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# A molecular epidemiological study of respiratory viruses detected in Japanese children with acute wheezing illness

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## Abstract

**Background:** Recent studies strongly suggest that some respiratory viruses are associated with the induction of acute wheezing and/or exacerbation of bronchial asthma. However, molecular epidemiology of these viruses is not exactly known.

**Methods:** Using PCR technology, we attempted to detect various respiratory viruses from 115 Japanese children. Furthermore, the detected viruses were subjected to homology, pairwise distance, and phylogenetic analysis.

**Results:** Viruses were detected from 99 (86.1%) patients. Respiratory syncytial virus (RSV) alone and human rhinovirus (HRV) alone were detected in 47 (40.9%) and 36 (31.3%) patients, respectively. Both RSV and HRV were detected in 14 (12.2%) patients. Human metapneumovirus (HMPV) alone and human parainfluenza virus (HPIV) alone were detected in 1 (0.9%) patient each, respectively. Homology and phylogenetic analyses showed that the RSV and HRV strains were classified into genetically diverse species or subgroups. In addition, RSV was the dominant virus detected in patients with no history of wheezing, whereas HRV was dominant in patients with a history of wheezing.

**Conclusions:** The results suggested that these genetically diverse respiratory viruses, especially RSV and HRV, might be associated with wheezing in Japanese children.

## Background

A range of respiratory viruses are known to cause acute respiratory infections (ARI), including the common cold, bronchiolitis, and pneumonia in humans [1]. The major pathogens are potentially respiratory syncytial virus (RSV), human rhinovirus (HRV), human metapneumovirus (HMPV), human parainfluenza virus (HPIV), enterovirus (EV), influenza viruses (InfV), adenoviruses (AdV), and human bocavirus (HBoV) [2,3]. Respiratory infections by RSV, HRV, and HPIV are implicated in the induction of wheezing and the exacerbation of asthma, although their mechanisms are not clearly known [4]. The prevalence of asthma in developed countries is around 10 to 15% in children, while the prevalence is lower but increasing rapidly in developing countries [5]. Accumulating evidence indicates that the etiology of most cases of asthma, namely virus-induced asthma, is linked to such respiratory virus infections [6-9]. In addition, other epidemiological studies suggest that about 70% of infants have experienced an RSV infection by the age of 1 year, and 100% by the age of 2 years; the host response to the virus varies greatly, but includes upper respiratory tract infections, typical bronchiolitis (with crepitations but no wheeze), and RSV-induced wheezy bronchitis [10,11]. In addition, HRV includes over 100 serotypes and most of these are epidemic, although their epidemiology is not known [12]. Similarly, most children are infected at least once with HPIV early in life, but reinfections occur throughout life [13]. HBoV and HMPV are recently discovered

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agents of ARI, and these viruses are also associated with the common cold, bronchiolitis, and pneumonia [14]. However, the relationships between these viruses and virus-induced wheezing are not exactly known.

Genetic analyses including sequence and phylogenetic analyses of various viruses enable detailed genetic characterization of these agents. With the use of these methods, detailed molecular epidemiological studies have been reported, even in non-culturable viruses such as HRV species C (HRV-C) or HBoV [15,16]. However, molecular epidemiology of various respiratory viruses with regard to virus-induced asthma is not exactly known. From these backgrounds, we detected various respiratory viruses and performed a molecular epidemiological study of them in Japanese children with acute wheezing illness.

## Methods

### Subjects

One hundred fifteen wheezy Japanese children were enrolled in the present study. A summary of patient data is shown in Table 1. All patients visited the National Hospital Organization Yokohama Medical Center from November 2007 to March 2009. Of these patients, 39 had a history of wheezing, while the other 76 patients had no such history. In addition, 66 patients had viral bronchitis and/or bronchiolitis at consultation. These patients were treated with infusion, oxygen, and β2-agonist or epinephrine nebulization. Informed consent was obtained from the parents of all subjects for the donation of the nasopharyngeal swabs used in this study.

### DNA/RNA extraction, PCR, and sequencing

For viral DNA/RNA extraction, RT-PCR, and sequence analysis, nasopharyngeal swab samples were centrifuged at 3000 × g at 4°C for 15 min, and the supernatants were used for RT-PCR and sequence analysis as described previously [17]. Viral nucleic acid was extracted from the samples using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany). The reverse transcription reaction mixture was incubated with random hexamers at 42°C for 90 min, followed by incubation at 99°C for 5 min, and then amplification by thermal cycling. The PCR procedures for amplification of various viral genes including RSV [18], HRV [19,20], HMPV [21], HPIV [22], EV [19,20], InfV [23], AdV [24], and HBoV [25] were conducted as previously described. The primers for PCR are shown in Table 2. To avoid carry over and cross-contamination in PCR, the extraction of viral RNA/DNA was conducted in a room physically separate from that used for performing PCR. Furthermore, positive and negative controls were included in all PCR assays. PCR products were determined by electrophoresis on 3% agarose gel. Purification of DNA fragments and nucleotide sequence determination procedures were performed as described previously [17].

### Phylogenetic analysis and calculation of pairwise distances

We performed homology and phylogenetic analysis of the *G* gene of RSV, and the *VP4/VP2* coding region of HRV, because these viruses were the most commonly detected strains. The nucleotide positions of the nucleotide positions of the *G* gene of RSV were 673-912 (240 bp, for subgroup A) or 670-963 (294 bp, for subgroup B), and the *VP4/VP2* coding region of HRV were 623-1012 (390 bp). We used the CLUSTAL W program on the DNA Data Bank of Japan (DDBJ) homepage http://clustalw.ddbj.nig.ac.jp/top-j.html and TreeExplorer (Version 2.12) http://evolgen.biol.metro-u.ac.jp/TE/. Evolutionary distances were estimated using Kimura's two-parameter method, and phylogenetic trees were constructed using the neighbor-joining (NJ) method [26]. The reliability of the tree was estimated using 1000 bootstrap replications. We selected the reference strains as previously described to construct the phylogenetic trees of RSV and HRV

**Table 1 Subject data in this study**

| No. of patients | Sex(M/F) | Age (months) | History of wheezing and/or asthma | No. of patients | Sex(M/F) | Age (months) | No. of inpatients and outpatients | | No. of cases of bronchitis and/or bronchiolitis | Age (months) | Hospitalization (days) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 115 | 70/45 | 20.8 ± 25.7 | No | 76 | 44/32 | 16.9 ± 23.9 | inpatients | 55* | 46* | 13.5 ± 21.0 | 7.1 ± 2.5 |
| | | | | | | | outpatients | 21 | 7 | 25.9 ± 28.9 | |
| | | | Yes | 39 | 26/13 | 28.5 ± 27.5* | inpatients | 16 | 8 | 18.1 ± 17.0 | 7.1 ± 1.2 |
| | | | | | | | outpatients | 23 | 5 | 35.7 ± 31.3 | |

Data are expressed as mean ± SD

M/F: male/female

*$p < 0.05$

**Table 2 Primers for PCR used in this study**

| Virus | Primer | Sequence | Reference no. |
|---|---|---|---|
| RSV | ABG490 | 5'-ATGATTWYCAYTTTGAAGTGTTC-3' | [22] |
| | F164 | 5'-GTTATGACACTGGTATACCAA CC-3' | |
| | AG655 | 5'-GATCYCAAACCTCAAACCAC-3' | [23] |
| | BG517 | 5'-TTYGTTCCCTGTAGTATATGT G-3' | |
| HRV | EVP4 | 5'-CTACTTTGGTGTCCGTGTT-3' | [24] |
| | OL68-1 | 5'-GGTAAYTTCCACCACCANCC-3' | [25] |
| HMPV | hMPV-1f | 5'-CTTTGGACTTAATGACAGATG-3' | [26] |
| | hMPV-1r | 5'-GTCTTCCTGTGCTAACTTTG-3' | |
| | hMPV-2f | 5'-CATGCCGACCTCTGCAGGAC-3' | [27] |
| | hMPV-2r | 5'-ATGTTGCAYTCYYTTGATTG-3' | |
| HPIV | PIS1+ | 5'-CCGGTAATTTCTCATACCTAT G-3' | [28] |
| | PIS1- | 5'-CTTTGGAGCGGAGTTGTTAAG-3' | |
| | PIS2+ | 5'-CCATTTACCTAAGTGATGGAAT-3' | |
| | PIS2- | 5'-GCCCTGTTGTATTTGGAAGAGA-3' | |
| | PIS3+ | 5'-ACTCCCAAAGTTGATGAAAGAT-3' | |
| | PIS3- | 5'-TAAATCTTGTTGTTGAGATTG-3' | |
| InfV A | M30F2/08 | 5'-ATGAGYCTTYTAACCGAGGTCGAAACG-3' | [29] |
| | M264R3/08 | 5'-TGGACAAANCGTCTACGCTGCAG-3' | |
| InfV B | BHA1F1 | 5'-AATATCCACAAAATGAAG GCAATA-3' | [29] |
| | BHAR1166 | 5'-ATCATTCCTTCCCATCCTCCTTCT-3' | |
| AdV | AdnU-S'2 | 5'-TTCCCCATGGCNCACAAYAC-3' | [30] |
| | AdnU-A2 | 5'-TGCCKRCTCATRGGCTGRAAGTT-3' | |
| HBoV | 188F | 5'-GACCTCTGTAAGTACTATTAC-3' | [31] |
| | 542R | 5'-CTCTGTGTTGACTGAATACAG-3' | |

RSV: respiratory syncytial virus; HRV: human rhinovirus; HMPV: human metapneumovirus; HPIV: human parainfluenza virus; InfV A: influenza virus subtype A; Inf B: influenza virus subtype B; AdV: adenovirus; HBoV: human bocavirus

[17,27]. Moreover, we calculated subgroup or species frequency distributions using pairwise genetic distances for each strain, as previously described [17].

### Statistical analysis

Data were analyzed using SPSS software (SPSS for Windows, Version 10.0). All data are expressed as mean ± SD. We performed bivariate analyses using Pearson $\chi^2$ and Fisher exact tests to compare the prevalence of respiratory viruses and other variables between the study groups. The Student's *t*-test was used to compare mean age in the study group. Statistical significance was set at the level of $p< 0.05$.

### Ethics approval

All samples were collected after written informed consent was obtained from the subjects' parents. The study protocol was approved by the Ethics Committee on Human Research of National Hospital Organization Yokohama Medical Center.

## Results

### Viruses detected in the present subjects

We genetically detected RSV, HRV, HMPV, HPIV, EV, InfV, AdV, and HBoV in samples from 115 Japanese children with acute wheezing (Table 3). RSV alone was detected in 47 patients (40.9%). Among these, subgroups A (RSV-A) and B (RSV-B) were found in 27 and 20 patients, respectively. HRV alone was detected in 36 patients (31.3%), and among these, HRV species A (HRV-A), B (HRV-B), and C (HRV-C) were found in 17, 2, and 17 patients, respectively. Both RSV and HRV were detected in 14 patients (12.2%). Among these, combinations of RSV-A + HRV-A, RSV-A + HRV-B, and RSV-A + HRV-C were found in 5, 1, and 1 patient, respectively. In addition, RSV-B + HRV-A, RSV-B + HRV-B, and RSV-B + HRV-C were found in 2, 1, and 4 patients, respectively. HMPV alone and HPIV alone were detected in 1 patient each, respectively. Finally, no viral genes for RSV, HRV, HMPV, HPIV, EV, InfV, AdV, and HBoV were detected in 16 patients (13.9%). From these data, RSV was revealed to be the dominant species detected in patients with no history of wheezing and/or asthma (38 patients vs. 9 patients, $p< 0.05$), while HRV was dominant in those with a history of wheezing and/or asthma (12 patients vs. 24 patients, $p< 0.05$). These results suggested that RSV and HRV were the major causative agents of acute wheezing in the present study. Moreover, both RSV and HRV were detected in over 10% of patients with acute wheezing.

