Ca^{2+}/Calmodulin-Dependent Kinase IIδ Causes Heart Failure by Accumulation of p53 in Dilated Cardiomyopathy

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Background—Dilated cardiomyopathy (DCM), characterized by dilatation and dysfunction of the left ventricle, is an important cause of heart failure. Many mutations in various genes, including cytoskeletal protein genes and contractile protein genes, have been identified in DCM patients, but the mechanisms of how such mutations lead to DCM remain unknown.

Methods and Results—We established the mouse model of DCM by expressing a mutated cardiac α-actin gene, which has been reported in patients with DCM, in the heart (mActin-Tg). mActin-Tg mice showed gradual dilatation and dysfunction of the left ventricle, resulting in death by heart failure. The number of apoptotic cardiomyocytes and protein levels of p53 were increased in the hearts of mActin-Tg mice. Overexpression of Bcl-2 or downregulation of p53 decreased the number of apoptotic cardiomyocytes and improved cardiac function. This mouse model showed a decrease in myofilament calcium sensitivity and activation of calcium/calmodulin-dependent kinase IIδ (CaMKIIδ). The inhibition of CaMKIIδ prevented the increase in p53 and apoptotic cardiomyocytes and ameliorated cardiac function.

Conclusion—CaMKIIδ plays a critical role in the development of heart failure in part by accumulation of p53 and induction of cardiomyocyte apoptosis in the DCM mouse model. **(*Circulation*. 2010;122:891-899.)**

Key Words: apoptosis ■ CaMKII ■ cardiomyopathy ■ heart failure ■ genes, p53

Heart failure is an important cause of morbidity and mortality in many industrial countries, and dilated cardiomyopathy (DCM) is one of its major causes.[1] Although treatments for heart failure have been progressed well in both pharmacological and nonpharmacological aspects, mortality of DCM patients remains high, and the only treatment for DCM patients with severe symptoms is heart transplantation. Because the number of hearts for transplantation is limited, the development of novel therapies for DCM has been awaited.

Clinical Perspective on p 899

DCM, characterized by dilatation and impaired contraction of the left ventricle, is a multifactorial disease that includes both hereditary and acquired forms. The acquired forms of DCM are caused by various factors.[2] Twenty percent to 35% of patients have hereditary forms,[1] and advances in molecular genetic studies during the last decade have revealed many mutations of various genes in DCM patients.[3–5]

Several hypotheses have been reported on the mechanisms of how gene mutations lead to DCM phenotypes. Mutations in genes encoding cytoskeletal proteins such as desmin and muscle LIM protein might disturb the interaction between the sarcomere and Z disk, resulting in impaired force transmission from the sarcomere to the surrounding syncytium.[4,6] On the other hand, mutations in genes encoding contractile proteins such as α-tropomyosin and cardiac troponin T have been reported to induce the decrease in myofilament calcium (Ca^{2+}) sensitivity.[7] An increase in apoptotic cardiomyocytes and/or destruction of membrane structure by calpain activa-

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tion have been reported to play a critical role in mutant gene–induced cardiac dysfunction.[8–10] However, the precise mechanisms remain largely unknown as a result, at least in part, of a lack of good animal models of DCM.

Several animal models of DCM have been reported.[11–13] The *mdx* mouse is a model of Duchenne muscular dystrophy, which has mutations in the dystrophin gene.[11] Unlike humans, *mdx* mice rarely show cardiac abnormality, which has limited the utility of *mdx* mice as a model to examine the pathogenesis of DCM. Although Golden Retriever–based muscular dystrophy dogs show DCM phenotypes,[12] the muscular dystrophy dogs are very difficult to maintain and handle. Although BIO 14.6 hamsters lacking δ-sarcoglycan are a good model of DCM,[13] it is difficult to apply genetic approaches to the hamster. To elucidate the molecular mechanisms of how gene mutations cause DCM, appropriate animal models, particularly mouse models, are necessary. We established here a mouse model of DCM by expressing a mutated cardiac α-actin gene (mActin-Tg), which has been reported in patients with DCM, in the heart.[5] mActin-Tg mice showed gradual dilatation and dysfunction of the left ventricle, resulting in death by heart failure. These phenotypes of mActin-Tg mice were quite similar to those of human DCM. In this study, we examined the underlying mechanisms of how this gene mutation leads to DCM using the new mouse model of DCM.

