Wnt/β-catenin/CBP signaling maintains long-term murine embryonic stem cell pluripotency

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Embryonic stem cells (ESCs) represent an important research tool and a potential resource for regenerative medicine. Generally, ESCs are cocultured with a supportive feeder cell layer of murine embryonic fibroblasts, which maintain the ESCs' capacity for self-renewal and block spontaneous differentiation. These cumbersome conditions, as well as the risk of xenobiotic contamination of human ESCs grown on murine embryonic fibroblasts, make it a priority to develop chemically defined methods that can be safely used for the expansion of ESCs. Using a high-throughput, cell-based assay, we identified the small molecule IQ-1 that allows for the Wnt/β-catenin-driven long-term expansion of mouse ESCs and prevents spontaneous differentiation. We demonstrate that IQ-1, by targeting the PR72/130 subunit of the serine/threonine phosphatase PP2A, prevents β-catenin from switching coactivator usage from CBP to p300. The increase in β-catenin/CBP-mediated transcription at the expense of β-catenin/p300-mediated transcription is critical for the maintenance of murine stem cell pluripotency.

IQ-1 Maintains Murine ESC Self-Renewal Independently of LIF. Here we report that the small molecule IQ-1, which we identified from a chemical library screen, maintains the pluripotency of murine ESCs in long-term culture in a Wnt-dependent manner. Subsequently, we determined that IQ-1 binds to the PR72/130 subunit of the serine/threonine phosphatase PP2A. The binding of IQ-1 to PR72/130 leads to decreased phosphorylation of the coactivator protein p300 at Ser-89, through an as yet undetermined mechanism. We also demonstrate that the phosphorylation of p300 at Ser-89 enhances the binding affinity of β-catenin to p300. IQ-1 thereby diminishes the β-catenin/p300 interaction and prevents β-catenin coactivator switching from CBP to p300. The increase in β-catenin/CBP-mediated transcription at the expense of the β-catenin/p300 interaction is critical for the maintenance of pluripotency.

Results

IQ-1 Maintains the Undifferentiated State of ESCs. We first screened murine ESCs (D3 ES) with Asahi Kasei's proprietary chemical library searching for compounds that enhanced alkaline phosphatase production, a marker of undifferentiated ESCs (13). From this screen, we identified IQ-1 (molecular weight = 362.42, Fig. 1a), which dose dependently increased alkaline phosphatase activity (Fig. 1b), in media containing 15% FCS without the addition of exogenous leukemia inhibitory factor (LIF). Treatment with IQ-1 resulted in enhanced expression of the undifferentiated ESC marker, stage-specific embryonic antigen 1 (SSEA-1) in a dose dependent fashion (Fig. 1c).

IQ-1 allowed for long-term expansion of ESCs in culture without MEFs and without the addition of exogenous LIF. Mouse D3 ESCs were cultured in media containing 15% FCS plus 4 μg/ml IQ-1 for 65 days. The ESCs continued to proliferate at a steady rate with an approximate 2 log increase every ten days (Fig. 1d) and maintained their pluripotency as judged by alkaline phosphatase staining (Fig. 1e).


The authors declare no conflict of interest.

Abbreviations: ESC, embryonic stem cell; MEF, murine embryonic fibroblast; LIF, leukemia inhibitory factor; SSEA-1, stage-specific embryonic antigen 1.

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that the effects of IQ-1 were not mediated via Stat3 signaling, we used a Stat3/luciferase reporter gene construct. Although IQ-1 significantly elevated Nanog expression, it did not affect Stat3/luciferase expression unlike LIF, which as anticipated, elicited a significant response (Fig. 2c). We conclude that the affects of IQ-1 on the maintenance of murine ESC pluripotency are independent of the LIF/Stat3 pathway.

