2.9. Cellular transformation

The classical PARP inhibitors were shown to modulate cell transformation after DNA damage. PARP-10 has been demonstrated to mono (ADP-ribosylate) PARP-10 itself and modify core histones (Kleine et al., 2008; Yu et al., 2005a). PARP-10 suppressed EIA-involved cellular transformation possibly through interaction with c-Myc (Yu et al., 2005a). PARP-10 is reported to shuttle between the cytoplasm and nucleus, and involvement in autophagy is suggested (Kleine et al., 2012). However, its function in carcinogenesis awaits further studies.

2.10. Cell death control

Cell death pathways are important for suppressing carcinogenesis. During apoptosis, PARP-1 is cleaved while PARP-1 activity is not essential for induction of apoptosis in most cases. After massive DNA damage and PARP-1 activation, PAR formation causes AIF (apoptosis inducing factor)-dependent cell death induction, in neuronal cells and also in cancer cells (Yu et al., 2006), whereas induced NAD depletion and subsequent ATP depletion also cause a type of necrosis. In these types of cell death, PARP-1 activity is essential and PARP inhibitor was shown to suppress cell death.

Autophagy-associated cell death is also reported to be involved in suppression of cancer. PARP-1 is reported to act in induction of autophagy (Munoz-Gamez et al., 2009; Rodriguez-Vargas et al., 2012). For example, PARP-1 was shown to be involved in induction of autophagy caused by cigarette smoke and when PARP-1 is inhibited, SIRT1 is activated and suppresses autophagy (Hwang et al., 2010). PARP10 is also suggested to be involved in autophagy processes by interacting with poly-ubiquitin receptor p62 (Kleine et al., 2012).

Of note, it has been recently reported that bcl-2 binds to PARP-1 and suppresses its activity and nonapoptotic cell death (Dutta et al., 2012). Therefore, bcl-2 overexpressing B-cell lymphomas and chronic lymphocytic leukemia showed decreased PARP-1 activity, and because cell death is blocked by bcl-2 these tumor cells may show accelerated genomic instability and may develop malignant phenotypes.

3. Tumor cell biology

Tumor malignancy depends highly on migratory and invasive abilities of cancer cells. In some cases, cancer cells gain these abilities during the process of EMT induced by TGF-β signaling factors or inflammation stress through introduction of additional mutations or by causing transcriptional aberration.

3.1. Cell migration, invasion and metastasis regulation

Under stimulation with erythropoietin, produced by cancer during anemia and hypoxia, PARP-1 induced cell migration through transcription of *c-fos*, *Egr-1* in breast cancer cells MDA-MB-435 (Inbar et al., 2012). Indeed, high expression levels of PARP-1, chromatin assembly factor-1 and nestin are regarded as the worst prognosis phenotypes of oral squamous cell carcinomas (Mascolo et al., 2012). It is reported that PARP-1 inhibition caused transcriptional suppression of NF-κB and MMP-2/9 in colon carcinoma cells CT26 (Li et al., 2009) and PARG dysfunction also suppressed metastasis via NF-κB downregulation in colon carcinoma cells (Li et al., 2012).

Furthermore, the PARP family is involved in angiogenesis. In fact, low doses of PARP inhibitor inhibited the migration of human umbilical vein endothelial cells HUVEC (Caldini et al., 2011) and angiogenesis (Pyriochou et al., 2008; Rajesh et al., 2006; Tentori et al., 2007). PARP inhibition down-regulated transcriptional factors, *OCT-1* and *CREB* leading to reduced expression of endothelial cell migration and adhesion associated genes, *SDC4* and *ID-1* (Lacal et al., 2009). *PARG*-knockdown caused downregulation of NF-κB and ERK/p38 activities in colon carcinoma cells and decreased the level of angiogenic factors such as VEGF and inhibited migration and proliferation of co-cultured HUVEC (Pan et al., 2012).

Functional inhibition of PARG also suppressed metastastic properties of colon carcinoma cells in a model of liver metastasis with increased levels of the Akt pathway, and a decreased expression of MMP2/9 and NF-κB pathway (Li et al., 2012).

3.2. Cell survival signaling

PARP-9/14/15 are Bal (B-aggressive lymphoma 1) family proteins (Aguiar et al., 2005), which have macrodomains. PARP-9 was found to be overexpressed in B cell aggressive lymphoma, including diffuse large B-cell lymphoma. PARP-9 contains two macrodomains, which bind both an ADP-ribose monomer and poly(ADP-ribose). PARP-9 was also able to promote tumor cell invasion (Aguiar et al., 2000). PARP-14 recruited histone deacetylase (HDAC)2/3 to IL14 responsive genes and promoted binding of Stat6 and further ADP-ribosylated HDACs to replace them by histone acetyl transferase and activated transcription (Mehrotra et al., 2011), thus showing an IL14-dependent transcriptional switch property. PARP-14 activated IL14-dependent glycolysis in B cells and also suppressed apoptosis. PARP-14 thus supported IL-14-induced survival during Myc-induced B cell lymphomagenesis (Cho et al., 2011). PARP-14 was also highly expressed in multiple myeloma and its high expression was associated with the poor diagnosis. PARP-14 showed binding to JNK1 and inhibited its activity and

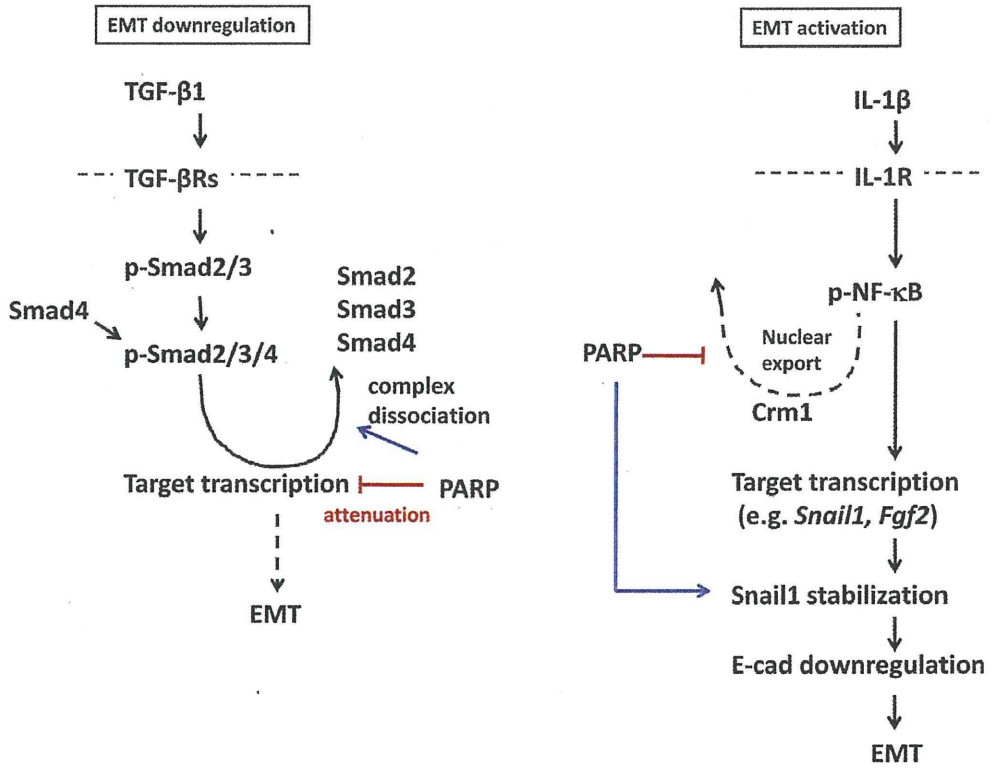

Fig. 3. The model for EMT suppression and activation by PARP-1. PARP-1 suppresses the TGF-β dependent EMT by Smad degradation but accelerates the Snail 1 dependent EMT by E-cad suppression via Snail l stabilization. The bilateral opposing characteristics of PARP-1 in EMT induction are consistent with its buffering roles for maintaining cellular homeostasis.

enhanced JNK-2-dependent survival signaling (Barbarulo et al., 2012), which counteract JNK1-mediated apoptosis. Bal family PARPs thus widely support survival signaling and also promote tumor malignancy through their macro domain function.

3.3. EMT and cancer stem cell maintenance

In the EMT pathway, E-cadherin (E-cad) is the main target to be suppressed by the TGF-β signaling pathway (Brandl et al., 2010; Nieto, 2002). In TGF-β/Smad regulation, PARP-1 dependent dissociation of Smad complexes from DNA by PARylating Smad3/4 attenuated Smad-specific gene responses and TGF-β-induced EMT (Huang et al., 2011; Lonn et al., 2010) as shown in Fig. 3 (left panel). On the other hand, under conditions of Snail/E-cad suppression, PARP-1 binding to integrin-linked kinase responsive element maintained Snail 1 transcription and resulted in downregulation of E-cad transcription (McPhee et al., 2008), resulting in EMT activation (Fig. 3, right panel). Furthermore, NF-κB and PARP-1 activated fibronectin gene transcription, which is important for cancer cell maintenance and migration ability (Stanisavljevic et al., 2011). Taken together, it is suggested that PARP-1 suppresses the TGF-β dependent EMT by Smad degradation but accelerates the Snail 1 dependent EMT by E-cad suppression via Snail l stabilization (Fig. 3).

The involvement of PARPs in cancer stem cell (CSC) maintenance has been also suggested; PARP inhibitor showed anti-CSC activity in basal-like breast cancer cell lines (Shimo et al., 2012). This could be related to the coactivator function of PARP-1 for NF-κB because constitutive NF-κB activation correlated to proliferation of basal-like subtype breast cancer cells (Yamaguchi et al., 2009). Furthermore, PARP inhibitor blocked development of cancerous stem cells by suppressing senescence induction process of differentiating ESCs (Fujimori et al., 2012). Further studies are required to elucidate the roles of PARP family molecules in CSC regulation.

4. Animal models and genetic studies in human cancers

4.1. Animal models

With classical types of PARP inhibitors, the roles of PARP in carcinogenesis were previously investigated using various carcinogens in animal models. Using streptozotocin, the incidence of insulinoma in the pancreas increased by the initial combinational treatment with benzamide or 3-aminobenzamide (Yamagami et al., 1985), suggesting the involvement of PARylation in the suppression of tumorigenesis. Potent PARP inhibitors are now in clinical trials for cancer therapy. By single

treatment or in combination with carcinogens, the effects of PARP inhibitors on carcinogenesis are expected to be evaluated in the future to consider long-term carcinogenic risks.