Methods

Detailed experimental methods are described in the online-only Data Supplement.

Mice

We generated transgenic mice (mActin-Tg) that expressed a mutated cardiac α-actin (R312H) with an HA tag in the heart. This mutation has been reported in patients with DCM.[5] Generation of transgenic mice with cardiac-restricted overexpression of human Bcl-2, AC3-I, or nuclear factor of activated T cell (NFAT)–luciferase has been described previously.[14–16] Heterozygous p53-deficient mice were purchased from The Jackson Laboratory (Bar Harbor, Me).[17] Wild-type littermates served as controls for all studies. KN-93 (10 $\mu mol \cdot kg^{-1} \cdot /d^{-1}$) was used to inhibit activation of Ca^{2+}/calmodulin-dependent kinase II (CaMKII). Echocardiography was performed on conscious mice.

Histology

For detection of apoptotic cardiomyocytes, we performed terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) staining, along with immunostaining for dystrophin.

Western Blot Analysis

Whole-cell lysates were resolved by SDS-PAGE. Western blot analyses were performed with some antibodies. The intensities of Western blot bands were measured with NIH ImageJ software (National Institutes of Health, Bethesda, Md).

Luciferase Assay

Left ventricles were homogenized in luciferase assay buffer as described previously.[15]

Force Measurements

A small fiber was dissected from the skinned left ventricular papillary muscle, and isometric force was measured as described previously.[7]

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction Analysis

Quantitative real-time polymerase chain reaction was performed with the LightCycler with the Taqman Universal Probe Library and Light Cycler Master. Relative levels of gene expressions were normalized to the mouse GAPDH expression with the ΔΔCt method.[18]

Statistical Analysis

Data are shown as mean±SEM. Multiple-group comparison was performed by 1-way ANOVA followed by the Bonferroni procedure for comparison of means. The F test was used to assess equal variances before comparison between 2 groups. Then, comparisons between 2 groups were performed with the Student t test (when $P>0.05$ in the F test) and the Welch t test (when $P<0.05$ in the F test). Survival rates were analyzed with the log-rank test. Values of $P<0.05$ were considered statistically significant.

Results

DCM Model Mouse

Because there are few useful DCM mouse models, we first generated transgenic mice that expressed a cardiac α-actin R312H mutant with an HA tag under the control of α-myosin heavy chain promoter (mActin-Tg). We obtained 3 independent founders of the transgenic mice (lines 301, 307, and 311). The protein levels of the cardiac α-actin R312H mutant were 1.6-fold in line 301, 3.3-fold in line 307, and 2.2-fold in line 311 compared with those of endogenous cardiac α-actin (Figure IA in the online-only Data Supplement). To confirm the expression of the transgene in cardiomyocytes, we performed immunohistological analyses with antibodies against HA and actinin. The mutated cardiac α-actin protein was colocalized with actinin, suggesting that the cardiac α-actin R312H mutant is incorporated into myofilaments (Figure IB in the online-only Data Supplement). Cardiac systolic function was decreased in mActin-Tg mice at 10 months of age, and the reduction was well correlated with protein levels of the cardiac α-actin R312H mutant (Figure IC in the online-only Data Supplement). To further investigate whether cardiac expression of the cardiac α-actin R312H mutant led to heart failure, we examined another transgenic mouse that expressed cardiac α-actin A331P mutant with an HA tag in the heart. This mutant has been reported to cause hypertrophic cardiomyopathy in human.[19] We obtained 2 independent founders of the transgenic mice that expressed almost the same levels of the cardiac α-actin A331P mutant protein. Although the protein levels of the mutant in the A331P mutant transgenic mice were almost same as those of the R312H mutant in line 307, which had the highest expression (Figure II in the online-only Data Supplement), echocardiography revealed that there were no significant differences in cardiac systolic function, wall thickness, and left ventricular dimension between cardiac α-actin A331P mutant transgenic mice and their wild-type littermates (Table I in the online-only Data Supplement). Although it is not known at present why the expression of cardiac α-actin A331P mutant did not induce hypertrophic cardiomyopathy, these results suggest that cardiac dysfunction of mActin-Tg mice is due to cardiac expression of the cardiac α-actin R312H mutant in the heart, not to high-level expression of the cardiac α-actin protein with the tag (lines 307 and 311).