IQ-1 Binds the PR72/130 Subunit of the Serine/Threonine Phosphatase PP2A. To identify the molecular target of IQ-1, whole cell lysates from P19 embryonic carcinoma cells were treated with biotinylated IQ-1. Compared with a control biotinylated compound (Fig. 3a, lane 2), biotinylated IQ-1 selectively bound 3 proteins (Fig. 3a, lane 3). The two bands at 72 and 130 kDa were identified by mass spectral sequencing as the differentially spliced regulatory subunits PR72/130 of the serine/threonine protein phosphatase, PP2A. This was subsequently confirmed by immunoblotting (Fig. 3b). A third band at 60 kDa was also detected by immunoblotting and is presumed to be a degradation product of PR72/130.

IQ-1 Modulates Wnt Signaling by Interaction with PR72/130. The serine/threonine phosphatase PP2A is involved in regulating intracellular signaling, gene expression and cell cycle progression. A major function of PP2A is to regulate signaling cascades by opposing the activity of serine/threonine kinases (21). PP2A consists of a multisubunit complex. The core components of this trimeric complex are a 36 kDa catalytic, a 65 kDa regulatory (PR65) and a third variable subunit, one of which is PR72/130. PR72/130 represents tissue selective differentially spliced forms of the same gene (22). PP2A regulates the Wnt signaling cascade at multiple levels (23, 24). Recently, PR72/130 has been shown to interact with the protein Naked cuticle (Nkd), a negative regulatory component of the Wnt signaling pathway (25), thereby modulating Wnt signaling (26). We hypothesized that the interaction of IQ-1 with PR72/130 results in the disruption of the PP2A/PR72/130/Nkd complex, and inhibits “negative” regulation of canonical Wnt/β-catenin signaling. In the event, communoprecipitation of PR72/130 with both PP2A and Nkd were significantly reduced in the presence of 10 μM IQ-1 (Fig. 3c, lane 3) compared with DMSO control (Fig. 3c, lane 2). We conclude that the molecular target of IQ-1 is the PR72/130 subunit of PP2A and that the binding of IQ-1 to PR72/130 disrupts the PP2A/Nkd complex.
We initiated our investigations of this hypothesis by using the Wnt/β-catenin reporter constructs TOPFLASH and FOPFLASH in wild-type 3T3 cells (wt), 3T3 CBP (+/−) cells and p300 (+/−) cells (29). A point mutant constitutively translocating β-catenin (pt-mut β-cat) was used to stimulate reporter activity (30). Although IQ-1 did not affect TOPFLASH or FOPFLASH activity in either the wt or CBP (+/−) cells, a 2- to 3-fold increase in TOPFLASH activity was observed in the p300 (+/−) cells (supporting information [SI] Fig. 6). This suggests that the effects of IQ-1 are coactivator specific and dependent on the expression level of p300.

To directly evaluate the effects of IQ-1 on β-catenin coactivator usage, we coimmunoprecipitated with antibodies to either CBP or p300 and immunoblotted for coactivator-associated β-catenin by using P19 embryonic carcinoma cells treated with Wnt3a in the presence or absence of IQ-1. Wnt3a treatment alone increased the level of β-catenin associated with both CBP and p300 (Fig. 4b, compare lane 2 to lane 1). Treatment with Wnt3a and IQ-1 caused a dramatic increase in the relative amount of β-catenin associated with CBP compared with cells treated with Wnt3a and either DMSO or the β-catenin/CBP antagonist ICG-001 (Fig. 4b Upper, compare lane 4 to lanes 2 and 3). ICG-001, which induces cellular differentiation (8), significantly enhances the β-catenin/p300 interaction at the expense of the β-catenin/CBP interaction (Fig. 4b Lower, compare lane 3 to lanes 2 and 4).