On the other hand, it is reported that PARP-1 acts as an enhancer of tumorigenesis in some experimental models. After 7,12-dimethylbenz(a)anthracene plus 12-O-tetradecanoylphorbol-13-acetate treatment, PARP-1 inhibition results in a delay in skin tumor formation. This could be related to compromised activation of AP-1, NF-κB and HIF-1α by PARP inhibition (Martin-Oliva et al., 2006). PARP-1 downregulation also abolished tumorigenicity of melanoma (Tentori et al., 2008) and suppression of PARG promoted tumorigenicity (Molloy-Simard et al., 2012). The susceptibility of knockout mice models suggests that multiple ADP-ribosylation-related proteins are involved in carcinogenesis (Table 1).

4.2. Genetic studies in human cancers

Genetic studies on the association of polymorphism of the *PARP-1* gene with the cancer have been carried out as summarized in Table 2. The *Ala/Ala* allele at *VAl762Ala* single nucleotide polymorphism (SNP) was shown to lead to lower PARP-1 activity (Wang et al., 2007) and this *Ala* allele was associated with the risk of prostate cancers in Caucasians (Lockett et al., 2004), for esophageal (Hao et al., 2004), and lung cancers (Zhang et al., 2005) in Chinese populations. *Val762Ala* polymorphism was also associated with the risk of cervical carcinoma in Chinese populations (Ye et al., 2012). However, recent meta-analysis showed that association of the *Val762Ala* polymorphism with an increased risk of cancer was found in Asians, whereas a decreased risk of cancer, such as glioma, was detected among Caucasians (Yu et al., 2012). No significant association was detected between *Val762Ala* polymorphism and cancer in an overall population, suggesting that the polymorphism could be affected by genes that may be different among ethnic populations. Investigation of breast cancers from French patients revealed the genetic variants and SNPs of *PARP-1* at 10%, in which one SNP (*Ala284Ala*) was likely associated with loss of estrogen- and progesterone-receptor expression (Cao et al., 2007).

When microRNA binding site polymorphisms in DNA repair genes were analyzed, SNP rs8679 at the 3'-untranslated region of the *PARP-1* gene was shown to be associated with an increased breast cancer risk. One of the candidate target miRNAs is *miR145*, and a further validation study is awaited (Teo et al., 2012). It is also notable that increased risk of melanoma with SNP rs321925, present in the intron of the *PARP-1* gene near the SNP *Val762*, was observed in Caucasian populations (Zhang et al., 2011), although no association with *Val762Ala* was observed. The biological significance is yet to be clarified.

Mutations of the *PARP-1* gene were quite rare in cancers, in contrast to the high mutation frequency in the DNA polymerase β, which is also involved in base excision repair (BER) (Chan et al., 2006), suggesting PARP-1 function may be required for cancer cell survival.

PARP-2 was reported to be involved in the risk of therapy-related acute myeloid leukemia, a secondary malignant leukemia developed after chemotherapy (Cahan and Graubert, 2010). The mutations and SNP association of other PARP family proteins, PARG, ARTCs, and ARHs have not been extensively studied yet.

On the other hand, the expression changes of PARP family proteins have been reported and from the early step of carcinogenesis. *PARP-1* expression became augmented, including in colorectal cancers (Nosho et al., 2006), and endometrial cancers (Postawski et al., 2011). In the lung cancers, PARP-1 expression is higher in small cell lung cancers (SCLC). In fact, PARP inhibitors reduced growth and augmented the effect of cytotoxic agents in SCLC (Byers et al., 2012). *PARP-1* expression was

Table 1
The susceptibility to carcinogenesis in mouse models.

Molecule	Spontaneous/induced	Outcome	Tissue or tumor type	References
Parp-1$^{-/-}$ (ex2)	Spontaneous (18–24 months)	Increased	Hepatocellular carcinoma	Tong et al. (2002)
Parp-1$^{-/-}$ (ex1)	Spontaneous (7 months)	No change	Various tissues	Tsutsumi et al. (2001)
	Spontaneous (15 months)	No change	Various tissues	Ogawa et al., 2006
Parp-1$^{-/-}$ (ex2)	Spontaneous	Increased	Uterine, lungs, hepatocellular carcinoma	Piskunova et al. (2009)
Parp-1$^{-/-}$ (ex2)	Spontaneous	Increased	Breast	Tong et al. (2007)
Parp-1$^{-/-}$ (ex1)	BHP	Increased	Hemangioma, hemangiosarcoma	Tsutsumi et al. (2001)
Parp-1$^{-/-}$ (ex1)	Azoxymethane	Increased	Colon, liver (nodule)	Nozaki et al. (2003)
Parp-1$^{-/-}$ (ex2)	Diethylnitrosamine	Increased	Hepatocellular carcinoma	Tong et al. (2002)
Parp-1$^{-/-}$ (ex1)	4-Nitroquinoline 1-oxide	No change	Oral cavity, esophagus	Gunji et al. (2006)
Parp-1$^{-/-}$ (ex1)	IQ	No change	Liver, forestomach	Ogawa et al. (2006)
Parp-1$^{-/-}$ (ex2)	*p53*$^{-/-}$	Increased	Colon, breast, brain	Tong et al. (2001, 2007)
Parp-1$^{-/-}$ (ex4)	*p53*$^{-/-}$	Decreased	Thymic lymphoma	Conde et al. (2001)
Parp-1$^{-/-}$ (ex2)	*Ku80*$^{-/-}$	Increased	Liver	Tong et al. (2002)
Parp-1$^{-/-}$ (ex2)	*SCID*	Increased	Thymus	Morrison et al. (1997)
Parp-1$^{-/-}$ (ex2)	*Ptc*$^{-/-}$	Increased	Medulloblastoma, basal cell carcinoma	Tanori et al. (2008)
Parp-1$^{-/-}$ (ex2)	*Wrn*$^{Dhel/Dhel}$	Increased	Various tissues	Lebel et al., 2003
Parp-2$^{-/-}$ *p53*$^{-/-}$		Increased	T-cell lymphoma	Nicolas et al. (2010)
Parp-4$^{-/-}$	Diethylnitrosamine	Increased	Colon	Raval-Fernandes et al. (2005)
Parp-4$^{-/-}$	Urethane	Increased	Lungs	Raval-Fernandes et al. (2005)
Parg$^{-/-}$ (110 kD)	Diethylnitrosamine	Increased	Liver	Min et al. (2010)
Arh1$^{-/-}$	Spontaneous	Increased	Lymphoma, adenocarcinoma	Kato et al. (2011)

Table 2
Polymorphisms in the *PARP-1* gene and its association with cancer risk.

SNP	Combination	Studied population	Risk	Cancer-type	References
Val762Ala	–	Caucasian	Increased	Prostate	Lockett et al. (2004)
	–	Han Chinese	Increased	Esophageal	Hao et al. (2004)
	–	Han Chinese	Increased	Lung	Zhang et al. (2005)
	Cag^{+}*H. pylori* infrction	Caucasian	Increased	Gastric	Silva-Fernandes et al. (2012)
	–	Chinese	Increased	Cervical	Ye et al. (2012)
	–	Caucasian	No difference	Breast	Cao et al. (2007)
	–	Japanese	No difference	Lung	Sakiyama et al., 2005
	–	Caucasian	Decreased	Glioma	Liu et al., 2009
	–	Han Chinese	No difference	Bladder	Wang et al., 2010
	–	Japanese	No difference	Soft-tissue	Nakayama et al., 2008
	XRCC1Arg399Gln	Han Chinese	Increased	Lung	Zhang et al. (2005)
	–	Caucasian	No difference	Melanoma	Zhang et al. (2011)
rs321925 (intron)	–	Caucasian	Increased	Melanoma	Zhang et al. (2011)
Lys940Arg	–	Japanese	No difference	Lung	Sakiyama et al., 2005
rs8679 (37-UTR)	–	Caucasian	Increased	Breast cancer	Teo et al., 2012

also associated with human papillomavirus positivity in precancerous high-grade squamous intraepithelial lesions (Hassumi-Fukasawa et al., 2012).

5. Cancer biomarkers and diagnosis

Cancer biomarkers are important for diagnosis and monitoring therapeutic effects and to determine the therapeutic strategies for individual cancers. There are various tumor biomarkers for different cancers, including CA-125, and α-fetoprotein. The amount of PARylated proteins in peripheral blood leukocytes was proposed to be a biomarker for head and neck, breast, and cervical cancers, because their levels decreased to less than half in the patients of these cancers (Lakadong et al., 2010). The underlying mechanism awaits further investigation.

6. Cancer prevention

When rats were fed a niacin (nicotinic acid)-deficient diet, NAD levels decreased in bone marrow and showed increased incidence of tumor development after treatment with ethylnitrosourea (Boyonoski et al., 2002). Non-lymphocytic leukemia was frequently observed among these tumors (Bartleman et al., 2008), which is a common secondary cancer after chemotherapy, suggesting that niacin supplementation may be useful for prevention of secondary cancer development. In skin keratinocytes, decreased levels of niacin led to a decrease in NAD levels, under which resulting lowered PARP activity enhanced the sensitivity to UV-damage (Benavente et al., 2009). A myristylnicotinate derivative of niacin is being evaluated for prevention of skin cancers in clinical trials (Tashtoush et al., 2007).

In clinical trials for ovarian, breast and prostate cancer patients, PARP inhibitors showed effectiveness to carriers of a *BRCA1* or *BRCA2* mutation with few side-effects (Audeh et al., 2010; Fong et al., 2009; Tutt et al., 2010). PARP inhibitors targeted the tumor cells with *BRCA1/2* mutations and specifically induced cancer cell death (Bryant et al., 2005; Farmer et al., 2005). This concept has been applied to several clinical strategies as 'synthetic lethality'. PARP inhibition in BRCA-deficient cells led to the persistence of DNA lesions, normally repaired by HR, resulting in cell-death induction. In *BRCA2* mutated breast carcinoma cells, the anti-tumor effect of PARP inhibitor was enhanced by additional inhibition of nicotinamide phosphoribosyltransferase (NAMPT), which mediates nicotinamide mononucleotide (NMN) production from nicotinamide (Bajrami et al., 2012). Under deficiency of *PTEN*, which is required for transcription of a key factor in HR repair, Rad51, PARP inhibitor also decreased the cancer cell survival (Mendes-Pereira et al., 2009). Furthermore, in androgen receptor (AR)-dependent prostate cancers, PARP-1 modulated the AR-dependent transcription as a coactivator and promoted tumor growth (Schiewer et al., 2012).