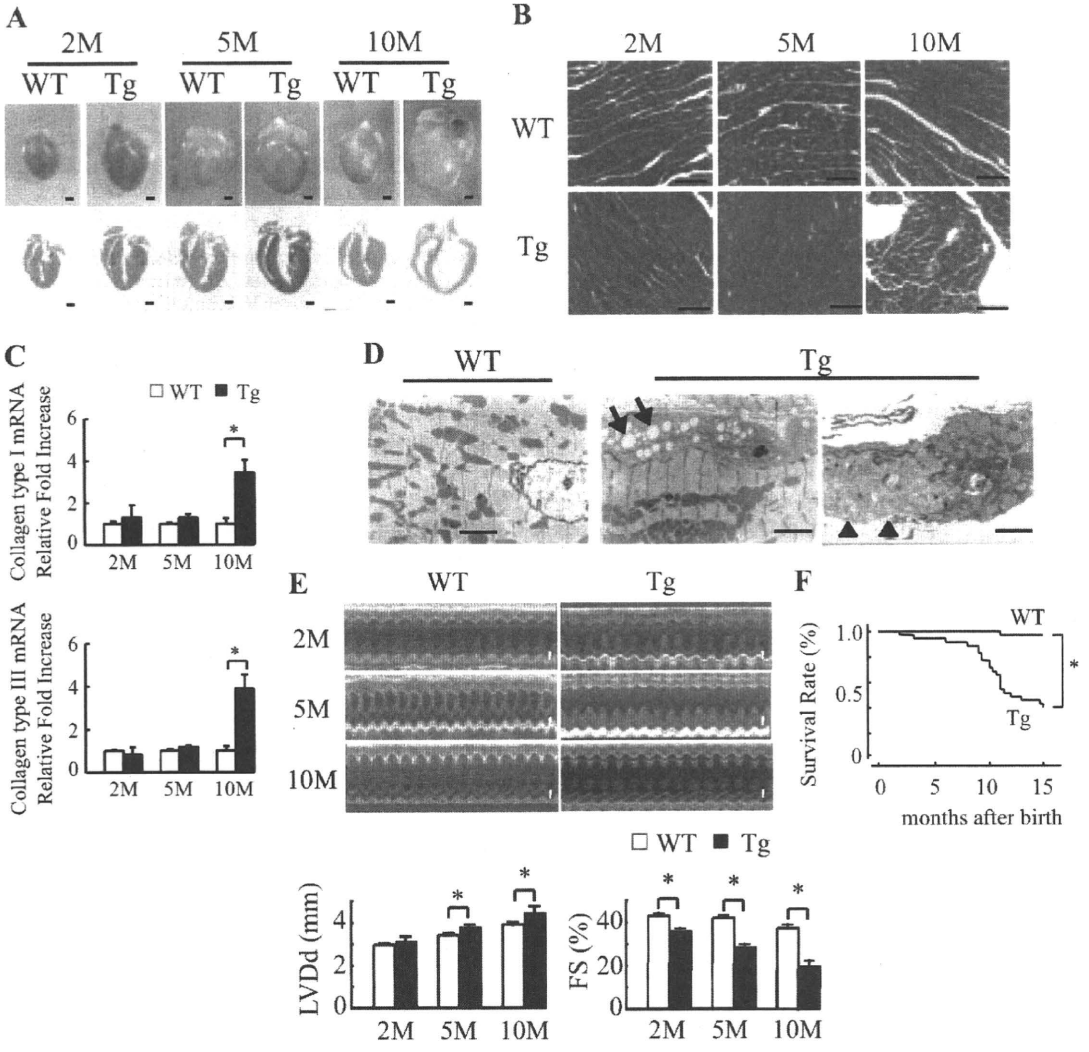

Figure 1. Mutated cardiac α-actin R312H transgenic mice. A, Gross morphology (top) and sections (bottom) of wild-type littermates (WT) or mActin-Tg (Tg) hearts at 2, 5, and 10 months (M) of age. Scale bar=1 mm. B, Masson trichrome staining. Scale bar=100 μm. C, Relative levels of collagen types I and III in hearts were normalized to GAPDH expression. *$P<0.05$ vs WT mice. n=4 in each group. D, Electron microscopic analyses. Cytoplasmic vacuolization (arrow) and lysis of myofibrils (arrowhead) were detected in the hearts of Tg mice. Scale bar=10 μm. E, Echocardiographic analysis. Scale bar=1 mm. LVDd indicates left ventricular end-diastolic dimension; FS, fractional shortening. *$P<0.05$. F, Kaplan-Meier survival curve. *$P<0.05$ vs WT mice. WT, n=32; Tg, n=37.

We used line 307, which expressed the cardiac α-actin R312H mutant at the highest levels, for further studies. The hearts in mActin-Tg mice were larger than those of wild-type littermates (Figure 1A), and heart weight and the ratio of heart weight to body weight were much increased in mActin-Tg mice (Table II in the online-only Data Supplement). Marked cardiac fibrosis was observed in mActin-Tg mice at 10 months of age, with increased expression of collagen types I and III (Figure 1B and 1C). Electron microscopic analyses showed that there were degenerated cardiomyocytes with an increase in vacuolar formation and lysis of myofibrils in mActin-Tg mice (Figure 1D). Echocardiography revealed that left ventricular dimension was gradually increased and that fractional shortening was reduced in mActin-Tg mice compared with wild-type littermates (Table II in the