1 hybrid cells. **B**. and **C**. Wnt Signaling Pathway qPCR Array assay lists the 34 up-regulated genes and 12 down-regulated genes in spheres, compared to HONE1 hybrid MCH4.4.

The stemness transition of HONE1 cells, driven by physiological Wnt/β-catenin signaling, appears to be a progressive process leading to a de-differentiated state in spheres, as demonstrated by analysis of up-regulated expression of core stem cell genes. Furthermore, genes that encode surface markers, such as CD9, CD24, CD44, CD90, and CD133, in spheres were expressed or clearly up-regulated compared to hybrid cells. Many Wnt signaling components in sphere-forming cells were identified as being involved in this stemness transition process, compared to hybrid cells. More than two-fold up-regulation of expression was detected in 34 genes, which included canonical, non-canonical, and Wnt/Ca^{+2} pathways.

Conclusions

These studies demonstrate that both dosage levels of β-catenin signaling and genetic background of tissues play critical roles in the regulation of self-renewal networks in human cells. There are very limited methods to explore physiological levels of Wnt/β-catenin signaling in current biology studies and the results of these studies indicate that haploid levels of expression of transferred β-catenin can induce signaling cascades that include multiple known pathways associated with reprogramming and self-renewal processes. Appropriate Wnt signaling may drive cancer cells into a progressive de-differentiated state, which generates expression profiles for exposure of the regulatory mechanism in stemness

transition. These novel results clarify the relationships that are still controversial and rather unclear at present among Wnt signaling, stemness networks, EMT events, TSG pathways and expression of common cancer stem cell surface markers. These findings shed new light on a variety of cellular processes never before associated with physiological levels of β-catenin signaling that provide a framework for future studies to define the molecular mechanisms governing cell fate determination in human cells.

Methods

Cell lines and culture conditions

NPC HONE1 cells [44], mouse MCH903.1 cells containing human chromosome 3, three HONE1 hybrid cell lines (MCH4.4/4.5/4.6), and four TS cell lines (MCH4.5-1TS/4.5-2TS/4.6-1TS/4.6-2TS) were cultured as previously reported [11]. Esophageal carcinoma cell line, SLMT1 and its hybrid cells, MCH3.11/3.22, with transferred chromosome 3 were cultured as previously reported [36]. To minimize any possible change of gene expression influenced by culture conditions, environmental factors and genetic alterations in cultured cells [7], RNAs and proteins were obtained from early passages of hybrid cells and corresponding passages of parental cells for cell fusion. Human embryonic stem cells, H7, were obtained from the Stem Cell Core Facility of the University of Hong Kong and were maintained in Matrigel-covered plates and mTeSR1 culture medium,

as suggested by the manufacturer (Stemcell Technologies, Vancouver, BC, Canada).

BIO treatment
BIO (Stemgent, San Diego, USA) was stored and diluted following manufacturer's instructions. Cells were grown in T25 flasks to 50% confluence and treated with BIO at concentrations of 0.5 μM, 1.0 μM, 2.5 μM, 5 μM, 10 μM, and 20 μM. Twenty-four hours later, both BIO-treated and mock-treated cells were washed in PBS and harvested for total RNA extraction.

Sphere formation assay
HONE1, HONE1 hybrids, and HONE hybrids with infected β-catenin shRNA cells were plated in T25 flasks, coated with 0.1% gelatin at 10–30 viable cells/ml and grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS. Cells were allowed to grow until spheres appeared. Three independent experiments were performed. After spheres formed, non-spheroid cells surrounding the spheres were scratched off and washed away with PBS daily in order to maintain the sphere growth. Only 50% of the culture medium was changed each time. The number of spheres was recorded under microscopy from 45 to 90 days after seeding cells. The pooled large and small spheres were collected for further RNA analysis.

RT² Profiler PCR array analysis
Stem Cell Transcription Factors, Stem Cell Signaling, and Wnt Signaling Pathway PCR Arrays were obtained from SABIOSCIENCES (http://www.sabiosciences.com/Signal_Transduction.php), a Qiagen Company (Frederick, MD, USA). Multiple RNA expression (internal control and housekeeping genes) and genomic DNA contamination controls are included in these qPCR arrays. One μg of total RNA was used for the first strand cDNA synthesis reaction. All reaction procedures and data analyses were performed following the manufacturer's manual (RT² Profile PCR Array User Manual version 5.01) and provided analysis software (RT² Profiler RCR Array Data Analysis version 3.5).

Infection of shRNAs
Both shRNAs scramble [45] (Addgene plasmid 1864) and β-catenin [46] (Addgene plasmid 19761), were obtained from Addgene (Cambridge, MA USA). HEK-293T cells were incubated at 37°C overnight and transfected with shRNA plasmid, psPAX2 packaging plasmid, pMD2.G envelope plasmid, and FuGENE in DMEM medium, following Addgene's protocol. Lentiviral particle solution from cultured cells was harvested 48 hours later. HONE1 hybrid cells were plated in T25 flasks overnight and grown to approximately 80-90% confluence. The following day, 1 ml infection media was added in 3 ml culture medium containing 8 μg/ml polybrene for 48 hours. The selective drug puromycin was added 24 hours later. Infected cells were expanded and the third to fifth passages were collected for subsequent RNA and protein analyses.