Therefore, further investigation is necessary to establish whether prophylactic treatment with PARP inhibitors in one allele mutation carriers of *BRCA* or *PTEN* can efficiently remove the biallelic mutated precancerous cell populations and be used as a chemopreventive strategy in cancer. Further animal studies need to be carried out to assess the side effects of PARP inhibition on genomic stability during long term treatment.

7. Perspectives

As we reviewed in this article, PARylation is involved in carcinogenesis in many ways (Fig. 4), however, a fine control of PARylation at each stage will enable intervention in carcinogenesis. For this purpose, understanding the PARylation related proteins and the function of enzymes that use NAD, including SIRT family proteins, might be particularly important. By identifying the environmental carcinogens, the strategies for minimizing exposure to carcinogens may become possible. However,

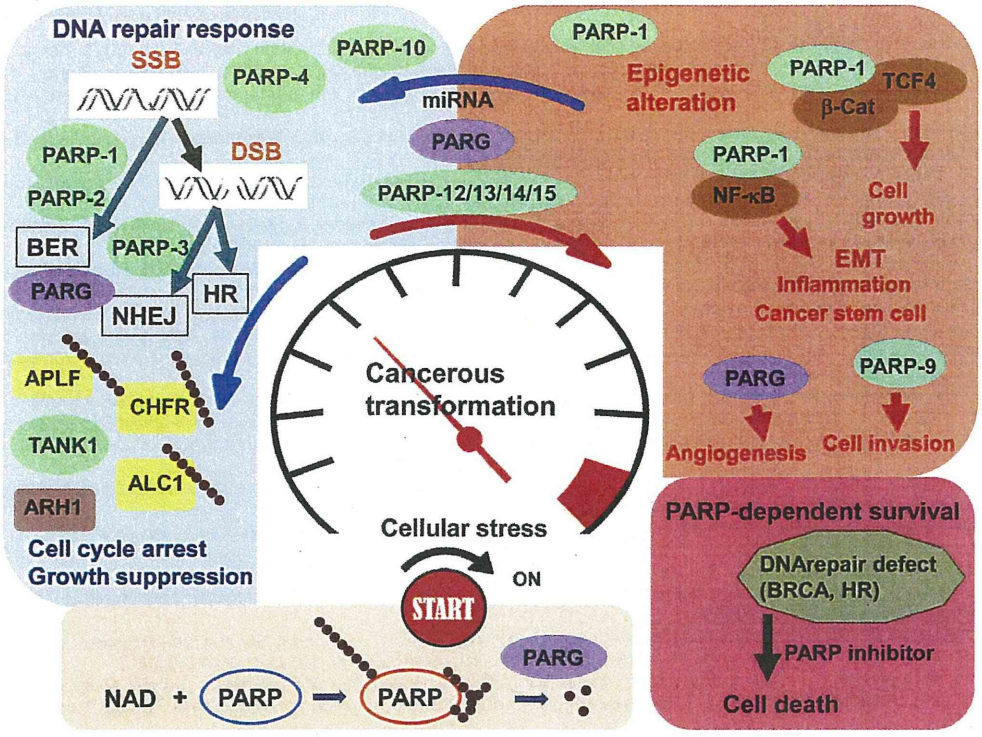

Fig. 4. Involvement of PARylation in cancer development. PARylation suppresses carcinogenesis through DNA repair and other DNA damage responses and downregulates cancer-associated pathways and these responses might serve as cancer barriers at various stages. On the other hand, PARPs support carcinogenesis through involvement in transcription, epigenetic regulation, EMT, cell invasion and signaling cascades. If the state of oncogene addiction is present, where cancer cells survive in a PARP-dependent manner, PARP inhibitor may be an effective therapeutic agent.

'aging' is one of the strongest inescapable risk factors for cancer. Because of this and other inescapable carcinogenic risk factors, various preventive and cancer suppressive strategies should be further developed. As observed in the TANK1, TANK2, and PARP-3 examples, some of the functions of PARP family proteins are considerably different among species, even between humans and mice. Therefore we also have to be careful in applying the results of animal models to human carcinogenesis.

Establishing strategies for prevention and complete cure of cancer may be difficult in the near future. Therefore, development of new strategies for delaying the onset of cancer development and delaying the progression of cancer might be also important.

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The *H19* induction triggers trophoblast lineage commitment in mouse ES cells

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ABSTRACT

Trophoblast lineage differentiation is properly regulated to support embryogenesis. Besides normal developmental process, during germ cell tumor formation or development of other reproductive system diseases, unregulated trophoblast differentiation is also observed and affects the pathogenesis of the diseases. During normal embryogenesis, cell fate of late-stage blastcyst is regulated by a reciprocal repression of the key transcriptional factors; Oct3/4 dominancy inhibits *Cdx2* expression in inner cell mass (ICM) and leads them to epiblast/primitive ectoderm but Cdx2 dominancy in trophectoderm (TE) leads them to trophoblast lineage. In contrast during early blastcyst stage, the *Cdx2* expression is restricted in TE and not present in ICM, although Oct3/4 signaling does not inhibit the *Cdx2* expression in ICM, implying that some factors could be inactivated leading to the suppressed *Cdx2* expression in ICM of early blastcyst.

ES cells (ESCs), which are derived from ICM, could be a unique model to study trophoblast differentiation in an ectopic context. We previously showed that poly(ADP-ribose) polymerase-1 (*Parp-1*) deficient ESCs highly expressed non-coding RNA *H19* and could differentiate into trophoblast lineage. The expression of *H19* is known to start at pre-blastcyst stage during mouse development, and the gene shows high expression only in trophoectoderm (TE) at blastcyst stage. However, its role in trophoblast differentiation has not been clarified yet. Thus, we hypothesized that the *H19* activation may act as a trigger for induction of trophoblast differentiation cascade in mouse ESCs. To investigate this issue, we asked whether a forced *H19* expression drives ESCs into trophoblast lineage or not. We demonstrated that the *H19* induction leads to trophoblast lineage commitment through induction of the *Cdx2* expression.

We also showed that the expression of *Cdx2* is induced in ESCs by forced *H19* expression even under a high level of Oct3/4, which could act as a suppressor for *Cdx2* expression. It is thus suggested that the *H19* induction promotes trophoblast lineage commitment against the repression pressure by Oct3/4 in differentiating ESCs. Taken together, this study suggests that the *H19* expression is able to function as a cascade activator of trophoblast lineage commitment possibly by overriding the Oct3/4 action in ESCs.

1. Introduction

Trophoblast lineage differentiation is properly regulated during embryogenesis. Besides normal developmental process, during development of germ cell tumors or other reproductive system disorders, unregulated trophoblast differentiation is also observed and malignancy of the tumors and pathogenic properties of the diseases are affected by the presence of trophoblasts [1]. The regulation mechanism of trophoblast differentiation during normal embryonal development and pathogenesis of the diseases, including tumorigenesis has not been fully elucidated.

Abbreviations: ESCs, embryonic stem cells; ICM, inner cell mass; PE, primitive ectoderm; TE, trophectoderm; Parp-1, poly(ADP-ribose) polymerase 1; Plf1, proliferin 1; Dnmt1, DNA methyltransferase 1; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; Tc, tetracycline; Zeo, zeocin; LIF, leukemia inhibitory factor; *Cdx2*, caudal-type homeobox protein 2; Fgf5, fibroblast growth factor 5; Tpbpa, trophoblast specific protein α; Igf2, insulin-like growth factor 2.

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Trophoblast differentiation is initiated with *Tead4* and *Klf5* activation in the outer layer of morulae, which specifies them to trophoblast lineage and induces genes for maintenance of trophoectoderm (TE) commitment in blastcyst stage [2]. However, one of the most significant regulations to define the TE and epiblasts has been suggested as a reciprocal expression pattern of *Cdx2* (caudal-type homeobox protein 2) in TE and Oct3/4 in epiblasts, which is established during the period of blastcyst stage. Previous reports demonstrated the repressive interaction between Oct3/4 and Cdx2 [2]. Furthermore, perturbed expression of the two genes induces the activation of TE-related genes in inner cell mass (ICM) cells/ES cells (ESCs) or that of ICM-related genes in TE cells during differentiation [3,4]. Accumulating *in vivo* studies show that; while Oct3/4 signaling does not inhibit *Cdx2* expression in ICM of early blastcyst, *Cdx2* expression is restricted in TE, implying that some factors could be inactivated or absent, that leads to suppression of the *Cdx2* expression in ICM [5].

We previously observed poly (ADP-ribose) polymerase-1 (*Parp-1*) deficient ESCs could differentiate to trophoblast lineage, while normal ESCs could not [6–8]. Mouse ESCs have been established from the ICM of blastocysts [9,10], which starts differentiation during mouse embryogenesis into the epiblast and primitive ectoderm (PE) on embryonic day 4.5; however, generally they do not give rise to trophectoderm (TE) derivatives [11,12]. The placenta of *Parp-1*$^{-/-}$ mouse also shows an increase in trophoblast giant cell number and a decrease in spongiotrophoblast number [8]. Parp-1 is involved in the regulation of transcription [13,14] and chromatin remodeling [15] through poly(ADP-ribosylation) of proteins and interaction with proteins. The absence of Parp-1 alters transcription of particular genes and induces trophoblast differentiation. *Parp-1*$^{-/-}$ ESCs shows early and enhanced expression of extraembryonic/trophoblast differentiation-associated gene, the *H19*, a non-coding RNA gene, and a homeobox transcription factor *Cdx2* gene upon ESC differentiation, and later a trophoblast specific gene, *Plf1* (proliferin 1) [16] is induced in *Parp-1*$^{-/-}$ ESCs. *Dnmt1* (DNA methyltransferase 1) deficient ESCs also differentiate into trophoblast lineage [17], accompanying induction of the *H19* expression [18].

The *H19* gene expression is initially activated at 2-cell stage, but from blastcyst stage, it is highly restricted in TE including primary trophoblasts and ectoplacental cone, although its role in trophoblast differentiation has not been clarified yet [19]. We hypothesized that the *H19* expression may induce trophoblast differentiation cascade and investigated here using ectopic context model of ESCs in this study. Our study showed that the *H19* gene expression initiates trophoblast differentiation commitment in mouse ESCs.