online-only Data Supplement and Figure 1E). The expression levels of ANP and SERCA2a were gradually increased and decreased in mActin-Tg mice, respectively (Figure III in the online-only Data Supplement). There was no significant difference in blood pressure, but heart rate was increased in mActin-Tg mice (Table II in the online-only Data Supplement), suggesting that the sympathetic nervous system is activated. Surface ECG monitoring showed low amplitude of the R wave in mActin-Tg mice (Table II in the online-only Data Supplement), which is often observed in human DCM patients. Many mActin-Tg mice died by 35 weeks of age (Figure 1F). Although telemetric ECG recording did not show life-threatening arrhythmia in mActin-Tg mice (data not shown), spontaneous Ca^{2+} sparks and Ca^{2+} waves were significantly increased in the cardiomyocytes of mActin-Tg mice (Table III in the online-only Data Supplement), suggesting that not only cardiac pump failure but also arrhythmia could be the cause of death. These phenotypes of mActin-Tg mice were quite similar to those of human DCM.

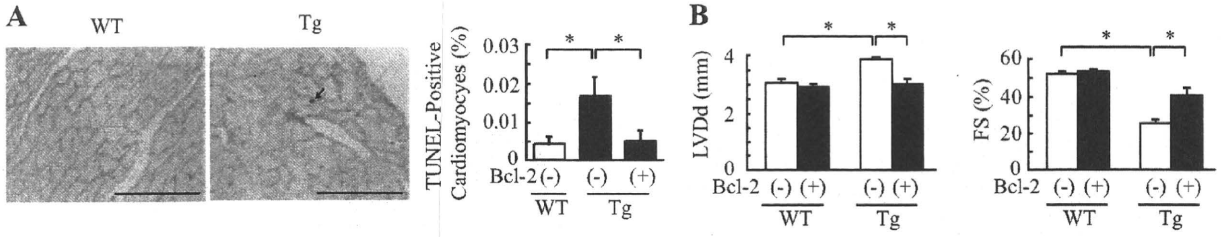

Figure 2. Increase in Bcl-2 preserves cardiac function in mActin-Tg mice. A, Double immunostaining for TUNEL (black) and dystrophin (red) of the heart (left). The graph indicates quantitative analyses of TUNEL-positive cardiomyocytes. Scale bar=100 μm. n=4 in each group. *$P<0.05$. B, Echocardiographic analyses at 5 months of age. *$P<0.05$. WT/Bcl-2(−), n=5; WT/Bcl-2(+), n=10; Tg/Bcl-2(−), n=10; Tg/Bcl-2(+), n=5. WT indicates wild-type littermates; Tg, mActin-Tg mice; LVDd, left ventricular end-diastolic dimension; and FS, fractional shortening.