### Seasonal variations of detected viruses

To address relationships between seasonal variations of respiratory viruses and acute wheezing, we showed detected viruses during investigation period as Figure 1. Prevalence of RSV was found from autumn to winter, while prevalence of HRV was found in all season. In addition, both viruses were detected from autumn to winter.

### Homology, phylogenetic analysis, and pairwise distances of RSV and HRV

We performed phylogenetic and homology analysis, and calculated the pairwise distances of RSV and HRV in the present cases. The phylogenetic tree based on *G* gene of RSV, and the *VP4/VP2* coding region of HRV are shown in Figure 2 and 3. The homology and pairwise distances are shown in Table 4. First, the RSV was classified into two subgroups, A and B. In addition, strains belonging to both subgroups were subdivided into three genotypes (GA2, GA5, and BA, Figure 2). HRV was classified into three species: HRV-A, -B, and -C. Strains belonging to these species were subdivided into many clusters in the phylogenetic tree (Figure 3). The homology of RSV-A was relatively high (over 80%), while it was quite low for other viruses and all species

**Table 3 Subtypes or species of detected viruses**

| | No history of wheezing and/or asthma | | Having history of wheezing and/or asthma | |
|---|---|---|---|---|
| Virus | No. of strains | Strain name | No. of strains | Strain name |
| RSV-A | 25 | RSV/YOK/07/14(AB551036), RSV/YOK/07/22(AB551037), RSV/YOK/07/26(AB551038), RSV/YOK/07/43(AB551039), RSV/YOK/07/51(AB551040), RSV/YOK/07/52(AB551041), RSV/YOK/07/53(AB551042), RSV/YOK/07/66(AB551044), RSV/YOK/08/79(AB551046), RSV/YOK/08/83(AB551047), RSV/YOK/08/113(AB551049), RSV/YOK/08/122(AB551053), RSV/YOK/08/123(AB551054), RSV/YOK/08/125(AB551056), RSV/YOK/08/127(AB551057), RSV/YOK/08/128(AB551058), RSV/YOK/08/130(AB551059), RSV/YOK/08/133(AB551060), RSV/YOK/08/134(AB551061), RSV/YOK/08/141(AB551065), RSV/YOK/08/142(AB551066), RSV/YOK/08/146(AB551069), RSV/YOK/08/148(AB551071), RSV/YOK/08/150(AB551072), RSV/YOK/09/162(AB551075) | 2 | RSV/YOK/08/73(AB551045), RSV/YOK/08/111(AB551048) |
| RSV-B | 13 | RSV/YOK/07/13(AB551078), RSV/YOK/07/16(AB551079), RSV/YOK/07/17(AB551080), RSV/YOK/07/21(AB551081), RSV/YOK/07/32(AB551083), RSV/YOK/07/33(AB551084), RSV/YOK/07/34(AB551085), RSV/YOK/07/38(AB551086), RSV/YOK/07/50(AB551092), RSV/YOK/07/56(AB551093), RSV/YOK/07/60(AB551095), RSV/YOK/07/62(AB551096), RSV/YOK/07/64(AB551097) | 7 | RSV/YOK/07/4(AB551076), RSV/YOK/07/59(AB551094), RSV/YOK/08/74(AB551102), RSV/YOK/08/80(AB551104), RSV/YOK/08/82(AB551105), RSV/YOK/08/84(AB551106), RSV/YOK/08/88(AB551107) |
| Subtotal | 38 | | 9 | |
| HRV-A | 5 | HRV/YOK/07/7(AB550346), HRV/YOK/07/61(AB550365), HRV/YOK/08/107(AB550377), HRV/YOK/08/110(AB550379), HRV/YOK/08/112(AB550380) | 12 | HRV/YOK/07/11(AB550348), HRV/YOK/07/15(AB550350), HRV/YOK/07/19(AB550352), HRV/YOK/07/24(AB550355), HRV/YOK/07/25(AB550356), HRV/YOK/07/36(AB550358), HRV/YOK/08/103(AB550374), HRV/YOK/08/131(AB550389), HRV/YOK/08/153(AB550396), HRV/YOK/08/167(AB550402), HRV/YOK/08/169(AB550403), HRV/YOK/08/171(AB550404) |
| HRV-B | 1 | HRV/YOK/08/129(AB550389) | 1 | HRV/YOK/08/154(AB550397) |
| HRV-C | 6 | HRV/YOK/07/5(AB550345), HRV/YOK/07/20(AB550356), HRV/YOK/07/41(AB550368), HRV/YOK/08/100(AB550379), HRV/YOK/09/163(AB550400), HRV/YOK/09/164(AB550401) | 11 | HRV/YOK/07/2(AB550343), HRV/YOK/07/10(AB550347), HRV/YOK/07/12(AB550349), HRV/YOK/07/18(AB550351), HRV/YOK/07/23(AB550353), HRV/YOK/07/55(AB550371), HRV/YOK/08/86(AB550377), HRV/YOK/08/106(AB550382), HRV/YOK/08/120(AB550386), HRV/YOK/08/126(AB550388), HRV/YOK/08/159(AB550398) |
| Subtotal | 12 | | 24 | |
| RSV-A +HRV-A | 2 | RSV/YOK/08/116(AB551050) + HRV/YOK/08/116(AB550381), RSV/YOK/08/145(AB551068) + HRV/YOK/08/145(AB550392) | 3 | RSV/YOK/07/1(AB551033) + HRV/YOK/07/1(AB550342), RSV/YOK/08/117(AB551051) + HRV/YOK/08/117(AB550382), RSV/YOK/08/119(AB551052) + HRV/YOK/08/119(AB550384) |
| RSV-A +HRV-B | | ND | 1 | RSV/YOK/08/140(AB551064) + HRV/YOK/08/140(AB550392) |
| RSV-A +HRV-C | | ND | 1 | RSV/YOK/07/3(AB551034) + HRV/YOK/07/3(AB550344) |
| RSV-B +HRV-A | 2 | RSV/YOK/07/42(AB551087) + HRV/YOK/07/42(AB550361), RSV/YOK/07/47(AB551090) + HRV/YOK/07/47(AB550363) | | ND |
| RSV-B +HRV-B | 1 | RSV/YOK/08/118(AB551108) + HRV/YOK/08/118(AB550363) | | ND |
| RSV-B +HRV-C | 4 | RSV/YOK/07/28(AB551082) + HRV/YOK/07/28(AB550365), RSV/YOK/07/45(AB551088) + HRV/YOK/07/45(AB550405), RSV/YOK/07/46(AB551089) + HRV/YOK/07/46(AB550370), RSV/YOK/07/67(AB551099) + HRV/YOK/07/67(AB550375) | | ND |
| Subtotal | 9 | | 5 | |
| HMPV-B2 | 1 | HMPV/YOK/07/44(AB565438) | | ND |
| HPIV-1 | | ND | 1 | HPIV/YOK/08/115(AB565748) |
| Total | 60 | | 39 | |

RSV-A, Respiratory syncytial virus subgroup A;RSV-B, Respiratory syncytial virus subgroup B; HRV-A, Human rhinovirus species A; HRV-B, Human rhinovirus species B; HRV-C, Human rhinovirus species C; HMPV, Human metapneumovirus; HPIV-1, Human parainfluenza virus type 1; ND, Not detected

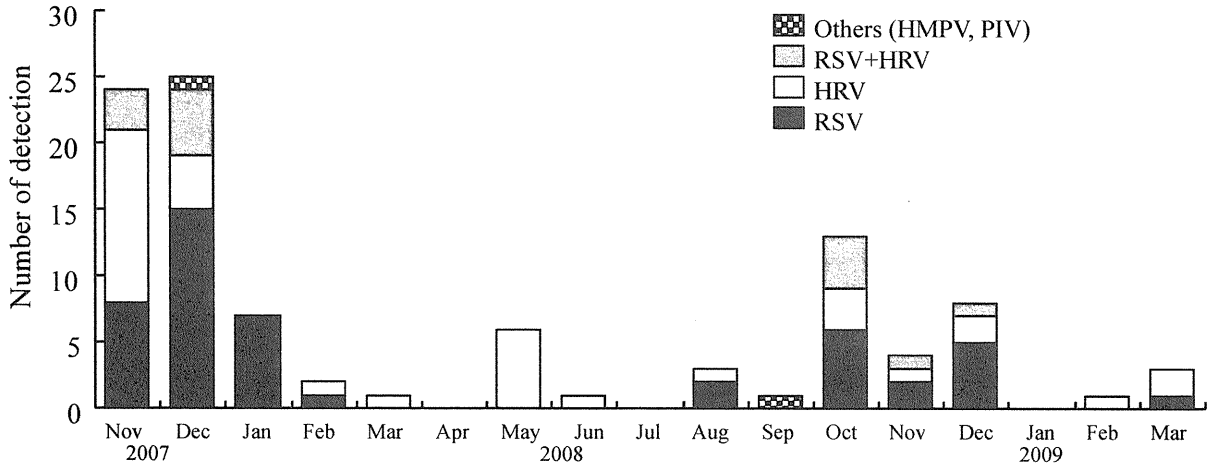


**Figure 1 Seasonal variations of respiratory viruses detected in this study.**

of HRV (over 30% divergence). Notably, the genetic diversity of HRV-C was wide (52 to 100%). In addition, the pairwise distances of HRV-A and HRV-C strains are high (over 0.2), while those for RSV-A and RSV-B strains are low. Based on these results it is suggested that acute wheezing-associated HRV has wide genetic diversity.

## Discussion

We detected and genetically analyzed major ARI viruses including RSV, HRV, HMPV, and HPIV in samples from 115 Japanese children with acute wheezing during a 17-month period (November 2007 and March 2009). These viruses were detected in over 80% of the patients. The dominant viruses were RSV and HRV, and both were detected in over 10% of the patients. In addition, these viruses were confirmed as belonging to various subgroups, genotypes, or species. All three species of HRV detected showed wide genetic diversity (more than 30% divergence). Interestingly, RSV was the dominant species detected in patients with no history of wheezing and/or asthma, while HRV was dominant in patients with a history of wheezing and/or asthma. The results suggested that RSV and HRV were major ARI viruses regarding virus-induced acute wheezing in the present study.

It is suggested that various respiratory viruses such as RSV, HRV, HMPV, HPIV, EV, InfV, AdV, and HBoV are detected in patients with lower respiratory tract infections including bronchiolitis and pneumonia [6,7]. These viruses are also detected in cases of acute wheezing [6,7]. Thus, they may be associated with both lower respiratory tract infection and acute wheezing in children [6,7]. At present, this disease status is recognized by physician and pediatrician as virus-induced asthma [28,29]. It may be important to address the genetic properties of ARI viruses associated with these diseases. However, few studies have been conducted into the genetic analysis of these viruses in acute wheezing. To better understand the relationships between viral properties and acute wheezing, it may be important to genetically analyze ARI viruses detected in the wheezy children. We studied the molecular epidemiology of these respiratory viruses detected in Japanese children with acute wheezing. To the best of our knowledge, the present study is the first to report the detection of RSV, and HRV-A, -B, -C with different genetic characteristics in Japanese children with acute wheezing.