2. Materials and methods

2.1. Cell culture

Wild-type J1 ESC clones and *Parp-1*$^{-/-}$ 210–58 and 226–47 ESC clones were used in this study [6]. These ES clones were cultivated as previously described [16]. Briefly, ES cells were cultured in the Dulbecco's Modified Eagle's Medium (Invitrogen) containing 20% fetal bovine serum (Thermo Fisher Scientific), non-essential amino acids (Invitrogen) and leukemia inhibitory factor (LIF), ESGRO (Millipore) on gelatin-coated dishes (AGC Techno Glass). For differentiation, ESCs were transferred to the differentiation condition 1 day after vector transfection and were cultured for 3 days. ZHTc6 ESCs were cultured as previously described [4]. Culture medium was supplemented with tetracycline (Tc, 40 ng/mL, Sigma) and zeocin (Zeo, 100 μg/mL, Invitrogen). For selection of Oct3/4 positive cells, ZHTc6 ESCs were inoculated in medium including Zeo, at least for 2 weeks. Exogenous *Oct3/4* induction in ZHTc6 ESCs was achieved by Tc withdrawal for 2 days in LIF containing condition.

2.2. Forced expression of the H19 in mouse ESCs

A 3170 bp fragment of the mouse *H19* (−252 to +2918 base from the transcription initiation site) was purified by digestion of cosmid 5-10-A [20]. The *Cyp7a1* enhancer/promoter in the pCyp7a1-GFP-Neo vector [21] was replaced by the *EF-1a* promoter fragment of the pEF/myc/nuc vector (Invitrogen) to yield pEF-GFP-Neo. Then, the *H19* fragment was inserted under the *EF-1a* promoter sequence. The resulting plasmid pEF-*H19*/GFP-Neo (Fig. 3A) was linearized with XbaI (Takara Bio) and then transfected into the ESC line J1 by using Lipofectamine 2000 (Invitrogen) according to the manufacture's protocol in serum-free condition. For establishment of stable cell-lines, transfected clones were selected by growth in the presence of 175 μg/mL G418 (Invitrogen). Following 9 days of selection, 24 colonies were collected. From them, ten ESC lines that proliferated with piled-up morphology were selected, and one clone showing a strong *H19* expression was used in the present study. For transient expression experiments, non-linearized *H19* vector (pEF-H19/GFP-Neo) or control vector (pEF-GFP-Neo) was transfected into control ESC clone or ZHTc6 ESCs as described above.

2.3. Northern blot analysis

RNAs of *H19* (2.3 kbps) and *Gapdh* (1.6 kbps) were probed as previously described [16]. The membrane was exposed to a Fuji Imaging Plate (Fuji Film), and the radioactivity was analyzed using BAS-2500 Bio-imaging analyzer (Fuji Film).

3. Results

3.1. H19 overexpression enhanced expression of trophoblast marker genes after LIF withdrawal in ESCs

Previously we reported that the *H19* gene expression is increased in undifferentiated ES cells and also further augmented in the differentiation condition under LIF removal by semi-quantified RT-PCR [16]. When analyzed by quantitative RT-PCR, we found that under undifferentiated condition in the presence of LIF, the increased expression level of the *H19* was found to be 6–10 folds in *Parp-1*$^{-/-}$ ESCs (Fig. 1A). Northern blot analysis also confirmed that the expression of full-length form of the 2.3 kb *H19* RNA is upregulated in *Parp-1*$^{-/-}$ ESCs in the presence of LIF and further augmented after LIF withdrawal (Fig. 1B). We thus hypothesized that the functional *H19* may act as a trigger and induce trophoblast differentiation cascade in mouse ESCs.

To investigate this issue, we asked whether a forced upregulation of *H19* expression promotes the commitment of ESCs to trophoblast lineage or not. As shown in Fig. 1C, we transfected wild-type J1 ESCs with an *H19* overexpression vector to obtain ESCs constitutively overexpressing the *H19* transgene. Real-time RT-PCR analysis confirmed the establishment of cells expressing the *H19* transgene (Fig. 1D) and enhanced expression of *Cdx2* in the ESCs (Fig. 1E, ESCs). Four days after differentiation condition induced by withdrawal of LIF, the *H19*-transduced clone showed an increased expression of the trophoblast marker genes, *Cdx2* and *Plf1*, but not the *Oct3/4* gene (Fig. 1E and F), compared to the control vector-transduced clone. The *Cdx2* is a marker gene for TE at an early stage of trophoblast differentiation, and induction of *Cdx2* is known to be sufficient for differentiation of ESCs into the TE [3]. The *Plf1* is a late stage marker of terminally differentiated

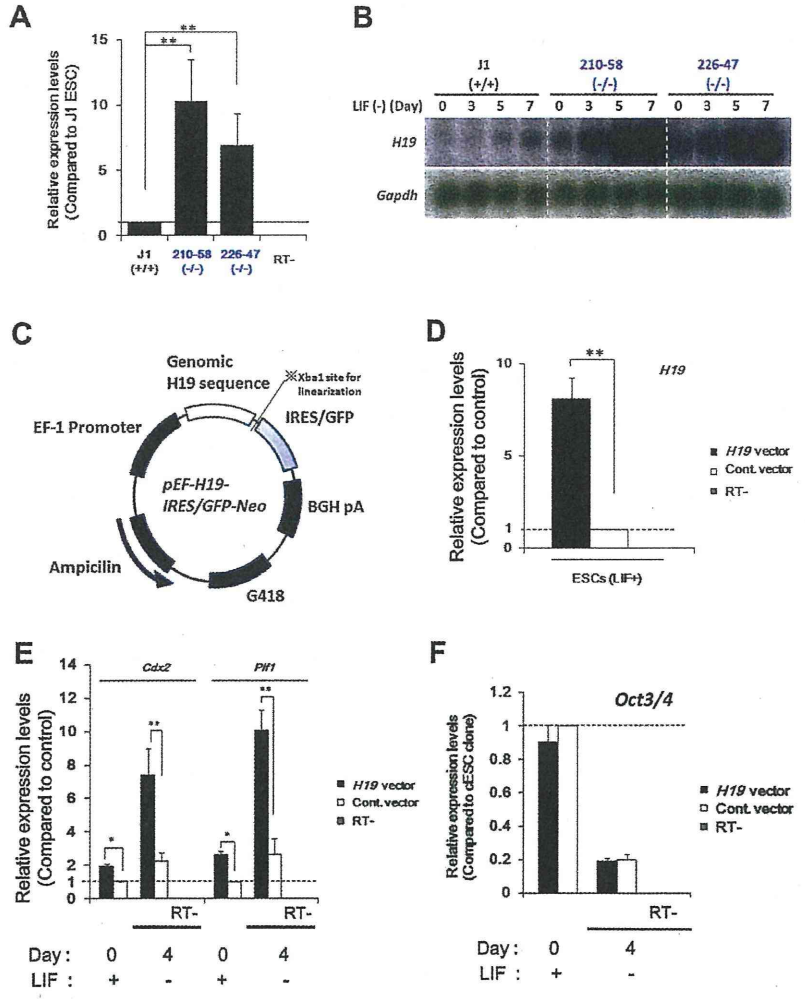

Fig. 1. Stable overexpression of *H19* activated expression of trophoblast related genes. (A and B) Upregulation of *H19* expression in *Parp-1*$^{-/-}$ ESCs. The expression level was analyzed by real-time PCR (A) and northern blot (B). The expression level was normalized using the level of *Gapdh* (**$P < 0.01$). RT- means without reverse transcription. (C) The vector construct for constitutive *H19* overexpression. (D) The *H19* overexpression in the *H19* vector-transfected clone. (E) Enhancement of trophoblast marker gene expression in the *H19*-overexpressing ESC clones. The ESC clone showed enhanced expression of trophoblast marker genes (*Cdx2* and *Plf1*), and these expression levels were higher at day 4 (D4) than in the control ESC clone (E). LIF withdrawal induced differentiation in the *H19* vector- or control vector-transfected ESC clones at day 4 (D4), but *Oct3/4* expression levels did not change under *H19* overexpression (F). Expression levels were analyzed by real-time PCR and were normalized using the expression level of *Gapdh* (*$P < 0.05$ and **$P < 0.01$, respectively).

trophoblast giant cells [22,23]. The *Oct3/4* level was not affected in the *H19*-transduced clone, confirming that *Oct3/4* is not regulated by the *H19* (Fig. 1F). Taken together, a forced expression of the *H19* triggered commitment to trophoblast lineage in ESCs under differentiation condition by LIF withdrawal.

To exclude potential bias through the cloning process in the above experiments, which used a stable clone, we transiently overexpressed the *H19* in a wild-type ESC clone, and cultured under differentiation conditions after LIF withdrawal (Fig. 2A and B). Although there was no difference in the expression level of *Fgf5*, a marker of PE, three days after differentiation induction (Fig. 2D), expression of the trophoblast marker genes, *Cdx2*, *Tpbpa* (trophoblast specific protein α) and *Plf1* were enhanced in the *H19*-transduced population (Fig. 2D). These results confirmed that under differentiation pressure by LIF withdrawal in ESCs, forced expression of the *H19* acts as a trigger for induction of expression of the *Cdx2*, a main transcription factor required for commitment to trophoblast lineage.