IQ-1 Indirectly Decreases the Phosphorylation of p300 Ser-89 and Thereby the β-Catenin/p300 Interaction. It is known that signaling through the “noncanonical” Wnt pathway can increase PKC activity (31) and that Ser-89 of p300 can be phosphorylated in a PKC-dependent fashion (32). Therefore, we decided to evaluate the effects of PKCa phosphorylation of p300 Ser-89 on the binding of p300 to β-catenin. We first phosphorylated recombinant p300 (1–110 aa) with purified PKCa. The in vitro phosphorylated p300 was then added to cell lysates from P19 cells and the β-catenin/p300 complexes were coimmunoprecipitated with antisera to PR72/130 and immunoblotted for PP2A and Nkd (described in Experimental Procedures). Lane 1 (control) was immunoblotted by using rabbit IgG. Lane 2 shows PP2A (Upper) and Nkd (Lower) from DMSO control-treated P19 cells. Lane 3 demonstrates the disruption of the PR72/130 complex, as judged by the diminished signal for PP2A (Upper) and Nkd (Lower) from P19 cells exposed to 10 μM IQ-1.

IQ-1 Maintenance of ESCs Is β-Catenin/CBP-Dependent. Wnt/β-catenin signaling has been demonstrated to inhibit neuronal differentiation and maintain pluripotency in stem cell populations (2–4) and is critical for the expansion of progenitors (5). However, Wnt/β-catenin signaling is also required for neural differentiation of ESCs and neural stem cells (6, 7). Using ICG-001, a recently characterized specific antagonist of the β-catenin/CBP interaction, we have developed a model to explain the divergent activities of Wnt/β-catenin signaling (8, 9, 27, 28). The key feature of this model is that β-catenin/CBP-mediated transcription is critical for “stem/progenitor” cell proliferation and maintenance of a pluripotent/multipotent state, whereas a switch to β-catenin/p300-mediated transcription is critical to initiate a differentiation program with more limited proliferative capacity. Based on this model, we hypothesized that IQ-1, by targeting the PR72/130 subunit with PP2A, was selectively increasing β-catenin’s usage of CBP as a coactivator at the expense of p300, thereby maintaining the ESCs in the undifferentiated state (Fig. 4a).
ESC pluripotency long term (48 days), similar to results obtained with IQ-1 and 15% FCS (SI Fig. 7). Neither Wnt3a nor IQ-1 alone was sufficient to maintain the undifferentiated status of ESCs in knockout serum replacement media (SI Fig. 8). Wnt3a alone induced proliferation, but was not sufficient to maintain the expression of the stem cell markers Oct4, Nanog or Rex1. However, the combination of Wnt3a and IQ-1 allowed for proliferation and maintenance of pluripotency as judged by the expression of Oct4, Nanog and Rex1 (SI Fig. 9a–c). Treatment of ESCs with IQ-1 and Wnt3a maintained the pluripotency of ESCs as judged by their ability to form embryoid bodies after day 48 (Fig. 5aLeft) and the ability to differentiate to all three germ layer-derived tissues (Fig. 4. IQ-1 Maintenance of ESCs is Wnt/β-catenin/CBP-dependent. (a) Wnt/β-catenin coactivator switching model. A key feature of this model is that β-catenin/CBP-mediated transcription is critical for ‘stem/progenitor’ cell proliferation, whereas coactivator switching to β-catenin/p300-mediated transcription is critical to initiate a differentiative program (25, 26). TCF, T cell factor. (b) IQ-1 increases the β-catenin/CBP complex at the expense of the β-catenin/p300 complex. P19 cells were treated with Wnt3A supplemented with IQ-1, the β-catenin/CBP antagonist ICG-001, DMSO, or DMSO-only control. Nuclear lysates were co-immunoprecipitated with anti-CBP or anti-p300 antibody and immunoblotted for β-catenin. (c) Phosphorylation of p300 Ser-89, in a PKC-dependent manner, increases the β-catenin/p300 interaction. After in vitro phosphorylation with PKCα, wild-type p300 (1–110 aa) and the mutant p300 (p300 S89A) were mixed with P19 lysates and co-immunoprecipitated by using a β-catenin-specific antibody. Western blot analysis for p300 (Upper) or β-catenin loading control (Lower) was performed. Lane 1, β-catenin/p300 binding; lane 2, PKCα phosphorylated p300/β-catenin binding; lane 3, S89A p300/β-catenin binding; lane 4, PKCα phosphorylated S89A p300/β-catenin binding. (d) IQ-1 decreases the phosphorylation of p300 Ser-89. P19 cells were treated with IQ-1 or DMSO (control) and exposed to purified Wnt3A for 24 h. Cell lysates were immunoblotted by using antibodies specific for p300 or p300 phosphorylated at position Ser-89. Lane 1, negative control (vector control (VC) and DMSO); lane 2, Wnt3a plus DMSO control: phospho Ser-89 p300 immunoblot (Top) and p300 immunoblot (Middle); lane 3, Wnt3a plus 10 μM IQ-1: phospho Ser-89 p300 immunoblot (Top) and p300 immunoblot (Middle). α-Tubulin is the loading control (Bottom).
Discussion
This study demonstrates that IQ-1 in conjunction with purified Wnt3a is sufficient to maintain murine ESC proliferation and pluripotency for extended periods of time in culture (at least 48 days) in the absence of serum. IQ-1 plus Wnt3a up-regulated the expression of the transcription factors Oct4 and Sox2, which are critical to ESC maintenance. Recently, Boyer et al. demonstrated that Oct4 and Sox2 co-occupy the promoters/enhancers of a substantial portion of the genes required for the maintenance of human ESCs (33). We also showed that IQ-1 down-regulates the expression of c-myc. C-myc appears to be a critical player in the balance between stem cell self-renewal and differentiation and is increased upon differentiation (35).