Many studies suggest that RSV is a major candidate as an inducer of acute wheezing [4,10,11] and it may infect all children under the age of 2 years [10,11]. Furthermore, some of these children may develop bronchiolitis and/or pneumonia with acute wheezing [10]. Sugai-Goto *et al.* demonstrated that genotypes and the major genes (*F, G,* and *N*) of RSV isolated from hospitalized children with bronchiolitis or bronchopneumonia accompanied by acute wheezing were not significantly different when compared with RSV strains detected from upper respiratory tract infections [27]. These viruses belong to subgroup A, genotype GA2 and subgroup B, genotype BA [27]. Furthermore, Nakamura *et al.* showed similar genetic data from various acute respiratory infections in Okinawa, Japan [30]. Our findings regarding the properties of *G* gene in the RSV strains detected were comparable with the above-mentioned reports. In contrast, it has been suggested that a specific genotype, GA3 type virus, might be associated with a significantly greater severity of illness [31]. Riccetto *et al.* demonstrated that the severity of illness of RSV infection in infants can be associated with other factors such as body weight and prematurity [32], and any

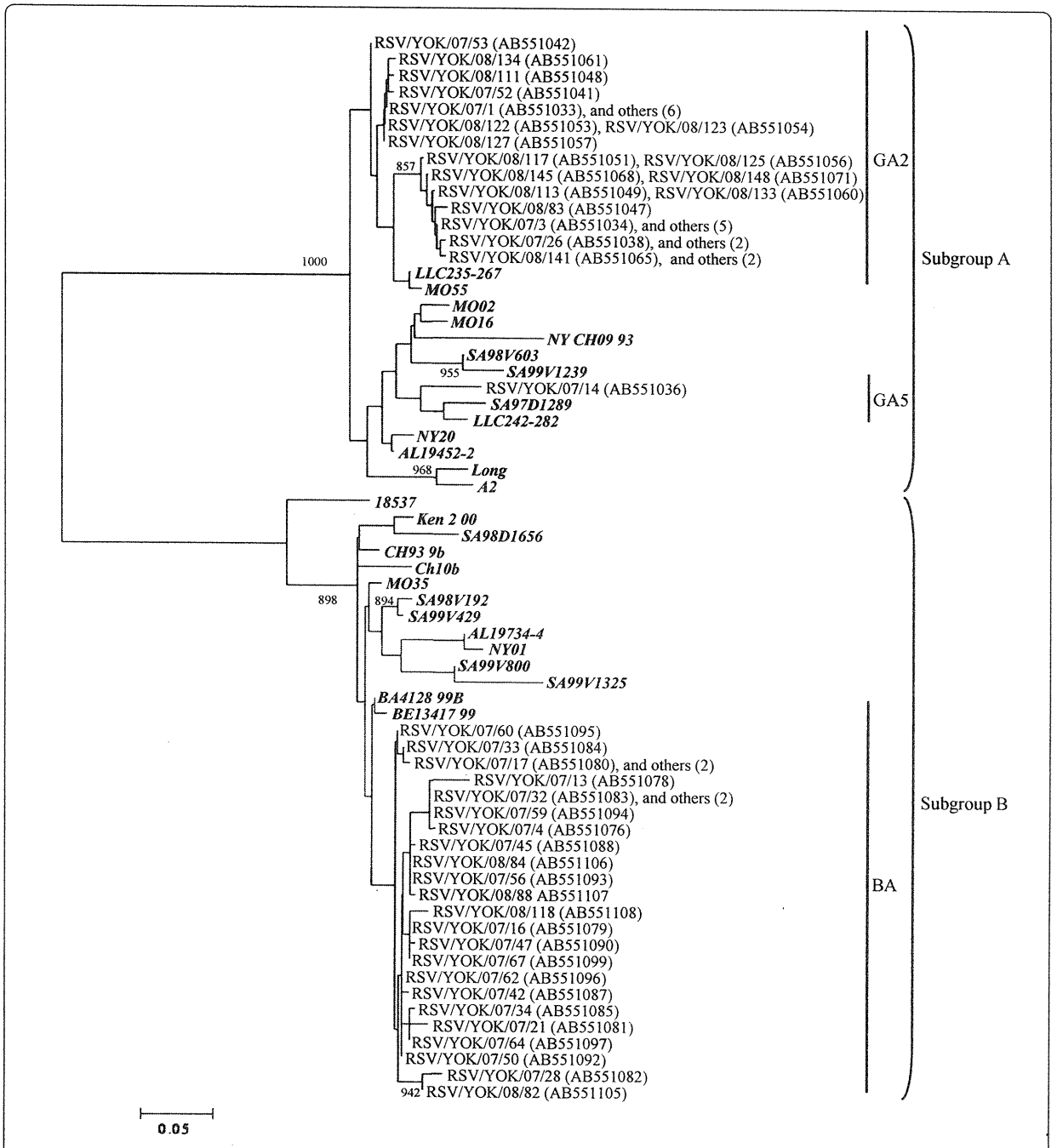


**Figure 2 Phylogenetic analysis of RSV (*G* gene)**. Detailed procedures and conditions of the phylogenetic tree are shown in the text. Numbers in parentheses indicate numbers of strains detected in other patients. Reference strains are shown in bold type. Bars, 0.05 substitutions per nucleotide position. Only bootstrap values more than 85% are shown at branch points.

association between the viral properties and pathogenicity of RSV has yet to be elucidated. Another report suggested that host immunity such as TLR4 polymorphism is linked to symptomatic RSV [33]. Thus, both the antigenicity of the viruses and host immune conditions may play important roles in the pathophysiology of severe respiratory infections such as bronchiolitis, pneumonia, and virus-induced asthma [1,34].

For a long time, HRV was simply thought to be causative agents of the mild common cold [12]. In general,

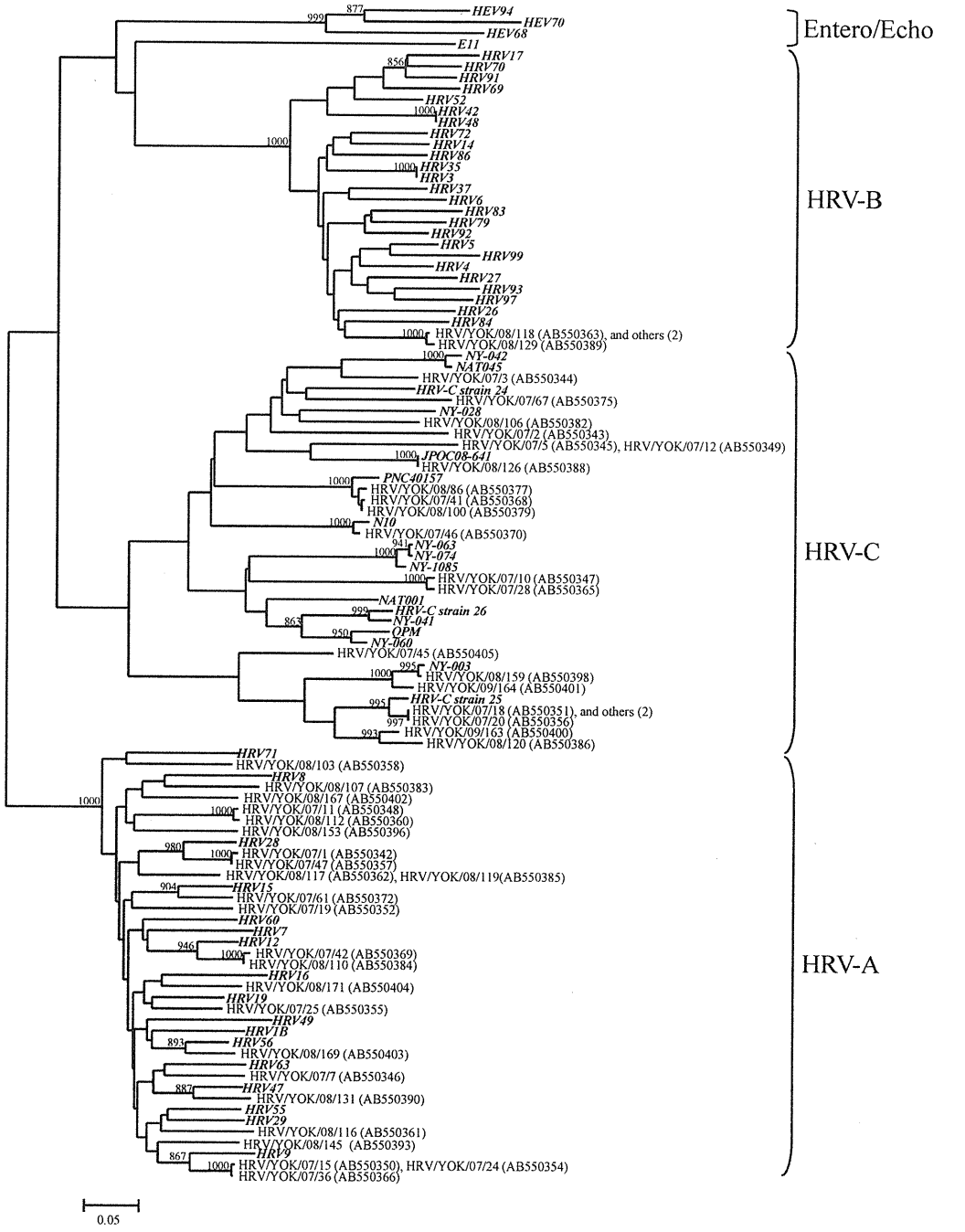


**Figure 3 Phylogenetic analysis of HRV (*VP4/VP2* coding region)**. Detailed procedures and conditions of the phylogenetic tree are shown in the text. Numbers in parentheses indicate numbers of strains detected in other patients. Reference strains are shown in bold type. Bars, 0.05 substitutions per nucleotide position. Only bootstrap values more than 85% are shown at branch points.

this acknowledgement may not be incorrect in non-asthmatic people [35]. However, it is suggested that HRV induces wheezing and exacerbation of symptoms in most asthmatics [12]. However, the molecular epidemiology of each HRV species is not yet known, because HRV is relatively difficult to isolate and detect. Thus, non-culturable HRV-C was only recovered a few years ago. Very recently, Mizuta *et al.* demonstrated

**Table 4 Pairwise distances and homology of RSV and HRV strains based on nucleotide sequences**

| | Homology (%) | | Pairwise distance | |
|---|---|---|---|---|
| Virus | All strains* | Present strains** | All strains* | Present strains** |
| RSV-A | 82.0 - 100 | 83.5 - 100 | 0.063 ± 0.043 | 0.035 ± 0.034 |
| RSV-B | 74.2 - 100 | 92.8 - 100 | 0.060 ± 0.040 | 0.029 ± 0.014 |
| HRV-A | 66.4 - 100 | 66.5 - 100 | 0.202 ± 0.031 | 0.200 ± 0.038 |
| HRV-B | 68.1 - 100 | 99.5 - 100 | 0.204 ± 0.039 | 0.002 ± 0.003 |
| HRV-C | 41.0 - 100 | 52.2 - 100 | 0.263 ± 0.069 | 0.254 ± 0.077 |

Data are expressed as mean ± SD

RSV-A, Respiratory syncytial virus subgroup A

RSV-B, Respiratory syncytial virus subgroup B

HRV-A, Human rhinovirus species A

HRV-B, Human rhinovirus species B

HRV-C, Human rhinovirus species C

* All strains: reference strains plus the strains detected in the present study.