3.2. Exogenous induction of the H19 leads to the expression of a trophoblast key regulator gene Cdx2, even under Oct3/4 overexpression

In the present study, we demonstrated that expression of the *Cdx2* is activated in undifferentiated ESCs by a forced expression of the *H19*, which could trigger trophoblast lineage commitment. It has been suggested that Oct3/4 could repress the *Cdx2* induction during ICM differentiation [2]. This led us to clarify whether the *H19* could override Oct3/4 suppressive pressure on *Cdx2* transcription or not. ZHTc6 ESC clone was employed in this experiment, in which exogenous *Oct3/4* is regulated under the control of the Tc responsive element (Tet-OFF system) [4] (Fig. 3A). Two days after Tc withdrawal in the presence of LIF, increased expression of *Oct3/4* slightly upregulated the *Fgf5* (fibroblast growth factor 5) but did not repress the *Cdx2* in ZHTc6 ESCs (Fig. 3B and C, block 3), which assured the Oct3/4 accumulation by Tc withdrawal and indicated that Oct3/4 upregulation is not sufficient to suppress

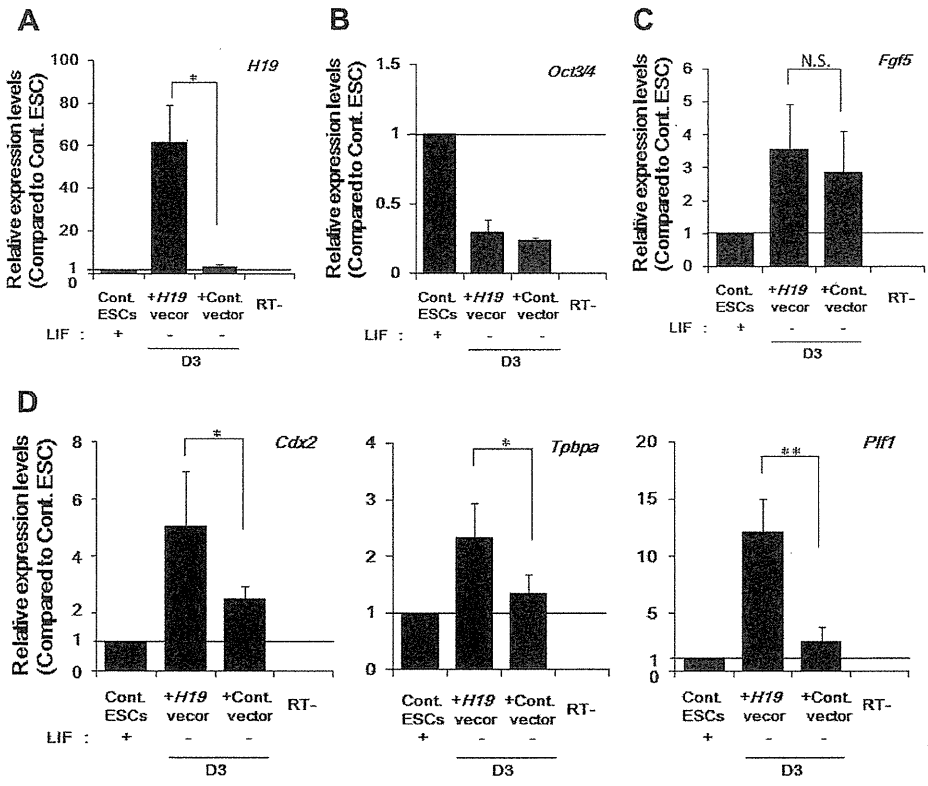

Fig. 2. The transient expression of exogenous *H19* enhanced trophoblast lineage differentiation during ESC differentiation. (A and B) Control ESC clones transfected with *H19* or control vector were induced to differentiate by LIF withdrawal. (C and D) Three days after differentiation (D3), trophoblast marker genes, *Cdx2*, *Tpbpa* and *Plf1*, were upregulated in *H19* vector-transfected ESCs (D), whereas no difference was observed in the levels of *Fgf5* between the *H19* vector- and control vector-transfected ESCs (C). Expression levels were analyzed by real-time PCR and were normalized using the expression level of *Gapdh* (*$P < 0.05$ and **$P < 0.01$, respectively). RT- means without reverse transcription.

the *Cdx2* at this period. The *Oct3/4* overexpression system successfully worked; the *Fgf5* expression is further augmented on day 6, which corresponds to 4 days after control vector transfection, indicating that the PE differentiation was further enhanced (Fig. 3B–D, block 5). On the other hand, expression of the *Nanog* (an epiblast marker) and *T* (a mesoderm marker) was not significantly altered (Fig. 3B and C, blocks 5 and 6) indicating that the PE differentiation is the main differentiation direction in this system. Of note, two days after Tc withdrawal, the *Oct3/4* forced expression repressed the *H19* expression prior to *Cdx2* suppression in the control vector transfected cells (Fig. 3C, the upper panel, block 3). Six days after Tc withdrawal, the *Oct3/4* forced expression reduced the *Cdx2* expression accompanying the attenuated level of *H19* expression (Fig. 3D, block 5). Importantly, forced expression of the *H19* to Oct3/4 overexpressing cells led to increased expression of *Cdx2* expression (Fig. 3D, block 6). Taken together, these results suggest that the *H19* expression is able to enhance the *Cdx2* expression even under the negative pressure of Oct3/4 on *Cdx2* in differentiating ESCs.

4. Discussion

In this study we demonstrated that the *H19* induction triggers trophoblast lineage commitment independently on Oct3/4 level in mouse ESCs cells. We showed that the expression of *Cdx2*, a key molecule for control of trophoblast differentiation, is induced by the *H19* expression. Our study also supported the notion that the *H19* upregulation in *Parp-1*$^{-/-}$ ESCs, which we previously observed, could activate the cascade of trophoblast lineage commitment by contributing to induction of the *Cdx2* expression (Fig. 4). Furthermore, during germ cell tumor formation, perturbed expression of the *H19* and trophoblast differentiation could be observed and affects malignancy of the tumors in some cases [1], suggesting that the trophoblast differentiation during tumorigenesis may be also initiated by the *H19* expression.

The role of non-coding RNA *H19* in trophoblast lineage commitment and differentiation regulation has not been fully elucidated. The *H19* expression depends on the DNA methylation state of the imprinting control region (ICR) located between the *H19* and *Igf2* (insulin-like growth factor 2) genes. A recent report demonstrated that *Dnmt1* deficient ESCs differentiated into trophoblast lineage [17]. *Dnmt1* deficiency induced DNA demethylation of the ICR and led to high level of the *H19* expression [18]. Although mouse ESCs do not generally differentiate into trophoblast lineage, it is suggested that the barrier for trophoblast lineage commitment in ESCs might be just dependent on epigenetic silencing.

The *H19* locus is paternally imprinted, and expressed only from the maternal allele [24]. The transcriptional product from the *H19* locus is a capped and polyadenylated non-coding RNA of 2.3 kb. This RNA is co-regulated with eight other imprinting genes co-organizing an imprinting gene network and is spliced and generates microRNA-675 but does not encode any protein [24]. In mouse models, the *H19* deficiency increases fetal weight and increases the frequency of teratoma formation [25]. Furthermore, the engineered expression of the *H19* could lead to complete parthenogenesis development [24]. These reports suggest the *H19* function as an

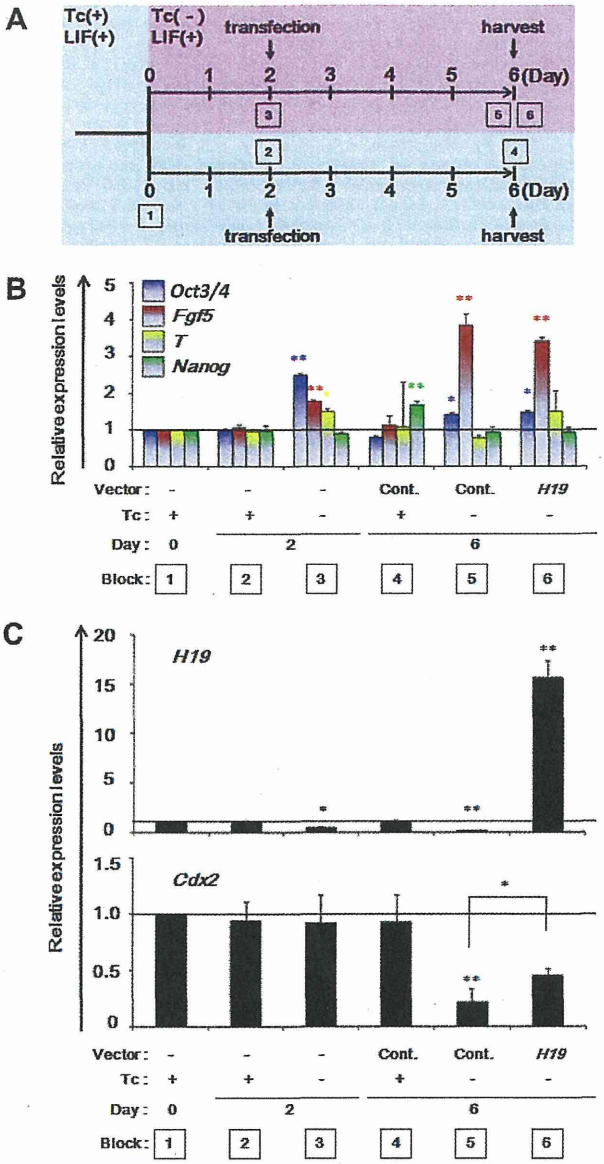

Fig. 3. The effect of *H19* overexpression in the presence of Tc-dependent Oct3/4 accumulation. (A) Experimental procedure. Each squared number indicates the RNA extraction points of the data shown in B and C. (B and C) Enhanced *Cdx2* transcription by *H19* overexpression in Oct3/4 accumulation conditions. Two days after Tc withdrawal in the presence of LIF, corresponding to the time when *Fgf5* transcription was increased (B), *H19* or control vectors were transfected into ZHTc6 ESCs (see A). Four days after transfection (the day 6 after Tc withdrawal), *H19* overexpression caused the augmented expression of *Cdx2* (C, block 5 and 6). The relative expression ratios of each gene (to the level at day 0) were shown after normalization to *Gapdh*. Unlinked asterisks indicate a significant difference in the expression level compared to that of ESCs (block 1) (*$P < 0.05$ and **$P < 0.01$, respectively).

important regulator of cell growth and differentiation. Further mechanistic role of the *H19* function in trophoblast lineage regulation should be elucidated.

In this study we also showed that forced expression of the *H19* even under a high level of Oct3/4 led to trophoblast lineage possibly acting as a cascade activator of trophoblast lineage by overriding the Oct3/4 action on the *Cdx2* in ESCs. Oct3/4 and Cdx2 are known to interact and regulate their levels each other. The main reported pathway of trophoblast lineage commitment is Oct3/4 down-regulation that leads to the *Cdx2* induction, which acts as a

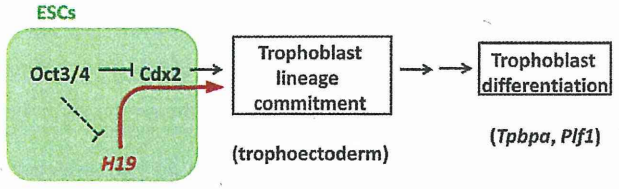

Fig. 4. A model; the *H19* upregulation could drive trophoblast lineage commitment in mouse ESCs. ESCs do not differentiate into trophoblast lineage because of the suppression of the *Cdx2* expression by Oct3/4. Forced expression of the *H19* is able to induce the *Cdx2* transcription (red letters and an arrow), which is critical for trophoblast lineage commitment of ESCs. Endogenous expression of the *H19* is downregulated by Oct3/4 (dotted lines) in ESCs. Forced expression of the *H19* drives *Cdx2* transcription even under the negative pressure by a high level of Oct3/4. This ectopic context model of ESCs suggests that *H19* upregulation might therefore contribute to induction of trophoblast lineage during tumorigenesis and pathogenesis of other reproductive system diseases. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

key transcription factor to induce Eomes, Mash-2, mSna, and Hand1 cascade. Our study suggested that the *H19* overexpression is able to promote trophoblast lineage commitment even under the suppressive pressure by Oct3/4. It is of question whether this Oct3/4 independent trophoblast lineage commitment by the *H19* could be involved in the differentiation control of trophoblast lineage in normal development and aberrant induction of trophoblast lineage during tumorigenesis and other pathogenesis of the reproductive system diseases.