Apoptotic Cardiomyocytes Are Increased in mActin-Tg Hearts

It has been reported that apoptosis of cardiomyocytes is observed in hearts of human DCM[10] and that cardiomyocyte death might cause cardiac dysfunction.[20] We thus examined apoptosis of cardiomyocytes by TUNEL labeling in left ventricular sections of wild-type littermates and mActin-Tg mice at 5 months of age. The number of TUNEL/dystrophin double-positive cardiomyocytes was significantly larger in mActin-Tg mice compared with wild-type littermates (Figure 2A). To examine whether the increase in apoptotic cardiomyocytes causes cardiac dysfunction in mActin-Tg mice, we generated double-transgenic mice by crossing mActin-Tg mice and the transgenic mice, which overexpress the antiapoptotic protein Bcl-2 in cardiomyocytes [mActin(+)/Bcl-2(+)-DTg].[14] The number of apoptotic cardiomyocytes in mActin(+)/Bcl-2(+)-DTg mice was significantly less compared with mActin-Tg mice (Figure 2A). Echocardiography revealed that the left ventricular dimension was smaller and fractional shortening was better in mActin(+)/Bcl-2(+)-DTg mice than in mActin-Tg mice at 5 months of age (Figure 2B), suggesting that the increase in apoptotic cardiomyocytes causes cardiac dysfunction in the DCM mouse model.

p53 Is Involved in Cardiomyocyte Apoptosis in mActin-Tg Mice

To clarify the mechanisms of how the cardiac α-actin R312H mutant induces apoptosis of cardiomyocytes, we examined expression levels of apoptosis-related proteins by Western blot analyses. The protein levels of p53 and Bax were higher in mActin-Tg mice compared with wild-type littermates (Figure 3A). Several key proapoptotic genes have been reported to be positively regulated by p53,[21] and increased expression of p53 induces left ventricular dilatation and dysfunction in several types of mice.[22,23] To determine the role of p53 in gene mutation–induced DCM, we crossed mActin-Tg mice and heterozygous p53-deficient mice [p53(+/−)]. Because many of homozygous p53-deficient mice [p53(−/−)] died of tumors before 5 months of age,[17] we used heterozygous p53-deficient mice [p53(+/−)] for this study. Echocardiography revealed that left ventricular dimension was smaller and fractional shortening was better in mActin-Tg/p53(+/−) mice than in mActin-Tg/p53(+/+) mice at 5 months of age (Figure 3B). Loss of a single p53 allele attenuated the increase of Bax (Figure 3C) and reduced the number of apoptotic cardiomyocytes in mActin-Tg mice (Figure 3D). These results suggest that p53-induced cardiomyocyte apoptosis induces dilatation and dysfunction of the left ventricle in the DCM mouse model.

Myofilament Calcium Sensitivity Is Decreased and Calcium-Dependent Enzymes Are Activated in mActin-Tg Mice

Many gene mutations associated with DCM have been reported to induce the decrease of myofilament Ca^{2+} sensi-