** Present strains: viruses detected in the present study.

that HRV-A isolates showed wide genetic diversity, and some viruses belonging to specific clusters of the phylogenetic tree of HRV-A isolates might be associated with bronchiolitis [17]. In addition, a new study suggested that HRV-C has a stronger link to virus-induced asthma than HRV-A and -B strains [36]. However, our results did not reveal a similar tendency, although the reasons for this are unknown.

In this study, both RSV and HRV were detected from over 10% of patients with acute wheezing. Chung *et al.* demonstrated that both RSV and HRV were detected in 3.9% of Korean children with acute wheezing [37]. Thus, our data and that of other studies may be comparable, although the percentages of virus detection differ. We additionally compared the severity of clinical symptoms between dual virus-detected patients and those in whom RSV or HRV was detected alone. However, there were no significant findings. In addition, RSV, HRV, HMPV, HPIV, EV, InfV, AdV, and HBoV were not detected in over 10% of patients. Although we were unable to explain why, it might be that other respiratory viruses and bacteria were involved.

It should be noted that some respiratory viruses might be detected in healthy children [1,38-40]. As mentioned above, various species of HRV have been relatively frequently detected in healthy children (around 10-20%) [39,40], although RSV was detected less frequently in healthy and asymptomatic persons [40]. Thus, to better understand the etiology of these viruses, it may be important to determine the prevalence of these viruses in healthy children. A limitation of this study is that we did not examine such prevalence in healthy children and instead focused mainly on detailed molecular epidemiological analysis of various respiratory viruses detected in children with acute wheezing. Additional molecular epidemiological studies of viruses detected in wheezy and healthy children would be of value.

In the present study, HMPV and HPIV were detected in samples from the subjects, albeit rarely (each virus was detected in one of only two patients). It is suggested that HMPV and HPIV are also associated with bronchiolitis and bronchopneumonia [41]. However, it is not known how these viruses are linked to the induction of wheezing and exacerbation of asthma [42]. A previous study suggested that sputum from HPIV infection contains tryptase due to activation (degranulation) of mast cells, and this activation may strongly induce an asthmatic attack [43]. Thus, HPIV infection may induce asthmatic conditions [7]. Additional studies regarding the relationships between HPIV and HMPV infection and virus-induced asthma are warranted.

## Conclusions

Our data suggested that both RSV and HRV with various genetic characteristics were associated with acute wheezing illness in Japanese children. In particular, HRV shows widely genetic diversity. Larger studies to examine the detailed genetic characteristics of the various respiratory viruses detected in wheezy and healthy children may be needed.

### Abbreviations

RSV: respiratory syncytial virus; HRV: human rhinovirus; HMPV: human metapneumovirus; HPIV: human parainfluenza virus; EV: enterovirus; InfV: influenza viruses; AdV: adenoviruses; HBoV: human bocavirus; DDBJ: DNA Data Bank of Japan; NJ: neighbor-joining

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### Authors' contributions

AF, HK, HT, MT, SY, and NO designed research; MA, KGS, HT, KM, MN, MY, and AN performed research; HT, AF, and MA contributed analytic tools, HK, AR, YO, YK, and MM analyzed data; HK, HT, and YO wrote the paper. All authors read and approved the final manuscript.

### Competing interests

The authors declare that they have no competing interests. The authors alone are responsible for the content and writing of the paper.

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# A Distinct Role for Pin1 in the Induction and Maintenance of Pluripotency*

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**The prominent characteristics of pluripotent stem cells are their unique capacity to self-renew and pluripotency. Although pluripotent stem cell proliferation is maintained by specific intracellular phosphorylation signaling events, it has not been well characterized how the resulting phosphorylated proteins are subsequently regulated. We here report that the peptidyl-prolyl isomerase Pin1 is indispensable for the self-renewal and maintenance of pluripotent stem cells via the regulation of phosphorylated Oct4 and other substrates. Pin1 expression was found to be up-regulated upon the induction of induced pluripotent stem (iPS) cells, and the forced expression of Pin1 with defined reprogramming factors was observed to further enhance the frequency of iPS cell generation. The inhibition of Pin1 activity significantly suppressed colony formation and induced the aberrant differentiation of human iPS cells as well as murine ES cells. We further found that Pin1 interacts with the phosphorylated $Ser^{12}$-Pro motif of Oct4 and that this in turn facilitates the stability and transcriptional activity functions of Oct4. Our current findings thus uncover an atypical role for Pin1 as a putative regulator of the induction and maintenance of pluripotency via the control of phosphorylation signaling. These data suggest that the manipulation of Pin1 function could be a potential strategy for the stable induction and proliferation of human iPS cells.**

Stem cells are characterized by their ability to self-renew through mitotic cell division and to differentiate into a diverse range of specialized cell types (1, 2). Human pluripotent stem cell proliferation is maintained through the action of several transcription factors including Oct4 (octamer 4), SOX2, Klf-4, Nanog, and c-Myc, which perform reprogramming functions under the stimulatory effects of stem cell-specific growth factors, including basic fibroblast growth factor (3–5). Basic fibroblast growth factor signaling has been shown to be essential for pluripotency as its depletion from cell culture media leads to aberrant cell differentiation and cell death (6, 7). Fibroblast growth factors produce mitogenic effects in targeted cells via signaling through cell surface receptor tyrosine kinases (8). These kinases can initiate intracellular signaling in cells, which is transmitted and diffused by tyrosine phosphorylation of the assembled proteins and of cellular substrates, including protein kinases with specificity for serine/threonine residues (8, 9). Although this intracellular phosphorylation signaling might indeed contribute to the self-renewal and pluripotency of stem cells (10, 11), it has not yet been fully determined how these phosphorylated proteins are further regulated.

Protein phosphorylation is a fundamental mode of intracellular signal transduction in a variety of key cellular processes such as cell proliferation, differentiation, and morphogenesis (12). A pivotal signaling mechanism that controls the function of phosphorylated proteins is the *cis-trans* isomerization of phosphorylated Ser/Thr-Pro motifs by the peptidylprolyl isomerase Pin1 (13, 14). This modification regulates multiple intracellular signaling pathways, including ErbB2/Ras, Wnt/β-catenin, and NF-κB, and thus plays an important role in the etiology of several human diseases (15–18). These include various cancers, Alzheimer disease, and immune disorders (14, 17, 18). However, the role of Pin1 in regulating the properties of pluripotent stem cells has not been adequately investigated to date.

In our current study, we investigated the role of Pin1 in the self-renewal and stemness of pluripotent stem cells. We reveal that Pin1 is induced upon cellular reprogramming and that its blockade significantly inhibits the self-renewal and maintenance of human iPS[2] cells in addition to murine ES cells. We find also that Pin1 can interact with phosphorylated Oct4 at the

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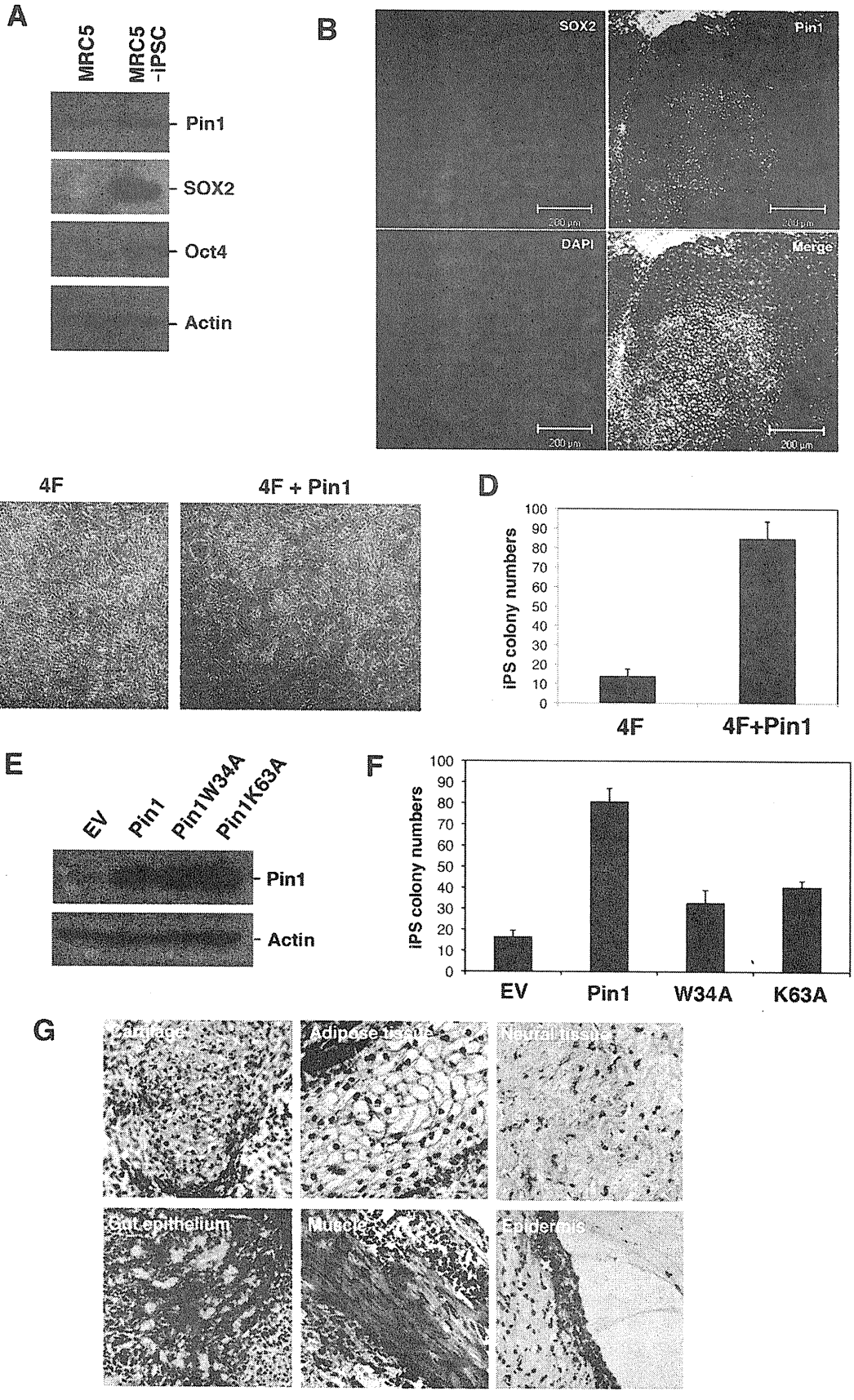
[2] The abbreviations used are: iPS, induced pluripotent stem; AP, alkaline phosphatase; dnPin1, dominant-negative Pin1; 4F, four reprogramming factors; DMSO, dimethyl sulfoxide; SUMO, small ubiquitin-like modifier; Oct4, Octamer 4.



$Ser^{12}$-Pro motif in this protein. This enhances the stability and hence the transcriptional activity of Oct4. Our present data thus suggest that Pin1 is indeed a putative regulator of the self-renewal and proliferation of pluripotent stem cells.