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Context-dependent activation of Wnt signaling by tumor suppressor RUNX3 in gastric cancer cells

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Key words

β-catenin, gastric cancer, RUNX3, TCF4, Wnt signaling

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RUNX3 is a tumor suppressor for a variety of cancers. RUNX3 suppresses the canonical Wnt signaling pathway by binding to the TCF4/β-catenin complex, resulting in the inhibition of binding of the complex to the Wnt target gene promoter. Here, we confirmed that RUNX3 suppressed Wnt signaling activity in several gastric cancer cell lines; however, we found that RUNX3 increased the Wnt signaling activity in KatoIII and SNU668 gastric cancer cells. Notably, RUNX3 expression increased the ratio of the Wnt signaling-high population in the KatoIII cells. although the maximum Wnt activation level of individual cells was similar to that in the control. As found previously, RUNX3 also binds to TCF4 and β-catenin in KatoIII cells, suggesting that these molecules form a ternary complex. Moreover, the ChIP analyses revealed that TCF4, β-catenin and RUNX3 bind the promoter region of the Wnt target genes, *Axin2* and *c-Myc*, and the occupancy of TCF4 and β-catenin in these promoter regions is increased by the RUNX3 expression. These results suggest that RUNX3 stabilizes the TCF4/β-catenin complex on the Wnt target gene promoter in KatoIII cells, leading to activation of Wnt signaling. Although RUNX3 increased the Wnt signaling activity, its expression resulted in suppression of tumorigenesis of KatoIII cells, indicating that RUNX3 plays a tumor-suppressing role in KatoIII cells through a Wnt-independent mechanism. These results indicate that RUNX3 can either suppress or activate the Wnt signaling pathway through its binding to the TCF4/β-catenin complex by cell context-dependent mechanisms.

RUNX3 is a member of the runt-related transcription factor RUNX family and was originally identified as a tumor suppressor of gastric cancer development.[1–4] In approximately 80% of gastric cancers, RUNX3 expression is lost due to epigenetic silencing and mislocalization in the cytoplasm.[1,4,5] Moreover, expression of RUNX3 in gastric cancer cells results in suppression of tumorigenicity, while expression of the mutant form of RUNX3 R122C found in human gastric cancer does not affect tumorigenicity.[1,6] Consistently, gastric epithelial cells derived from *Runx3* −/− p53 −/− mice form explanted tumors in nude mice.[7] The functional inactivation of RUNX3 is frequently observed in other solid tumors, including colon, pancreatic and lung cancers.[3,4] Taken together, these results indicate that RUNX3 plays a tumor suppressing role in a variety of cancers.

RUNX3 has multiple partners and is involved in diverse signaling pathways.[3,4] Wnt signaling suppresses phosphorylation of β-catenin by GSK-3β, leading to the accumulation of β-catenin in nuclei.[8] Accumulated β-catenin forms a complex with TCF4, which induces the transcription of Wnt target genes by binding to the promoter regions of these genes. The constitutive activation of Wnt signaling by genetic alteration leads to gastrointestinal tumor development.[9–11] It has previously been demonstrated in colon cancer cells that RUNX3 binds to TCF4 through the runt domain, forming a ternary complex of RUNX3, TCF4 and β-catenin, which inhibits the binding of the complex to the promoter region of Wnt target genes, thereby suppressing Wnt signaling.[12] The expression of Wnt target genes is significantly increased in *Runx3* −/− mouse intestinal mucosa without any alteration of the expression levels of TCF4 and β-catenin, and *Runx3* + /− mice develop intestinal tumors.[12] Notably, the association of the mutant RUNX3 R122C with TCF4 is weaker than wild-type RUNX3; thus, R122C cannot suppress Wnt signaling in *Runx3* −/− gastric tumor cells.[13] These results indicate that Wnt activation by RUNX3 downregulation contributes to tumorigenicity.

In contrast to these findings, we present the unexpected finding that RUNX3 activates Wnt signaling in KatoIII and SNU668 gastric cancer cells. Interestingly, RUNX3 binds TCF4 and β-catenin also in the KatoIII cells, and binding of the complex to Wnt target gene promoter is more stable in the presence of RUNX3, which may cause Wnt signaling activation. Accordingly, it is possible that RUNX3 can either suppress or activate Wnt signaling activity by binding to the TCF4/β-catenin complex, and the direction of Wnt signaling modulation may be regulated by a cell context-dependent mechanism.

Materials and Methods

Cell culture experiments. Human gastric cancer cell lines, AGS (ATCC), AZ521, MKN45, KatoIII, (RIKEN, BioResource Center, Tsukuba, Japan), SNU216, SNU484, SNU601, SNU638, SNU668 and SNU719 (Korean Cell Line Bank, Seoul, Korea) were cultured in RPMI1640 supplemented with 10% FBS. The cell proliferation rate was examined using the Alamar Blue Cell Viability Reagent (Invitrogen, Carlsbad, CA, USA). For the soft agar colony formation assay, cells were suspended in 0.33% agarose contained in the medium and seeded on 0.5% bottom agar. After 21 days of culture, soft agar was stained with Giemsa solution (Wako, Osaka, Japan) and colony numbers were scored.

Cells were transfected with pcDNA3, pcDNA-Flag-RUNX3 or pcDNA-Flag-RUNX3(R122C) vector.[6] KatoIII-R3 stable cell line was constructed by transfection with pcDNA-RUNX3 and selected with G418 (Wako) at 100 μg/mL. To knock down gene expression, cells were transfected with Silencer Select siRNA for RUNX3 or β-catenin (Ambion, Cambridge, MA, USA).

To examine the Wnt activation level, cells were cotransfected with super 8× TOPflash or Super 8× FOPflash (Addgene, Cambridge, MA, USA), together with pcDNA3, pcDNA-Flag-RUNX3 or pcDNA-Flag-RUNX3(R122C).[6] At 24 h after transfection, the luciferase activity was measured using a Luciferase assay system (Promega, Madison, WI, USA).

Wnt suppression and activation. To inhibit Wnt signaling, cells were treated with 10 μg/mL of C59 (provided by Dr David Virshup), which inhibits porcupine, a membrane-bound O-acyltransferase required for Wnt palmitoylation.[14] To activate Wnt signaling, conditioned media including Wnt3a and Rspondin were prepared from L cells expressing Wnt3a and 293T cells expressing Rspondin, respectively (provided by Dr Marc Leushacke), and the conditioned media were supplemented at 10% volume in the culture medium.

Western blotting. A total of 10 μg of protein samples were separated in 10% SDS-polyacrylamide gels. Antibodies for RUNX3[5] or unphosphorylated β-catenin (Millipore, Billerica, MA, USA) were used as the primary antibodies. The anti-β-actin antibody (Sigma, St. Louis, MO, USA) was used as an internal control, and the ECL detection system (GE Healthcare, Buckinghamshire, UK) was used to detect the signals.

Real-time RT-PCR. Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) and cDNA was constructed using the Prime Script RT Reagent Kit (Takara, Tokyo, Japan). Real-time RT-PCR was performed using the SYBR Premix Ex TaqII (Takara) and Stratagene Mx3000P (Agilent Technologies, Santa Clara, CA, USA). The primers were purchased from Takara.

Flow cytometry analysis. To examine the intracellular RUNX3 and β-catenin levels, permeabilized cells were incubated with the primary antibodies for total β-catenin (Sigma) or RUNX3,[5] followed by the secondary antibodies for rabbit IgG-conjugated with Alexa 488 (Molecular Probes, Grand Island, NY, USA) or mouse IgG-conjugated with Alexa 633 (Invitrogen), and examined using FACS Canto II (BD Biosciences, San Jose, CA, USA). Cells were transfected with a pcDNA-RUNX3-IRES-mGFP expression vector, in which internal ribosome entry site (IRES) fragment from pTRE3G-IRES (Clontech Laboratories, Mountain View, CA, USA) and maxGFP cDNA from pmaxGFP (LONZA, Allendale, NJ, USA) were subcloned to pcDNA-Flag-RUNX3, and RUNX3-expressing cells were isolated using the FACS Aria cell sorter (BD Biosciences, San Jose, CA, USA) to collect GFP-expressing cells.

Immunocytochemistry. The cells were seeded on cover slips and fixed in 4% paraformaldehyde, then permeabilized with 0.5% Triton X-100 in PBS. Antibodies against total β-catenin (Sigma) or RUNX3 were used as the primary antibodies, and anti-rabbit IgG Alexa 594 or anti-mouse IgG Alexa 488 (Molecular Probes) were used as the secondary antibodies.[5]

Immunoprecipitation. KatoIII cells were transfected with the pcDNA-Flag-RUNX3 or pcDNA-Flag, and the cell lysates were used for immunoprecipitation with anti-FLAG M2 agarose (Sigma). Western blotting was performed using antibodies against unphosphorylated β-catenin (Millipore), TCF4 (Santa Cruz Biotechnology, Santa Cruz, CA), FLAG peptide or β-actin (Sigma).

ChIP. The cells were treated with formaldehyde solution (Wako) for crosslinking. ChIP was performed using the ChIP Assay kit EZ ChIP (Millipore) and antibodies against TCF4 (Santa Cruz Biotechnology), unphosphorylated β-catenin (Millipore), RUNX3[5] and mouse normal IgG. The primer sequences for the c-*Myc* promoter were: 5′-TTGCTGGGTTAT TTTAATCAT-3′ and 5′-ACTGTTTGACAAACCGCATCC-3′.[15] For the *Axin2* promoter, conserved TCF/LEF binding sites are localized in intron 1,[16] and Simple ChIP Human Axin2 Intron 1 Primers (Cell Signaling, Danvers, MA, USA) were used.

Statistical analysis. Statistical analyses were performed using the unpaired Student's *t*-test, with *P*-values <0.05 considered significant.