Using an affinity chromatography approach, we determined that the molecular target(s) of IQ-1 are the differentially spliced regulatory subunits PR72/PR130 of the protein phosphatase PP2A. This is extremely interesting in that PR72 interacts with the protein Nkd (26), a negative regulatory component of the Wnt signaling pathway (25), associated with a switch from “canonical” Wnt/β-catenin signaling to the “noncanonical” Wnt signaling pathway (25). We demonstrate that the interaction of IQ-1 with PR72/130 results in the disruption of the PR72/130/PP2A/Nkd complex thereby modulating Wnt signaling (Fig. 5c). The question of the roles of the two splice variants PR72 and PR130 in the switch from “canonical” to “noncanonical” Wnt signaling was not addressed in these studies and will require further analysis.

We showed that in vitro phosphorylation of recombinant p300 by PKC, an enzyme coactivated via “noncanonical” Wnt signaling, increased the affinity of the β-catenin for p300. Furthermore in cells, IQ-1 significantly decreased Wnt-stimulated phosphorylation of p300 at Ser-89, without affecting the overall cellular level of p300. The mechanism by which IQ-1 can decrease the phosphorylation status of p300 at Ser-89 remains unclear and is the subject of ongoing investigations.

We demonstrate that IQ-1 selectively promotes the β-catenin/CBP interaction at the expense of the corresponding β-catenin/ p300 interaction. IQ-1 by enhancing β-catenin/CBP-mediated transcription and preventing the switch to β-catenin/p300-mediated transcription allows for the long-term expansion of murine ESCs while maintaining pluripotency without MEFs or serum. This mechanism rationalizes the controversies concerning the divergent effects of Wnt/β-catenin signaling on proliferation and differentiation in ESCs (1, 4).

Differential coactivator usage in Wnt/β-catenin signaling appears to be a critical regulator in the maintenance of the “stem/progenitor” state, the initiation of differentiation with a more restricted proliferative capacity (8), as well as potentially a switch from “canonical” to “noncanonical” Wnt signaling. IQ-1’s effects on Wnt-mediated pluripotency are associated with inhibition of the “negative” regulation of canonical Wnt/β-catenin signaling by the Nkd/PR72/PP2A complex, thereby increasing β-catenin/CBP-driven transcription at the expense of β-catenin/p300-driven transcription.