Luciferase assay
HONE1 and HONE1 hybrid cells were transiently transfected using 1 μg of luciferase reporter plasmids (STOP) and control plasmids (SFOP) with the Lipofectamine 2000 (Invitrogen) transfection reagent according to manufacturer's instructions. Cells were collected and lysed 48 hours post-transfection. The luciferase activity was assayed using the Luciferase Assay System (Promega). The β-galactosidase activity was assayed using Galacto-Light Plus System (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). Both luciferase and β-galactosidase activities were measured using a TD20/20 luminometer (Turner Biosystems). STOP/SFOP ratio of luciferase reporter activities was normalized using β-galactosidase activities [47,48].

RT-PCR and qPCR analyses
Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was reverse-transcribed with M-MLV Reverse Transcriptase (USB, Cleveland, OH, USA), as previously described [48]. All RNAs were treated with DNAse. The primers and RT-PCR and qPCR conditions are summarized in Additional file 1: Table S2. Human *GAPDH* was used as an internal control for all RT-PCR reactions. For qPCR analysis, triplicate PCR reactions were performed using the LightCycler 480 Real-Time PCR Instrument (Roche Diagnositics GmbH, Mannheim, Germany).

Flow cytometry
Antibodies against CD24 and CD44 (BD Pharmingen) were used for FACS analyses. To sort HONE1 and HONE1 hybrid cells, the collected cells were resuspended in Pharmingen Stain Buffer and stained with antibodies for 30 min. The antibody-positive cells were sorted by the BD FACSAria Cell Sorter (San Jose, CA, USA), according to the protocols provided by the manufacturer.

Cell proliferation assay
The cell proliferation ability was studied using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, 0.5×10^3, 2.0×10^3, and 4.0×10^3 cells were seeded in 96-well plate. The cell proliferation rate was measured consecutively for 5 days. Thirty μl of 5 mg/ml filtered MTT solution (Sigma Chemical Co., St. Louis, MO) was added into the cells and incubated at 37°C for 4 hours. The absorbance at 570 nm was recorded

with Multiskan FC Microplate Photometer (Thermo Scientific Inc., MA, USA).

Three-dimensional matrigel colony formation assays
A total of 1,000 cells was seeded on the top of Matrigel (BD Biosciences) and cultured for 14 days in 96-well plate. Images were captured at 4x and 10x magnification using Nikon Eclipse Ti inverted microscope (Nikon Instruments Inc., NY, USA) and analyzed with SPOT Advanced software (Diagnostic Instruments Inc., MI, USA). Colonies in 3 fields at 10x magnifications were counted and reported as the average number of colonies on the day 7 and 14.

Western blotting and immunofluorescence staining
Cells were seeded onto a 150 mm culture plate and were then scraped from the plate for Western blotting, which were performed as previously described [49]. The primary antibodies for Western blot analyses are summarized in Additional file 1: Table S3. Cells for immunofluorescence staining of β-catenin were grown on glass coverslips and incubated with primary and secondary antibodies for overnight and one hour, respectively, and analyzed by confocal microscopy (LSM 710, Zeiss, Germany).

Animal assay and immunohistochemical staining
Both HONE1 and HONE1 hybrid cells were injected into nude mice as previously described [11,12]. Tumors derived from both HONE1 and HONE1 hybrid cells were excised for both tissue culture in selective medium and paraffin-embedded for immunohistochemistry analysis using antibodies (β-catenin, E-cadherin, and Cyclin D1) (Additional file 1: Table S3), as described previously [48]. All animal experiments were approved by the Government of Hong Kong Special Administrative Region and University of Hong Kong.

Additional file

Additional file 1: Table S1. Expression changes in HONE1 hybrid Cells (compared to parental HONE1 cells) assayed by Stem Cell Signaling PCR Array. **Table S2.** Primers used in RT-PCR and qPCR analyses. **Table S3.** Antibodies used.

Abbreviations
BIO: Bromoindirubin-3'-oxime; CSC: Cancer stem cells; DMEM: Dulbecco's modified eagle medium; EMT: Epithelial-mesenchymal transition; FGF: Fibroblast growth factor; MHC: Microcell hybrid cell; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; NPC: Nasopharyngeal carcinoma; ShRNA: Short hairpin RNA; TGF-β: Transforming growth factor-β; TS: Tumor segregants; TSG: Tumor suppressor gene.

Competing interest
The authors declare that they have no competing interest.

Authors' contributions
YC designed research. YC performed molecular, cell culture and animal studies. AKLC, JMYK and YPP carried out Western and immunofluorescence staining. YPP and YC performed Luciferase, qPCR array and *in vitro* growth assays. PMC performed immunohistochemical staining. JMYK and PHYL performed studies in SLMT1 and SLMT1 hybrid cells. YC, MLL and MLW contributed cell lines and reagents. YC, AKLC, JMYK, YPP, MLL and MLW analyzed data. YC, MLL and MLW wrote manuscript. All authors have read and approved the final manuscript.

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