Results

Wnt activation by RUNX3 expression in KatoIII and SNU668 cells. RUNX3 expression was detected by western blotting in AZ521 and MKN45 cells, while it was not detected in other gastric cancer cell lines (Fig. 1a). Consistently, high levels of RUNX3 mRNA were detected in AZ521 and MKN45 cells by RT-PCR (Fig. S1). We transiently transfected the RUNX3 expression vector to all gastric cell lines, and examined Wnt signaling activity by luciferase reporter analysis. The Wnt signaling activity was significantly decreased in SNU216, SNU601, SNU638 and SNU719 cells, which was consistent with the previous results.[12] However, RUNX3 expression increased Wnt signaling activity in KatoIII and SNU668 cells (Fig. 1b). Importantly, the R122C mutant form of RUNX3 that is defective in the RUNX3 function did not change Wnt signaling activity in these cells.[1,6] The KatoIII-R3, stable RUNX3-expressing cells (Fig. S2) also exhibited an increased luciferase activity, which was suppressed by RUNX3 siRNA transfection (Fig. 1c). Moreover, Wnt activation levels increased gradually in accordance with the amount of the RUNX3 expression vector (Fig. 1d). Consistently, the expression levels of Wnt target genes, Sox4 and Axin2, increased significantly in KatoIII and SNU668 cells by RUNX3 expression (Fig. 1e).

Notably, inhibition of endogenous RUNX3 expression by siRNA in AZ521 significantly increased Wnt signaling activity, whereas RUNX3 siRNA transfection partially suppressed Wnt signaling in MKN45 cells (Fig. 1e,f). These results, taken together, suggest that RUNX3 suppresses or activates the Wnt signaling in a cell context-dependent mechanism.

Saturation of ligand-induced β-catenin stabilization in KatoIII cells. We further examined the RUNX3-induced Wnt activation mechanism using KatoIII cells. In KatoIII cells, Wnt signaling

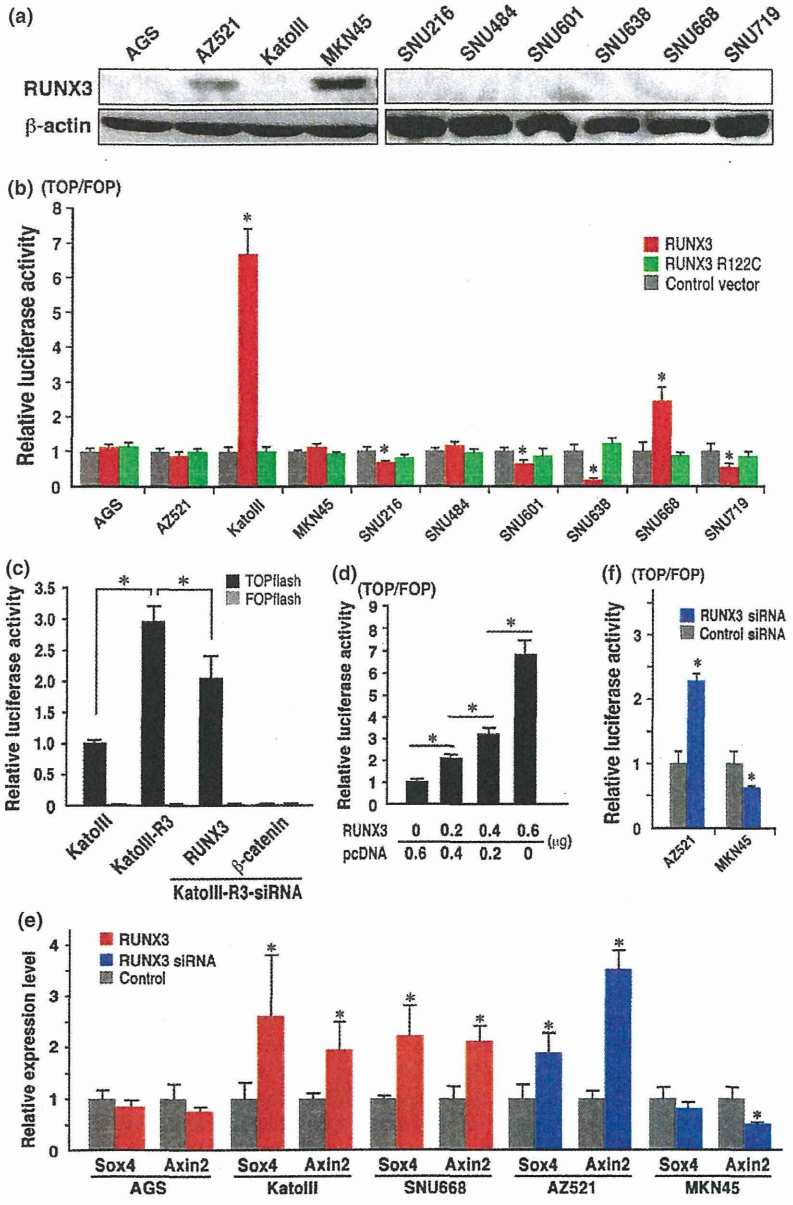

Fig. 1. RUNX3-induced Wnt signaling activation. (a) Western blotting of the RUNX3 expression in gastric cancer cells. (b) Relative luciferase activity of TOPflash/FOPflash in the respective gastric cancer cells transfected with RUNX3 or R122C mutant RUNX3 vector to the control vector-transfected cells. (c) Luciferase activity of the TOPflash and FOPflash in KatoIII-R3 cells and siRNA-transfected KatoIII-R3 cells relative to the level in the parental KatoIII cells. (d) Relative luciferase activity of TOPflash/FOPflash in KatoIII cells transfected with RUNX3 expression vector. (e) Relative expression levels of the Wnt target genes, SOX4 and Axin2, detected by RT-PCR in the RUNX3 vector-transfected cells (red) or RUNX3 siRNA-transfected cells (blue) to the control level. (f) Relative luciferase activity of TOPflash/FOPflash in AZ521 and MKN45 cells transfected with RUNX3 siRNA to the control level. (b–f) Bar graphs are shown as mean ± SD. $^*P < 0.05$ versus control levels otherwise indicated.

is activated by β-catenin gene amplification.[17] Treatment of control KatoIII cells with a Wnt ligand secretion inhibitor C59 significantly suppressed the endogenous Wnt signaling, indicating that Wnt ligand stimulation maintains the basal Wnt activation level (Fig. 2a). C59 treatment also decreased the luciferase activity in the RUNX3-expressing KatoIII cells. However, the ratio of the RUNX3-induced increase of luciferase activity in the C59-treated cells was similar to that in the control cells; that is, approximately 4.5-fold the control levels. Accordingly, it is possible that RUNX3 increases Wnt signaling activity in KatoIII cells through a ligand-independent mechanism.

We next examined the β-catenin levels of the RUNX3-transfected cells by western blotting. Although the active β-catenin levels were slightly increased both in the KatoIII-R3 cells and the RUNX3 vector-transiently transfected KatoIII cells (Fig. 2b, Figs S2 and S3), the increase was not sufficient to explain the marked increase of the TOPflash activity (Fig. 1b).

The Wnt activation level increased significantly in 293T cells following treatment with Wnt3a and/or Rspondin (Fig. 2c). However, treatment of KatoIII cells with Wnt3a /Rspondin did not change the luciferase activity (Fig. 2d). Consistently, the β-catenin high population measured by flow cytometry was significantly increased in the 293T cells following Wnt3a/Rspondin treatment, while the β-catenin high population in the KatoIII cells did not change following stimulation with Wnt3a/Rspondin (Fig. 2e,f). Therefore, it is possible that the Wnt ligand-induced β-catenin stabilization level is saturated in KatoIII cells, and RUNX3 activates Wnt signaling through mechanisms other than β-catenin stabilization.

Increase of β-catenin^{Hi} population by RUNX3 expression in KatoIII cells. We previously found that the Wnt activation level in individual KatoIII cells oscillates between a Wnt-high and

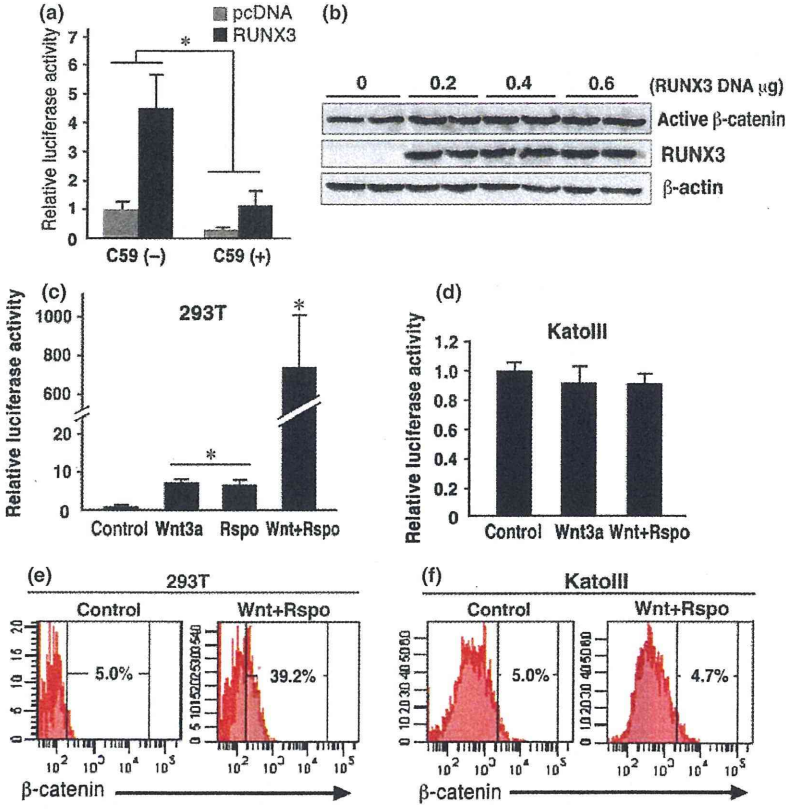

Fig. 2. Saturation of ligand-induced β-catenin stabilization in KatoIII cells. (a) Luciferase activity of TOPflash/FOPflash in KatoIII cells transfected with RUNX3 or control vector in the absence or presence of the C59 relative to the control cell level (mean ± SD). $*P < 0.05$. (b) Western blotting for active β-catenin and RUNX3 in KatoIII cells transfected with RUNX3 vector. (c,d) Relative luciferase activity of TOPflash/FOPflash in the 293T cells (c) and KatoIII cells (d) treated with Wnt3a and/or Rspondin. (mean ± SD). $*P < <0.05$ versus control level. (e,f) Flow cytometry analyses of β-catenin in the control cells (left) and cells treated with Wnt3a/Rspondin (right) in 293T (e) and KatoIII cells (f). The proportion (%) of the β-catenin^{Hi} population (top 5% level of the control) is indicated.