## EXPERIMENTAL PROCEDURES

*Colony Formation Analysis*—Human iPS cells were obtained from the RIKEN BioResource Center (clone no. 201B7) (19). Cells were cultured in human embryonic stem cell culture medium (KnockOut Dulbecco's modified Eagle's medium (Invitrogen)) supplemented with 20% KnockOut SR (Invitrogen), 1% GlutaMAX (Invitrogen), 100 μM nonessential amino acids (Invitrogen), 50 μM β-mercaptoethanol, and 10 ng/ml basic fibroblast growth factor). Murine ES cells were cultured in human embryonic stem cell culture medium (KnockOut Dulbecco's modified Eagle's medium supplemented with 15% KnockOut SR, 1% GlutaMAX (Invitrogen), 100 μM nonessential amino acids, 50 μM β-mercaptoethanol, and 1000 units/ml recombinant human leukemia inhibitory factor) (20). Colony formation was scored by counting the number of alkaline phosphatase (AP)-positive colonies as described previously (21). The number of cells per colony was determined by manually counting the number of DAPI-stained cells (21).

*Cell Reprogramming*—MRC5 fibroblasts were transduced with retroviral vectors encoding reprogramming factors as described previously (19). Briefly, the retroviral vector plasmids pMXs-hOct4, pMXs-hSOX2, pMXs-hKLF4, pMXs-hcMYC (Addgene), and pVSV-G were introduced into Plat-E cells using Effectene transfection reagent (Qiagen). After 48 h, virus-containing supernatants were passed through a 0.45-μm filter and supplemented with 10 μg/ml hexadimethrine bromide (polybrene). Cells were seeded at $6 \times 10^5$ cells per 60 mm dish at 24 h before incubation in the virus/polybrene-containing supernatants for 16 h. After 6 days, cells were plated on irradiated mouse embryonic fibroblasts, and culture medium was replaced with the hESC culture medium 24 h later. Cells were maintained at 37 °C and 5% $CO_2$ for 30 days.

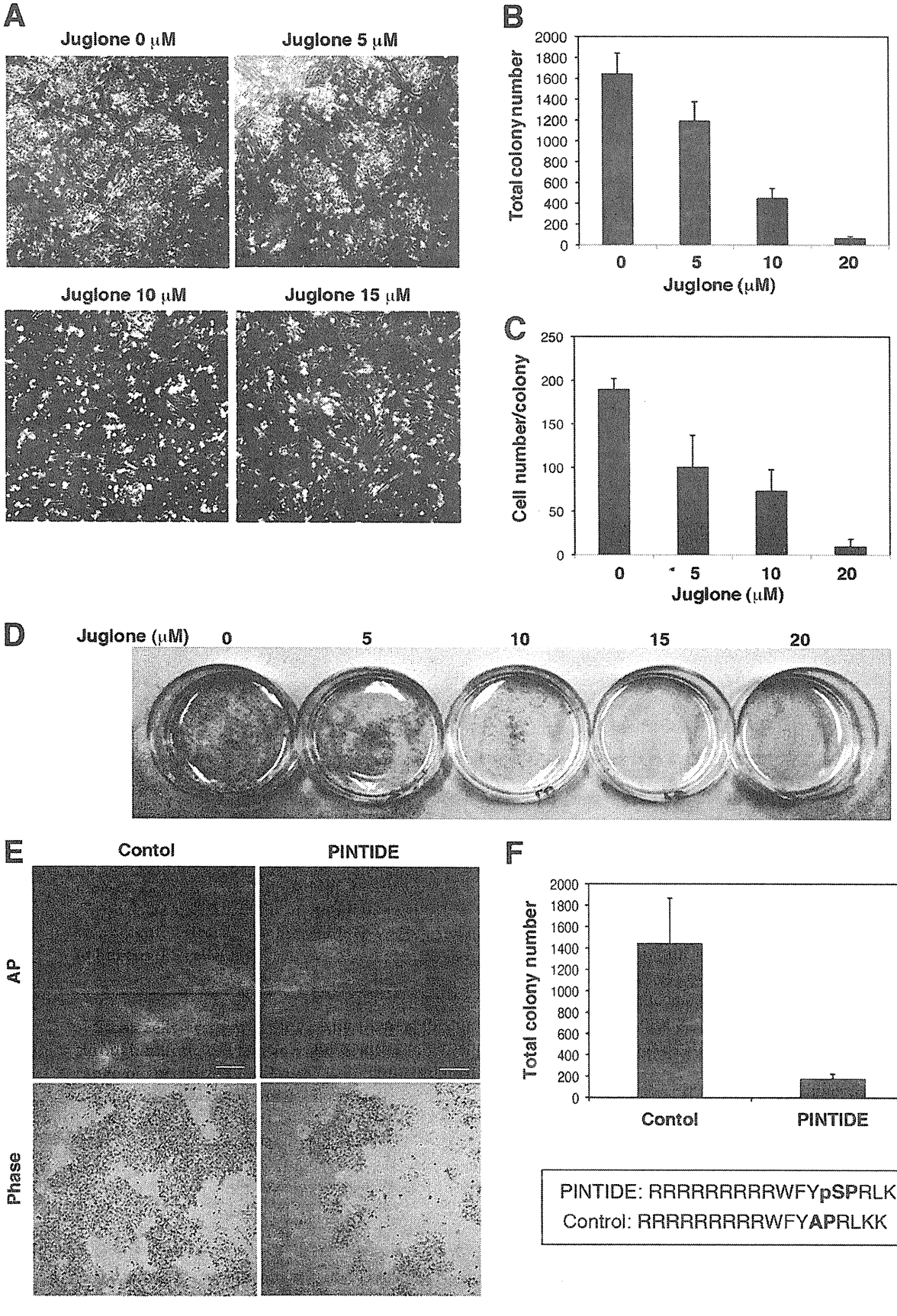
*Construction of Expression Vectors*—Oct4 cDNA was subcloned into pcDNA3-HA expression vector (Invitrogen). Expression constructs of Oct4 were as follows: pcDNA-HA-Oct4 wild-type, amino acids 1–360; pcDNA-HA-Oct4 ΔC, amino acids 1–297; pcDNA-HA-Oct4 ΔN1, amino acids 138–360; pcDNA-HA-Oct4 ΔN2, amino acids 113–360; and pcDNA-HA-Oct4 ΔN3, amino acids 34–360. pcDNA-HA-Oct4-S12A was generated by KOD-Plus Mutagenesis Kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The primers were 5′-CGCCCCCTCCAGGTGGT-3′ (forward) and 5′-CGAAGGCAAAATCTGAAGCC-3′ (reverse).

*Gene Reporter Assay*—A pGL3-fgf4 reporter plasmid containing an Oct-SOX binding cassette and the firefly luciferase gene was transfected with pRL-CMV (22). The −2601/+1 (nucleotide positions indicated with respect to the +1 translation start site) genomic fragment of the Oct4 promoter upstream region was amplified by PCR from human lymphocyte genomic DNA and cloned into the KpnI/HindIII sites of the pGL4-basic reporter plasmid (Promega, Madison, WI) as described previously (23). The primer sets were as follows: 5′-CCTGGTACCAGGATGGCAAGCTGAGAAACACTG-3′ and 5′-TCGCAAGCTTGCGAAGGGACTACTCAAC-3′. Cells were transfected with reporter plasmid vectors using Effectene (Qiagen) or Xfect Stem (Clontech). One day after transfection, the cells were resuspended in passive lysis buffer (Promega) and incubated for 15 min at room temperature. Luciferase activities were measured with a Dual-Luciferase reporter assay system (Promega) in accordance with the manufacturer's instructions.

*GST Pulldown Assay and Immunoprecipitation Analysis*—Cells were lysed with GST pulldown buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 1 mM $Na_3VO_4$, 1 mM DTT, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.2 mM PMSF) and incubated with 30 μl of glutathione-agarose beads containing either GST-Pin1 or GST at 4 °C for 2 h. The precipitated proteins were then washed three times with lysis buffer and subjected to SDS-PAGE. For immunoprecipitation, cells were lysed with Nonidet P-40 lysis buffer (10 mM Tris HCl (pH 7.4), 100 mM NaCl, 0.5% Nonidet P-40, 1 mM $Na_3VO_4$, 100 mM NaF, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.2 mM PMSF). Cell lysates were incubated for 1 h with protein A/G-Sepharose-nonimmunized IgG complexes. Supernatant fractions were recovered and immunoprecipitated with 5 μg of anti-Myc antibody and 30 μl protein A/G-Sepharose. After washing three times with lysis buffer, the pellets were analyzed by SDS-PAGE.

*Proteomics Analysis*—Human iPS cell lysates were processed for immunoprecipitation with a monoclonal anti-Pin1 antibody (clone 257417, R&D Systems) at 4 °C for 3 h followed by SDS-PAGE. Gel lanes corresponding to the region from ~30 to 150 kDa were systematically excised, and the pieces were reduced, alkylated, and trypsinized. Peptides were analyzed by the linear ion trap Orbitrap hybrid mass spectrometer (Thermo Scientific). Protein identification was performed by peptide

FIGURE 1. **Pin1 is preferentially expressed in human iPS cells.** *A*, immunoblotting analysis of Oct4, SOX2, and Pin1 in MRC5 and MRC5-derived iPS cells. Actin was used as a loading control. *iPSC*, induced pluripotent stem cells; *EV*, empty vector. *B*, immunofluorescent analysis of Pin1 and SOX2 in human iPS cells. Representative images of phase-contrast microscopy and fluorescent immunocytochemistry for SOX2 (*red*) and Pin1 (*green*) are shown. Nuclei are indicated by DAPI staining (*blue*). Note that Pin1 is highly expressed in SOX2-positive pluripotent stem cells. *C* and *D*, Pin1 expression enhances 4F (Oct4, SOX2, Klf4, and c-Myc)-induced iPS cell induction. MRC5 fibroblasts were infected with retrovirus vectors encoding 4F and co-infected with those encoding either empty vector or Pin1. A representative picture of colony formations stained with AP is shown (*C*). The numbers of AP-positive colonies were scored in three independent experiments (*D*). Note that the co-introduction of Pin1 with 4F increases the frequency of iPS colony formation. *E* and *F*, MRC5 fibroblasts were infected with retrovirus vectors encoding 4F and co-infected with those encoding empty vector, HA-tagged wild-type Pin1, or its W34A or K63A mutants. The expression levels of HA-Pin1 or its mutants in infected MRC5 cells were analyzed by immunoblotting analysis with anti-HA antibody (*E*). The number of AP-positive colonies was scored in three independent experiments (*F*). *G*, teratoma tissue derived from human iPS cells induced by 4F and Pin1. iPS cells were transplanted subcutaneously into immunodeficient mice ($2 \times 10^6$/mouse). Representative images of hematoxylin and eosin stained tumor with light microscope (200×) are shown.



mass fingerprinting with the Mascot and Aldente search algorithms.

*Quantitative Real-time PCR*—Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized using a cDNA synthesis kit (Toyobo, Osaka, Japan) and subjected to RT-PCR analysis with the SYBR Premix Ex gent Kit TaqII (Takara Bio, Shiga, Japan) using an Applied Biosystems 7300 real-time PCR System. The primer sets used were as follows: mOct4, 5′-CGTGTGAGGTGGAGTCTGGAGACC-3′ and 5′-ACTCGAACCACATCCTTCTCTAGCC-3′; mGAPDH, 5′-CCATGGAGAAGGCTGGGG-3′ and 5′-CAAAGTTGTCATGGATGACC-3′.

*Teratoma Formation*—Cells were harvested using accutase, collected into tubes, and centrifuged. The pellets were then suspended in human ESC culture medium. Fox Chase severe combined immunodeficiency mice (CREA, Tokyo, Japan) were injected with $2 \times 10^6$ cells mixed with an equal volume of Matrigel (BD Biosciences). Frozen tumor tissues embedded in optimum cutting temperature compound were sliced by cryosectioning and stained with hematoxylin and eosin.