The ability of ESCs to self-renew can be viewed as a combination of proliferation with prevention of differentiation and cell death. Wnt signaling has been associated with both proliferation and differentiation of ESCs and therefore, the role of Wnt signaling in ESCs is controversial (1, 4). Differential coactivator usage and the model outlined in Fig. 4e can rationalize the divergent effects of Wnt signaling. The ability to expand “stem/progenitor” populations under defined growth conditions has important ramifications in the area of regenerative medicine. The controlled proliferation of “stem/progenitor” cells will be a critical component to the success of this endeavor.

Experimental Procedures
Cell Culture. ESCs, D3ES (ATCC CRL-1934) were maintained on mitomycin C treated MEFs in mouse ESC medium containing DMEM (Invitrogen, Carlsbad, CA) supplemented with 15% FBS (Invitrogen), 0.1 mM MEM nonessential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma, St. Louis, MO), 2 mM L-glutamine (Invitrogen) and 1,000 units/ml LIF (CHEMICON, Temecula, CA). To remove MEFs, cells were collected by trypsinization and plated on gelatin-coated culture dishes for 20 min. Nonadherent cells remaining on the plates were replated on gelatin-coated culture dishes again. Nonadherent cells were used for further experiments. P19 cells were cultured according to conditions recommended by the American Type Culture Collection. Cells were incubated at 37°C in a 5% CO₂ incubator. Purified Wnt3a is commercially available (R&D Systems, Minneapolis, MN). Knockout serum replacement medium (Invitrogen) was used according to the manufacturer’s instructions.

Compound Screens. For the small molecule screen, alkaline phosphatase activity of ESCs was determined. ESCs were plated into 96-well tissue culture plates at a density of 316–1,000 cells per well in ESC medium without LIF. Compounds were added at a final concentration of 4 μg/ml. Cells were treated with compounds for 7 days and test cell populations were washed with PBS and 100 μl of p-nitrophenyl phosphate solution (MOSS, Pasadena, MD) was added. The absorbance at 405 nm was measured by spectrophotometry (Spectramax; Molecular Devices, Sunnyvale, CA).

Flow Cytometry. Analysis of SSEA-1 expression of ESCs was performed by flow cytometry according to Zandstra et al. (37). The test cell population was washed in ice cold HBSS (Invitrogen) containing 2% FCS (HF) and resuspended for 10 min in HF containing anti-mouse CD16/CD32 monoclonal antibody at 1 μg/100 μl (PharMingen, San Diego, CA) to block nonspecific binding. Blocked cells were then incubated at 1 × 10⁵ cells/ml for 40 min on ice with anti-SSEA-1 (Kyowa Medex, Tokyo, Japan) followed by FITC-goat anti-mouse IgM antibody (ZYMED, South San Francisco, CA). Cells to be analyzed for their SSEA-1 expression were then washed twice in HF with 2 μg/ml propidium iodide (Dojindo, Kumamoto, Japan) added into the final wash. The cells were then resuspended in HF for analysis on a FACS Calibur (Becton Dickinson, Franklin Lakes, NJ).

Real-Time RT-PCR. Total RNA was isolated and reverse-transcribed by using SuperScript III (Invitrogen). Real-time RT-PCR (Sybr Green; BioRad, Hercules, CA) was performed by using with the following gene-specific primers: Nanog-F, aggtgtctactgagatactgctg; Nanog-R, caaccactgtgctttegacgc; GAPDH-F, gtggaggtgctgtaagac; GAPDH-R, tettgactgcttgctg.