Wnt-low state.[18] We thus examined the β-catenin levels of the RUNX3-expressing cells using flow cytometry. When the RUNX3 vector was transfected, the β-catenin^{Hi} population (top 50% level of the control in Fig. 3a, [left]) increased significantly in the RUNX3^{Hi} cells (78%: Q2/(Q1 + Q2)), but not in the RUNX3^{Lo} cells (45%: Q4/(Q3 + Q4)) (Fig. 3a [right], b). Notably, the maximum β-catenin level in the RUNX3-transfected cells was similar to that observed in the control (Fig. 3a). We confirmed that transfection efficiency was not different between β-catenin^{Hi} and β-catenin^{Lo} KatoIII cells (Fig. S4). Immunocytochemistry of the RUNX3 vector-transfected KatoIII cells showed that approximately 73% of the RUNX3-positive cells were also positive for β-catenin, whereas approximately 44% of the RUNX3-negative cells were β-catenin positive (Fig. 3c). These results indicate that RUNX3 expression increases the number of cells in the Wnt-high population of KatoIII cells.

Tumor suppressing function of RUNX3 in KatoIII cells. It has been reported that RUNX3 suppresses the tumorigenicity of KatoIII cells.[19] Therefore, we reexamined the role of RUNX3 in the tumorigenicity of KatoIII cells. The RUNX3 vector-transfected KatoIII cells exhibited slight but significant suppression of cell proliferation at 48 and 72 h after seeding (Fig. 4a), and RUNX3 siRNA increased proliferation KatoIII-R3 cells at 48 h after seeding (Fig. S5).

We next transfected the RUNX3-IRES-mGFP expression vector to KatoIII cells, and GFP^{Hi} and GFP^{Lo} cells were isolated by cell sorting, corresponding to RUNX3^{Hi}-expressing and RUNX3^{Lo}-expressing cells, respectively (Fig. 4b). Importantly, proliferation of GFP^{Hi} cells was significantly suppressed compared with that of the GFP^{Lo} cells (Fig. 4c), and we were unable to establish a GFP^{Hi} (RUNX3^{Hi}) cell line due to the limited proliferation. Moreover, RUNX3-transfected KatoIII cells exhibited significant suppression of soft agar colony formation (Fig. 4d), and the number of colonies larger than 0.1 mm in diameter was significantly decreased to 5.6% of the control (Fig. 4e). Moreover, expression of cell cycle inhibitor *p21$^{WAF1/Cip1}$* was increased significantly in KatoIII cells by RUNX3 expression (Fig. 4f), which was consistent with previous report.[20] These results indicate that RUNX3 plays a tumor-suppressing role in KatoIII cells, and that RUNX3-dependent Wnt activation is not sufficient to maintain the tumorigenicity of RUNX3^{Hi} KatoIII cells.

Binding of the RUNX3 and TCF4 complex to the Wnt target gene promoters in KatoIII cells. It has previously been shown that RUNX3 binds to TCF4/β-catenin complex, which suppresses the binding of the complex to the Wnt target gene promoters.[12,13] Notably, immunoprecipitation experiments revealed that RUNX3 bound β-catenin and TCF4 also in the RUNX3-transfected KatoIII cells (Fig. 5a), suggesting that RUNX3, TCF4 and β-catenin form a ternary complex also in KatoIII cells.

. We next performed ChIP analyses to examine whether the binding of the TCF4/β-catenin complex to the promoter of the Wnt target genes was suppressed in the RUNX3-expressing KatoIII cells. In the control KatoIII cells, TCF4 and β-catenin bound to the *Axin2* and c-*Myc* gene promoters (Fig. 5b, Lanes 1 and 4). Importantly, TCF4 and β-catenin also bound to the *Axin2* and c-*Myc* gene promoters in the RUNX3-expressing KatoIII cells, and their band intensities were higher than those of the control cells (Fig. 5b, Lanes 2 and 5). Consistently, ChIP-based real-time PCR analysis showed that the quantified DNA amount of the *Axin2* and

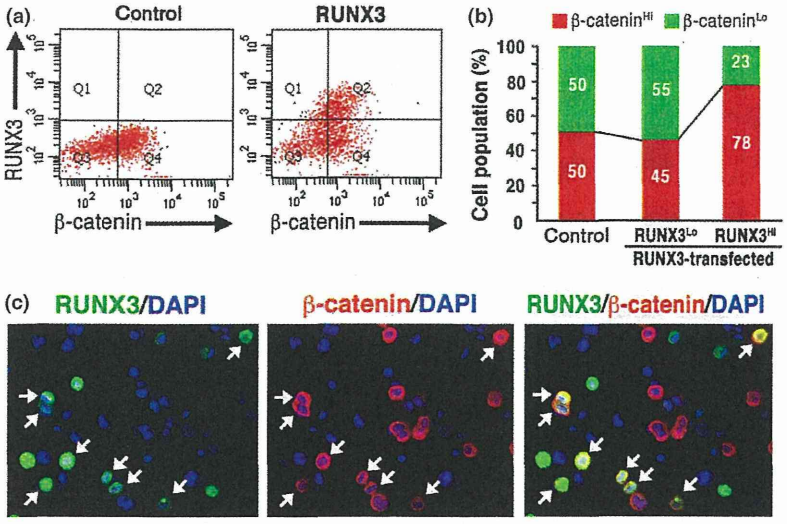

Fig. 3. The increased β-catenin^{Hi} population in RUNX3-expressing KatoIII cells. (a) Flow cytometry analyses of β-catenin and RUNX3 expression in the control (left) and RUNX3-transfected (right) KatoIII cells. The β-catenin^{Hi} population (Q2 and Q4) was set as the top 50% level in the control KatoIII cells. (b) Proportion of the β-catenin^{Hi} and β-catenin^{Lo} cells in the control cells (control) or RUNX3^{Lo} and RUNX3^{Hi} of the RUNX3-transfected cells. (c) Immunocytochemistry for RUNX3 (green, left) and β-catenin (red, center) and a merged image (right) with DAPI staining (blue) of RUNX3-transfected KatoIII cells. Arrows, RUNX3 and β-catenin double-positive cells.

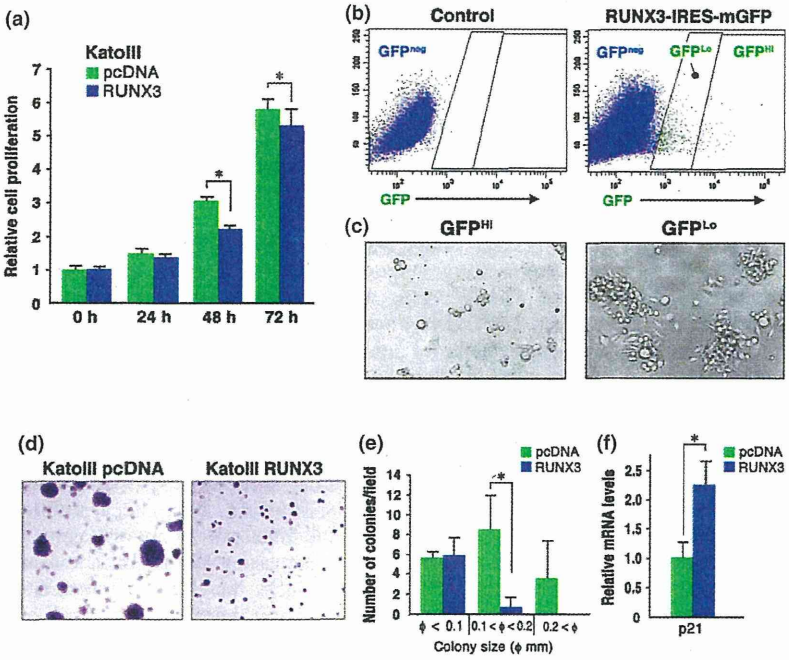

Fig. 4. Suppression of tumorigenicity of KatoIII cells by RUNX3. (a) Relative cell proliferation rates of RUNX3 vector-transfected KatoIII cells and control vector-transfected cells at the indicated time points (mean ± SD). *$P < 0.05$. (b) Flow cytometry analyses for GFP expression in the control (left) and RUNX3-IRES-mGFP-transfected (right) KatoIII cells. (c) Representative photographs of GFP^{Hi} (left) and GFP^{Lo} (right) cells sorted from RUNX3-IRES-mGFP-transfected KatoIII cells after culture for 6 days. (d) Representative photographs of the soft agar colonies of the control (left) and RUNX3-transfected KatoIII cells (right). (e) The number of soft agar colonies per dissecting microscopic field. *$P < 0.05$. (f) Relative p21 mRNA expression level in RUNX3 vector-transfected KatoIII cells to the control cells (mean ± SD). *$P < 0.05$.

c-*Myc* gene promoters that bound TCF4 and β-catenin was significantly higher in the RUNX3-expressing KatoIII cells compared with the control cells (Fig. 5c,d). Moreover, we confirmed that RUNX3 bound the *Axin2* and c-*Myc* gene promoters in the RUNX3-expressing KatoIII cells (Fig. 5b, Lanes 2 and 5, and Fig. 5c,d). In contrast, the R122C mutant RUNX3 did not significantly increase the binding of TCF4 and β-catenin to the *Axin2* and c-*Myc* gene promoters (Fig. 5b, Lanes 3 and 6, and Fig. 5c,d). These results indicate that the complex consisting of RUNX3, TCF4 and β-catenin binds to the promoter of Wnt target genes in KatoIII cells in a more stable fashion than the TCF4/β-catenin complex, which may increase the proportion of the Wnt-high cells in the RUNX3-expressing KatoIII cells.

Discussion

It has been demonstrated that RUNX3 binds to TCF4 and β-catenin, resulting in suppression of the binding of TCF4 to the Wnt target gene promoters, thereby suppressing the Wnt signaling.[12] In the present study, we found that RUNX3 increased the Wnt activity in KatoIII and SNU668 cells. RUNX3 bound TCF4 and β-catenin also in the KatoIII cells, and the complex was stabilized to bind Wnt target gene promoter, which is in contrast to the previous findings.[12,13]

The important question is how RUNX3 activates Wnt signaling in KatoIII and SNU668 cells. It is known that β-catenin replaces the transcriptional repressor, Groucho, from