## RESULTS

*Pin1 Is Induced upon Cellular Reprogramming and Enhances Generation of iPS Cells*—To examine the role of Pin1 in cellular reprogramming and pluripotency, we initially investigated the expression levels of this prolyl isomerase in human iPS cells. Pin1 was found to be significantly induced upon the generation of iPS cells derived from MRC5 human fibroblasts (Fig. 1*A*). Immunofluorescent analysis further revealed that Pin1 is selectively expressed in SOX2-positive pluripotent stem cells, whereas its expression was found to be significantly suppressed in the surrounding SOX2-negative differentiated cells (Fig. 1*B*). These results indicate that Pin1 is preferentially expressed in reprogramming stem cells.

We next evaluated whether Pin1 affects the reprogramming of somatic cells into iPS cells. The co-infection of a Pin1-encoding retrovirus vector with those encoding four defined reprogramming factors (4F; SOX2, Oct4, Klf-4, and c-Myc) (24) notably boosted the generation of AP-positive iPS cell colonies compared with an induction of human fibroblast MRC5 cells with only four iPS factors (Fig. 1, *C* and *D*). We next performed a parallel experiment using either a WW-domain (binding domain) mutant (W34A) or a peptidyl prolyl isomerase-domain (catalytic domain) mutant (K63A) of Pin1. We confirmed the equivalent expression of each of these mutants and wild-type Pin1 (Fig. 1*E*). Neither of these mutants could boost iPS cell colony formation to the level seen with wild-type Pin1 (Fig. 1*F*), indicating that both the WW and PPIase domains are required for this function.

To test pluripotency *in vivo*, we transplanted 4F plus Pin1-introduced iPS cells subcutaneously into the dorsal flanks of immunodeficient mice. Nine weeks after injection, we observed teratoma formation composed of various tissues including gut-like epithelial tissues (endoderm), striated muscle (mesoderm), cartilage (mesoderm), neural tissues (ectoderm), and epidermal tissues (ectoderm) (Fig. 1*G*). These results indicate that the expression of Pin1 with defined reprogramming factors accelerates the frequency of iPS cell generation.

*Pin1 Is Required for Pluripotent Stem Cell Self-renewal and Colony Formation*—We next addressed whether Pin1 indeed plays any roles in the self-renewal of human iPS cells. iPS cells were dissociated with accutase and then plated at a clonal density in the presence of several concentrations of the selective Pin1 inhibitor juglone (5-hydroxy-1,4-naphthoquinone) (25, 26). The blockade of Pin1 by juglone considerably reduced both the numbers and size of the colonies in a dose-dependent manner (Fig. 2, *A–C*). It was notable also that the concentration of juglone used did not illicit nonspecific toxic effects in the feeder mouse embryonic fibroblast cells (Fig. 2*A* and data not shown). The effect of Pin1 inhibition upon colony formation was also confirmed in feeder-free cultures of human iPS cells by AP staining (Fig. 2*D*). Moreover, treatment with the Pin1 inhibitory phosphopeptide PINTIDE (27), but not a nonphosphorylated control peptide, significantly reduced the colony formation of human iPS cells (Fig. 2, *E* and *F*).

We next investigated the effects of Pin1 inhibition upon colony formation in murine ES cells. The blockade of Pin1 by juglone significantly reduced the colony numbers in two different murine ES cell types, BDF2 and R1 (Fig. 3*A*). The adenovirus-mediated transduction of a GFP-fused dominant-negative Pin1 (GFP-dnPin1) (28), but not a GFP control, significantly suppressed colony formation in murine ES (R1) cells manifesting as a considerable reduction in both the numbers and colony size of the murine ES cells (Fig. 3, *B–D*). These results together demonstrate that Pin1 is indispensable for the self-renewal and proliferation of pluripotent stem cells.

*Pin1 Functions in Maintenance of Pluripotency*—We next asked whether Pin1 has any roles in the maintenance of pluripotency in stem cells. Human iPS cells were dissociated and then cultured for 5 days to form colonies. When human iPS cells are cultured in hES medium supplemented with basic fibroblast growth factor, the overwhelming majority of the cells in the colonies are undifferentiated (Fig. 4*A*). However, treatment with juglone resulted in aberrant cell differentiation resulting in a "mosaic pattern" of iPS cell colonies following AP staining (Fig. 4*A*). Similarly, the adenovirus-mediated transduction of GFP-dnPin1, but not a GFP control, prominently reduced the number of AP-positive undifferentiated cells in murine ES cell colonies (Fig. 4*B*). These results together indicate that Pin1 can sustain pluripotent stem cells in an undifferentiated state in addition to the enhancement of self-renewal.

FIGURE 2. **Defective self-renewal of human iPS cells caused by Pin1 inhibition.** *A–C*, human iPS cells were dissociated with accutase and then plated on a feeder cell layer at a clonal density in the presence of the indicated concentrations of juglone for 3 days. Colony formation was analyzed by phase-contrast microscopy (*A*). The number of colonies was counted at 3 days after treatment (*B*). The number of cells per colony was determined by manually counting the DAPI-stained cells (*C*). Data are the mean ± S.E. *D*, human iPS cells were plated at a clonal density on the feeder-free culture in the presence of the indicated concentrations of juglone followed by AP staining. *E* and *F*, human iPS cells were dissociated with accutase and then plated on feeder-free dishes at a clonal density in the presence of 50 μg/ml of the Pin1 inhibitory phosphopeptide PINTIDE (RRRRRRRRRWFYpSPRLKK) or a nonphosphorylated control peptide (RRRRRRRRRWFYAPRLKK) for 48 h (*E*). AP-positive colony numbers were scored (*F*). Data are the mean ± S.E. *Scale bar*, 50 μm.

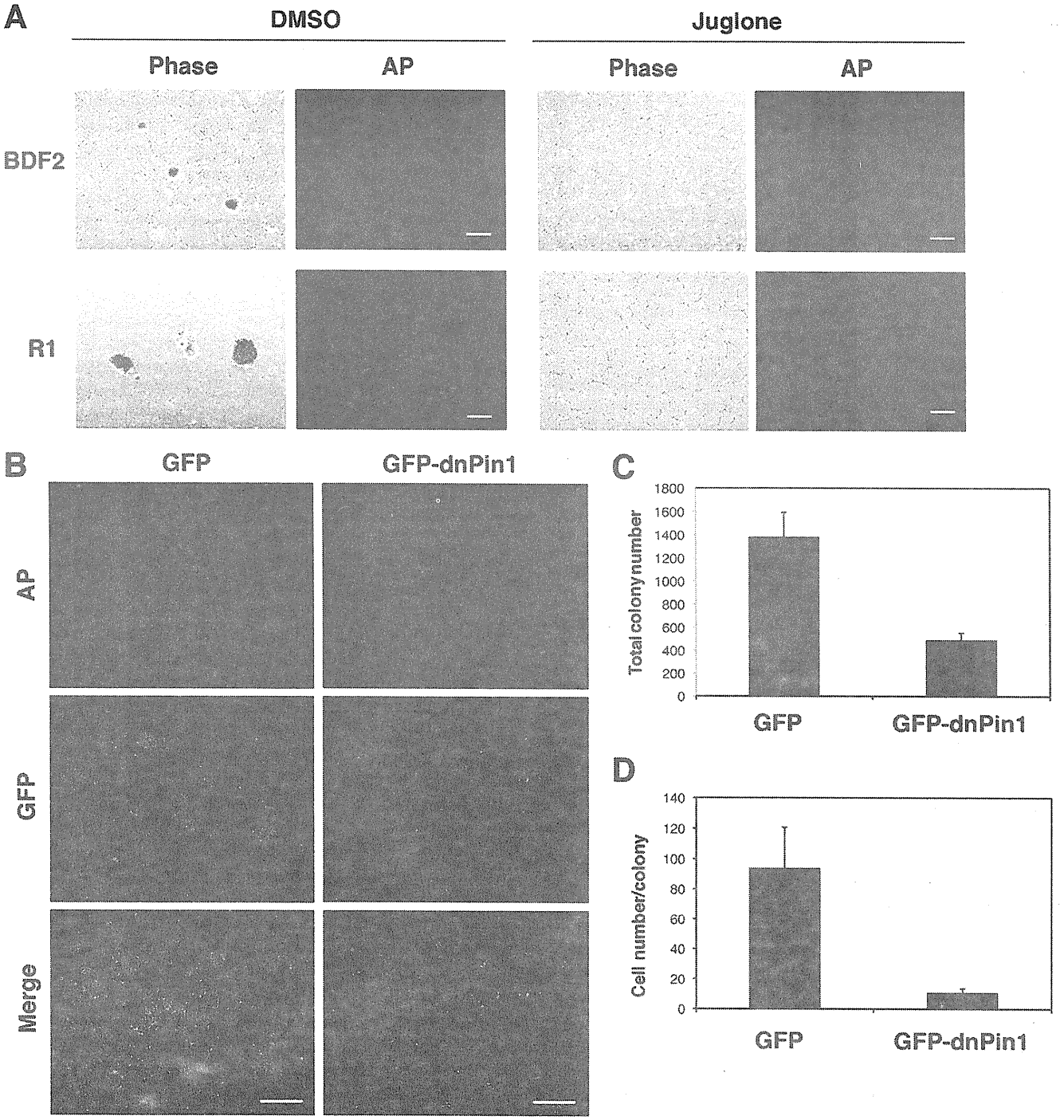



FIGURE 3. **Pin1 inhibition suppresses colony formation in murine ES cells.** *A*, two different murine ES cell types (BDF2 and R1) were plated on gelatin-coated dishes and treated with either DMSO or juglone (10 $\mu$M). Colonies were stained with AP (*red*). *Scale bar*, 200 $\mu$m. *B–D*, murine ES cells (R1) were infected with an adenovirus vector encoding either GFP or GFP-dnPin1 (3000 viral particles/cell). The cells were then stained with AP (*red*) and DAPI and analyzed by immunofluorescent microscopy (*B*). *Scale bar*, 200 $\mu$m. The total colony number (*C*) and the number of cells per colony (*D*) were then determined. Data are the mean ± S.E.