Transfection and Luciferase Assay. ESCs were cultured in 96-well cell culture dishes coated with a 0.1% aqueous gelatin solution and transfected with 0.2 μg/well of pSTAT3-TA-Luc (Clontech, Palo Alto, CA), by using Lipofectamine 2000 (Invitrogen). After 6 h, cells were washed and exposed to either IQ-1 at the indicated doses, or LIF, for 24 h. Transfection efficiencies were normalized with pRL-null luciferase plasmid. Luciferase assays were performed...
Affinity Purification of Target Proteins. P19 cells were cultured to 90–100% confluency. Cells were lysed in protein-binding buffer [PB; 20 mM Hepes, pH 7.9/10 mM NaCl/0.5 mM EDTA/0.5% Nonidet P-40/6 mM MgCl2/5 mM 2-mercaptoethanol/one tablet of Complete protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN)]. Biotinylated IQ-1 was bound overnight at room temperature to a 50% slurry of streptavidin-agarose beads (Amersham Pharmacia, Piscataway, NJ) in buffer containing 50% DMSO and 50% PB. Beads were washed to remove unbound IQ-1 and then incubated with whole cell lysates. Proteins eluted by boiling in SDS were Coomassie stained to detect target proteins or immunoblotted.

Detection of CBP, p300, and Phospho-Serine89-p300 in P19 Cells. P19 cells exposed to either Wnt5A or vehicle control, after which IQ-1 was added to a final concentration of 10 μM. Control DMSO was 0.025%. Cells were incubated for 24 h. At the end of this incubation period, cells were washed, lysed and subjected to SDS/PAGE. CBP and p300 were detected by using the rabbit polyclonal antibody (A-22) (1:5,000); and (C-20) (1:5,000) (Santa Cruz Biotechnology, and p300 were detected by using the rabbit polyclonal antibody 0.025%. Cells were incubated for 24 h. At the end of this incubation period, cells were washed, lysed and subjected to SDS/PAGE. CBP and p300 were detected by using the rabbit polyclonal antibody (A-22) (1:5,000); and (C-20) (1:5,000) (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. Phospho-serine89-p300 was detected by using custom antisera at a dilution of 1:100.

Coimmunoprecipitation Experiments. Coimmunoprecipitation was performed as described in (28). For detection of PP2A and Nkd that were associated with PR72/130, P19 cells were treated with or without 10 μM IQ-1 (control DMSO, 0.025%) for 24 h. Nuclear fraction was isolated and precleared with PR72/130 antisera. Immunoblottting of CBP was performed with a mouse monoclonal (Chemicon; 1:200), anti-MAP2 (m-20) (1:100), and an IgG mouse monoclonal antibody (Becton Dickinson) at a dilution of 1:1,000.

**Immunocytochemistry and Antibodies.** Cells were fixed for 20 min with 4% paraformaldehyde in PBS. Immunostaining was carried out by using standard protocols. Primary antibodies were used at the following dilutions: anti α-fetoprotein mouse monoclonal (R&D Systems; 10 μg/ml), anti-Actin, smooth muscle Ab-1 mouse monoclonal (1:1; Lab Vision, Fremont, CA), anti-GATA4 mouse monoclonal (Santa Cruz Biotechnology; 1:100), anti-MAP2 (microtubule-associated protein) mouse monoclonal (Chemicon; 1:700), anti-tubulin mouse monoclonal (Chemicon; 1:200), anti-oligodendrocyte mouse monoclonal (Chemicon; 1:1,000). Secondary antibodies were Alexa Fluor 488 or 594 goat anti-mouse IgG (H+L) (Invitrogen; 1:200). Cells were imaged by using an Olympus (Melville, NY) IX70 microscope with ×40–×200 magnification.

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Table 1. Effects of IQ-1 treatment on genes associated with stem cell maintenance by RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>ΔΔ CT IQ-1</th>
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</thead>
<tbody>
<tr>
<td>Sox-2</td>
<td>4.31</td>
</tr>
<tr>
<td>MDR-1</td>
<td>1.69</td>
</tr>
<tr>
<td>Oct4</td>
<td>0.39</td>
</tr>
<tr>
<td>c-myc</td>
<td>-2.01</td>
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</tbody>
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The comparison is between the two conditions Wnt3a+IQ-1 versus Wnt3a +DMSO. Values are represented as ΔΔCT from the control gene beta-actin.