*Identification of Pin1 Binding Proteins in Human iPS Cells*—Our initial data indicated that Pin1 could enhance the function of reprogramming factors during the induction and maintenance of pluripotency. We next identified the substrates targeted by Pin1 in human iPS cells. Using a monoclonal Pin1 antibody, we co-immunoprecipitated proteins from human iPS cell lysates treated with a phosphatase inhibitor mixture. These isolated immune complexes were then boiled and resolved by one-dimensional SDS-PAGE, and the proteins were visualized using silver staining. Continuous regions of the gel corresponding to proteins of ~30 to 150 kDa in size were systematically excised (Fig. 5*A*), digested with trypsin, and analyzed in a linear

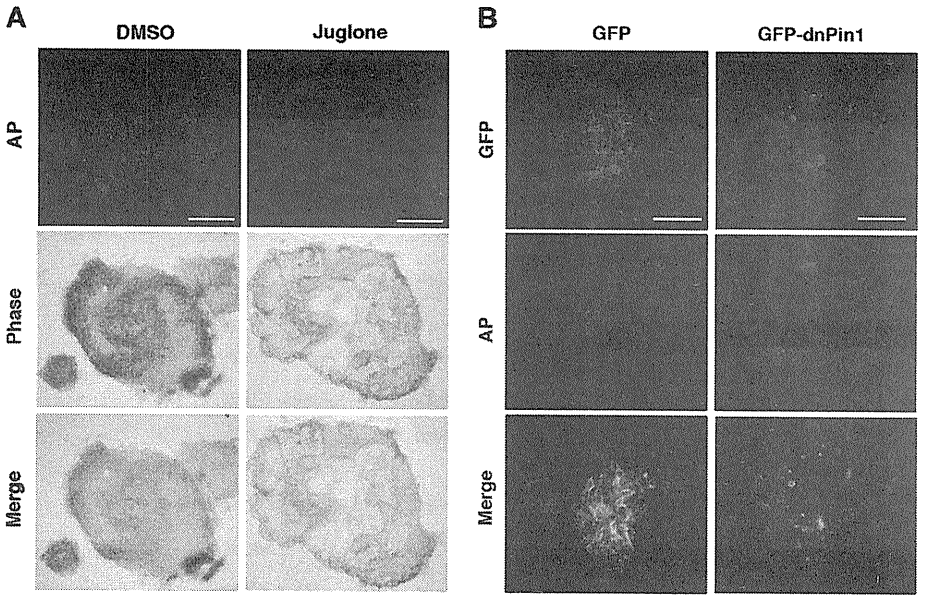



FIGURE 4. **Pin1 inhibition leads to the aberrant cell differentiation of human iPS cells.** *A*, human iPS cells were cultured for 5 days before forming colonies and then treated with either DMSO or juglone (10 $\mu$M) for 3 days. The cells were then stained with AP (*red*). Representative images of phase-contrast microscopy and fluorescent immunocytochemistry are shown. *Scale bar*, 200 $\mu$m. *B*, mouse ES cells were cultured for 2 days before forming colonies and then infected with an adenovirus vector encoding either GFP or GFP-dnPin1 (3000 viral particles/cell). After 48 h, the cells were then stained with AP (*red*) and DAPI (*blue*) and analyzed by immunofluorescent microscopy. *Scale bar*, 50 $\mu$m.

ion trap (LTQ) Orbitrap hybrid mass spectrometer. Peptide mass fingerprinting with the Mascot and Aldente search algorithms subsequently identified 23 Pin1 interacting proteins in human iPS cells (Fig. 5*B*). Notably, these Pin1-binding proteins included the pluripotent transcription factor Oct4. Because Oct4 has been shown to be a master regulator of pluripotency (29), we decided to further analyze the Oct4-Pin1 interaction.

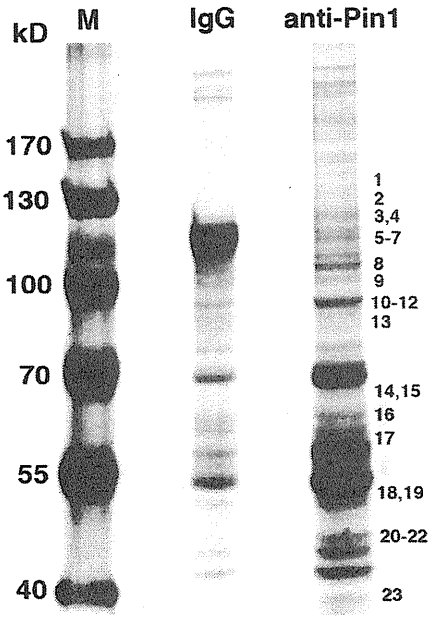
*Pin1 Binds and Regulates Protein Stability of Oct4*—To further characterize the Oct4-Pin1 interaction, a GST pulldown analysis was initially performed. We found that recombinant GST-Pin1, but not GST alone, binds Oct4. This association was completely abolished by pretreatment of the cell lysates with calf intestine alkaline phosphatase (Fig. 6*A*), indicating that Pin1 binds phosphorylated Oct4. Immunofluorescence analysis further demonstrated that Pin1 co-localizes with Oct4 in the nuclei of iPS cells (Fig. 6*B*). Pin1 has been shown to regulate the stability of its substrate proteins upon binding (17), and we thus addressed whether this was the case for Oct4. Cycloheximide analysis using HeLa cells transfected with Oct4 alone or co-transfected with Oct4 and Pin1 revealed that the protein half-life of Oct4 is significantly enhanced in cells co-expressing Pin1 (Fig. 6*C*). Moreover, immunoprecipitation analysis with cells co-transfected with Oct4 and Myc-tagged ubiquitin, with or without Pin1 co-transfection, further revealed that Pin1 overexpression significantly reduces the polyubiquitination of the Oct4 protein (Fig. 6*D*). Consistently, the Oct4 protein expression level was significantly reduced in human iPS cells treated with juglone as compared with control cells (Fig. 6*E*). These results together confirm that Pin1 enhances the protein stability of Oct4 by suppressing ubiquitin proteasome-mediated proteolysis.

We next investigated the gene expression profile of Oct4 during the inhibition of Pin1. Murine ES cells were transfected with pGL4-Oct4-2601 promoter (harboring a genomic fragment of the Oct4 gene 5′-upstream region) and treated or not with juglone. Pin1 inhibition by juglone did not affect the transcriptional activity of the Oct4 promoter (Fig. 6*F*). Consistently, the results of parallel quantitative RT-PCR analysis demonstrated that the Oct4 mRNA level was not significantly altered by Pin1 inhibition (Fig. 6*G*), whereas the Oct4 protein level was significantly reduced by juglone treatment, as revealed by immunoblot analysis (Fig. 6*H*). These results together indicate that Pin1 regulates the protein stability of Oct4 but not Oct4 transcription.

We next addressed whether Pin1 enhances the transcriptional activity of the Oct4 protein. A luciferase reporter assay using the Oct-Sox enhancer region derived from the FGF4 gene was performed in HeLa cells co-transfected with Oct4, SOX2 or Pin1. Although the sole expression of Pin1 had no significant effects, the co-expression of Oct4 and Pin1 produced a significant increase in reporter activity in a dose-dependent fashion (Fig. 6*I*). This indicated that Pin1 promotes Oct4-mediated transcriptional activation. We performed a parallel experiment using the W34A and K63A Pin1 mutants. Neither of these mutants up-regulated the transcriptional activity of Oct4 to the levels seen with wild-type Pin1 (Fig. 6*J*), indicating that both the WW and PPIase domains are required for this function.

*Pin1 Interacts with Ser$^{12}$-Pro motif of Oct4*—To identify the specific Pin1 binding site within the Oct4 protein, we generated several Oct4 deletion mutants and performed GST-pulldown analysis. These experiments revealed that a C-terminal Oct4 deletion mutant (representing amino acids 1–297) could still bind Pin1, but that three extended N-terminal deletion mutants (amino acids 138–360, 113–360, or 34–360) failed to do so (Fig. 7*A*). These data indicate that Pin1 binds to Oct4 in the region between amino acids 1 and 34. Previous reports have indicated

A




B

| No. | Accession No. | Gene description | Predicted size |
|---|---|---|---|
| 1 | ADCY5_HUMAN | Adenylate cyclase type 5 | 138818 |
| 2 | CCD40_HUMAN | Coiled-coil domain-containing protein 40 | 130033 |
| 3 | PK3CA_HUMAN | Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha | 124203 |
| 4 | ZEB1_HUMAN | Zinc finger E-box-binding homeobox 1 | 123997 |
| 5 | VINC_HUMAN | Vinculin | 123722 |
| 6 | RADIL_HUMAN | Ras-associating and dilute domain-containing protein | 117351 |
| 7 | UBP2L_HUMAN | Ubiquitin-associated protein 2-like | 114465 |
| 8 | DSG1_HUMAN | Desmoglein-1 | 113644 |
| 9 | ENPP3_HUMAN | Ectonucleotide pyrophosphatase/phosphodiesterase family member 3 | 100059 |
| 10 | ZN337_HUMAN | Zinc finger protein 337 | 86819 |
| 11 | NASP_HUMAN | Nuclear autoantigenic sperm protein | 85186 |
| 12 | ZY11B_HUMAN | Protein zyg-11 homolog B | 83921 |
| 13 | MPEG1_HUMAN | Macrophage-expressed gene 1 protein | 78587 |
| 14 | FA13C_HUMAN | Protein FAM13C | 65687 |
| 15 | VPS45_HUMAN | Vacuolar protein sorting-associated protein 45 | 65036 |
| 16 | ANR53_HUMAN | Ankyrin repeat domain-containing protein 53 | 59493 |
| 17 | RPA34_HUMAN | DNA-directed RNA polymerase I subunit RPA34 | 54951 |
| 18 | VIME_HUMAN | Vimentin | 53619 |
| 19 | KCAB1_HUMAN | Voltage-gated potassium channel subunit beta-1 | 46534 |
| 20 | PRS8_HUMAN | 26S protease regulatory subunit 8 | 45597 |
| 21 | FKBP8_HUMAN | Peptidyl-prolyl cis-trans isomerase FKBP8 | 44534 |
| 22 | PO5F1_HUMAN | POU domain, class 5, transcription factor 1 (OCT4) | 38571 |
| 23 | THAP1_HUMAN | THAP domain-containing protein 1 | 24928 |

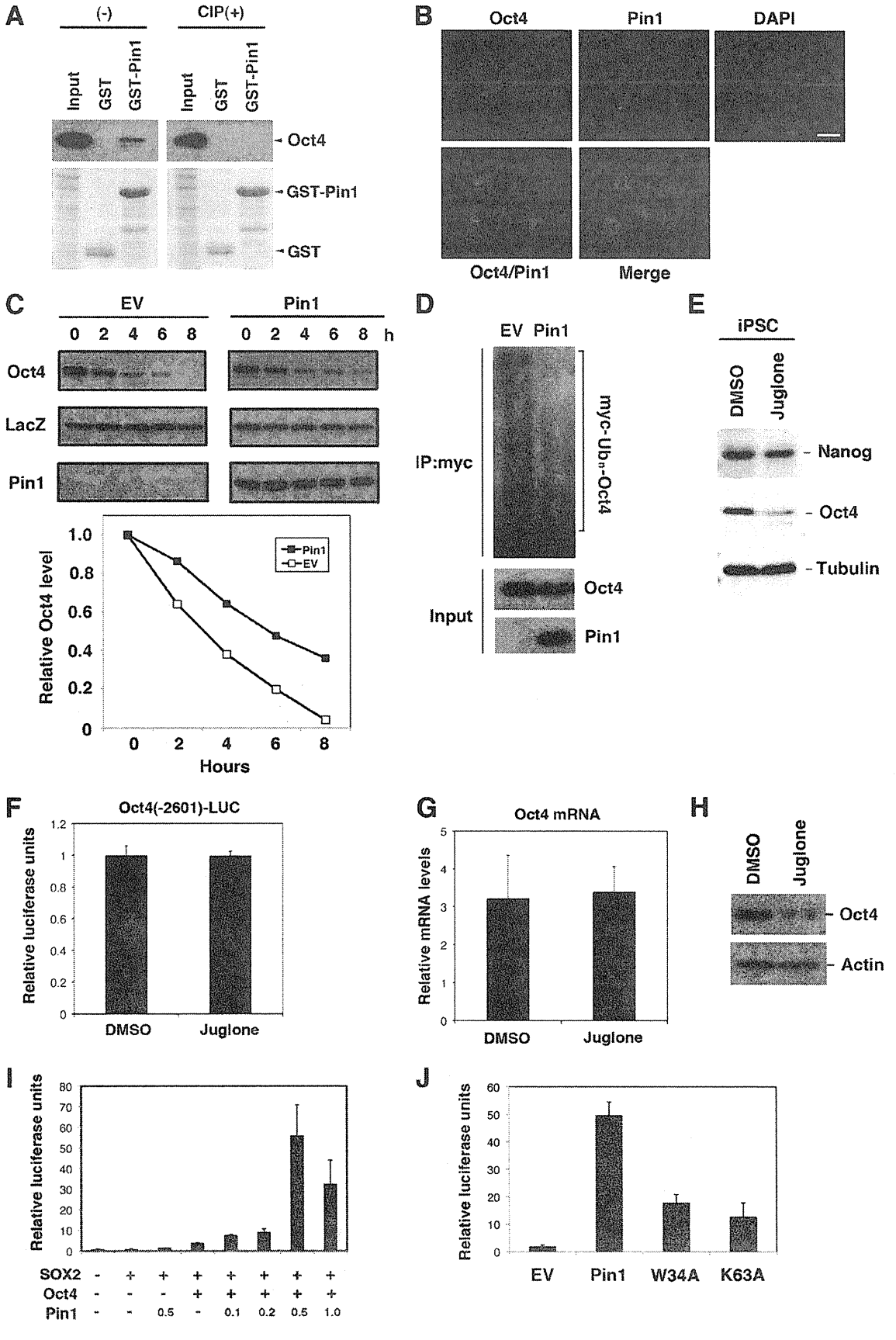
FIGURE 5. **Identification of Pin1-binding proteins in human iPS cells.** *A* and *B*, lysates of human iPS cells were subjected to immunoprecipitation with either non-immunized control mouse IgG (IgG) or mouse anti-Pin1 monoclonal antibodies. Proteins bound to protein A/G-agarose beads were isolated, resolved by SDS-PAGE, and detected by silver staining (*A*). *M* indicates protein marker. Excised gel bands were digested with trypsin and analyzed on a linear ion trap (LTQ) Orbitrap hybrid mass spectrometer followed by peptide mass fingerprinting with the Mascot and Aldente search algorithms (*B*).

that Pin1 can bind only phosphorylated Ser/Thr-Pro motifs (17, 27) of which only one ($Ser^{12}$-Pro) exists between residues 1 and 34 in the Oct4 protein. Interestingly, this motif is conserved between various species including human, mouse, rat, and rabbit (Fig. 7*B*). We generated an Oct4 site-directed mutant at this site by substituting serine 12 for alanine (S12A). GST pulldown analysis subsequently revealed that Pin1 binds wild-type Oct4, but not its S12A mutant (Fig. 7*C*). These results confirm that Pin1 indeed bind the phosphorylated $Ser^{12}$-Pro motif of Oct4.

To further examine the functional interactions between Pin1 and Oct4 on this site, we next investigated the nature of the S12A mutant in terms of its protein expression in the presence of Pin1. HeLa cells were transfected with either wild-type Oct4 or its S12A mutant and co-transfected with Pin1. This was followed by immunoblotting analysis. We found that Pin1 increased the expression levels of wild-type Oct4, but not the S12A mutant (Fig. 7*D*).

## DISCUSSION

In our present study, we report that Pin1 is an essential regulator of the self-renewal and maintenance of pluripotent stem cells. We further found the following: 1) Pin1 is induced upon the induction of human iPS cells; 2) the co-expression of Pin1 with defined reprogramming factors significantly enhances the



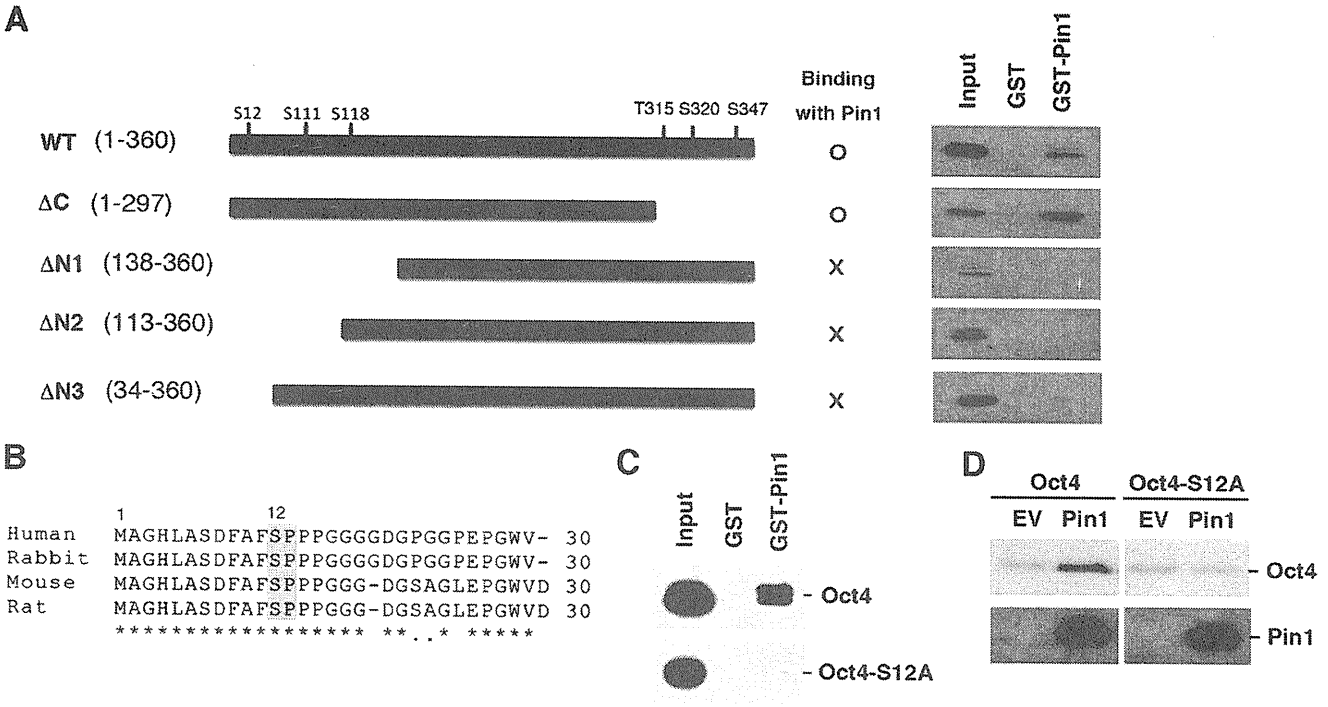



FIGURE 7. **Pin1 interacts with the Ser[12]-Pro motif of Oct4.** *A*, schematic representation of the Oct4 deletion mutants generated in this study (*left panel*). HeLa cells were transfected with the indicated Oct4 deletion mutants for 24 h. Cell lysates were then prepared and subjected to GST pulldown analysis with either GST or GST-Pin1 followed by immunoblotting analysis with Oct4 antibodies (*right panel*). *B*, amino acid sequence alignment of the human, rabbit, mouse, and rat Oct4 proteins. The conserved Ser[12]-Pro motifs are *boxed*. *C*, HeLa cells were transfected with the Oct4 site-directed mutant Oct4-S12A and subjected to GST pulldown analysis. *D*, HeLa cells were transfected with wild-type Oct4 or its S12A mutant with or without Pin1. After 24 h, the cells were subjected to immunoblotting analysis with an anti-Oct4 antibody.

frequency of iPS cell induction; 3) the blockade of Pin1 significantly inhibits the colony formation of dissociated human iPS cells and murine ES cells; 4) Pin1 inhibition leads to the aberrant cell differentiation in human iPS cells and murine ES cells after forming colonies; 5) Oct4 is a putative Pin1 substrate in human iPS cells; and 6) Pin1 interacts with Oct4 at its Ser[12]-Pro motif and facilitates its stability and enhanced transcriptional activity. Our findings thus uncover a novel role of Pin1 as a putative regulator of the self-renewal and survival of pluripotent stem cells via Oct4 function.

Our current results add to previous findings indicating that Pin1 is a multifunctional protein that mediates various phosphorylated proteins involved in divergent cellular processes (17). This implicates Pin1 as a modulator of multiple signaling pathways depending on the cell type and biological context. Indeed, we demonstrate in our present study that Pin1 is a crucial regulator of the phosphorylation-dependent intracellular signaling network that controls cellular stemness and pluripotency. Moreover, iPS cells induced by the expression of four Yamanaka factors (Oct4, SOX2, Klf4, and c-Myc) led to a high expression level of Pin1, and these cells were found to be dependent on Pin1 function. This suggests that Pin1 could be one of the crucial factors in the induction of iPS cells from somatic cells that functions by cooperating with reprogramming transcription factors.

FIGURE 6. **Pin1 interacts with phosphorylated Oct4 and enhances its transcriptional activity.** *A*, human iPS cell lysates treated or untreated with calf intestine alkaline phosphatase were subjected to GST pulldown analysis with either GST or GST-Pin1, followed by immunoblotting analysis with anti-Oct4 antibody (*upper panel*). Coomassie staining for the GST or GST-Pin1 used in the assay is shown in the *lower panel*. *B*, human iPS cells were fixed with 4% paraformaldehyde and then co-immunostained with monoclonal antibodies against Oct4 (*green*) and polyclonal antibodies against Pin1 (*red*). Cells were then analyzed by confocal microscopy. *Scale bar*, 10 μm. *C*, HeLa cells transfected with the indicated vectors and HA-LacZ cells were treated with cycloheximide and harvested at the indicated time points. This was followed by immunoblotting analysis with Oct4, Pin1, and HA antibodies (*upper panel*). Quantitative data are shown in the *lower panel*. *D*, HeLa cells were transfected with Myc-tagged ubiquitin, Oct4, and co-transfected with either empty vector (*EV*) or Pin1. Cells were then treated with MG-132 for 12 h, and lysates were prepared and immunoprecipitated with anti-Myc antibody followed by immunoblotting analysis with anti-Oct4 antibody. Total cell lysates prior to immunoprecipitation (input) were immunoblotted with anti-Pin1 or anti-Oct4 antibody. *E*, human iPS cells were plated on Matrigel-coated feeder-free dishes and treated with either DMSO or juglone (20 μM) for 24 h. Cell lysates were then processed for immunoblotting analysis with anti-Nanog, anti-Oct4, or anti-tubulin antibodies. *F*, a plasmid containing the luciferase (*LUC*) gene flanked with 2601 bp of the Oct4 5′-upstream region was transfected into murine ES cells. The resulting cells were cultured in Matrigel-coated feeder-free dishes and treated with either DMSO or juglone (10 μM) for 24 h, and analyzed by gene reporter assay. *G*, murine ES cells were cultured in Matrigel-coated feeder-free dishes and treated with either DMSO or juglone (10 μM) for 24 h. Total RNAs were then extracted and reverse-transcribed. These preparations were then subjected to quantitative RT-PCR analysis for Oct4. The transcript levels were normalized using GAPDH. *H*, murine ES cells were cultured in Matrigel-coated feeder-free dishes and treated with either DMSO or juglone (10 μM) for 24 h. Cell lysates were then processed for immunoblotting analysis with either anti-Oct4 or anti-β-actin antibody. *I*, HeLa cells were transiently transfected with plasmids encoding Oct4, SOX2, or Pin1 and co-transfected with Oct-SOX reporter gene and pRL-CMV. At 24 h post-transfection, the cells were collected and subjected to a gene reporter assay. *J*, HeLa cells were transiently transfected with an Oct-SOX reporter gene and co-transfected with plasmids encoding wild-type Pin1 or its W34A or K63A mutants, together with Oct4 and SOX2. At 24 h post-transfection, the cells were collected and subjected to a gene reporter assay.