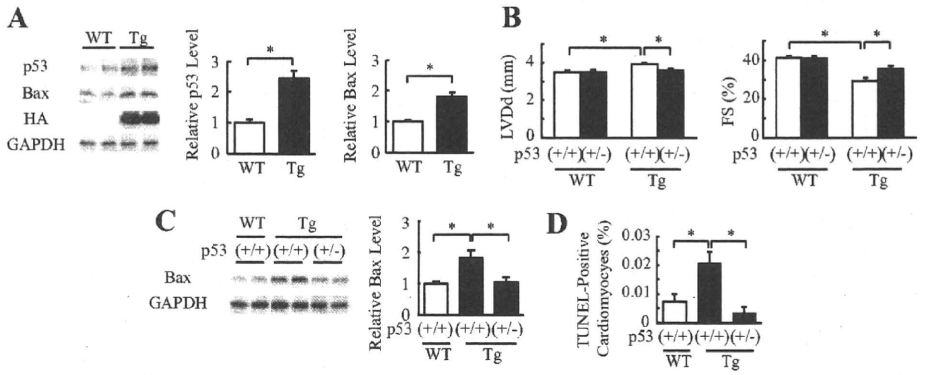

Figure 3. Inhibition of p53 preserves cardiac function in mActin-Tg mice. A, Western blot analyses in the hearts of wild-type littermates (WT) or mActin-Tg (Tg) mice at 5 months of age. The graph indicates relative protein levels of p53 (n=8 in each group) or Bax (n=10 in each group). *$P<0.05$. B, Echocardiographic analyses at 5 months of age. WT/p53(+/+), n=12; WT/p53(+/−), n=10; Tg/p53(+/+), n=19; Tg/p53(+/−), n=14. *$P<0.05$. C, Western blot analyses in the hearts. The graph indicates relative protein levels of Bax. n=6 in each group. *$P<0.05$. D, Quantitative analyses of TUNEL-positive cardiomyocytes. n=5 in each group. *$P<0.05$.

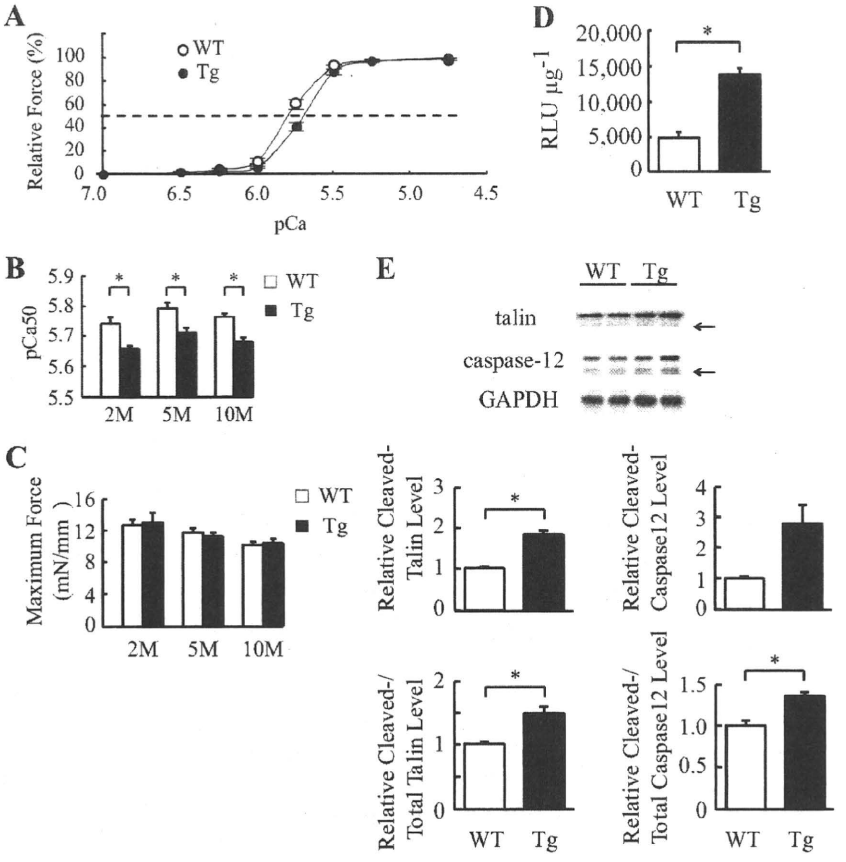

Figure 4. Myofilament Ca^{2+} sensitivity is decreased and Ca^{2+}-dependent enzymes are activated in mActin-Tg mice (Tg). A, Force-pCa relationship in skinned cardiac muscle fiber at 5 months of age. The broken line indicates pCa50. Wild-type (WT; n=11) and Tg (n=10) fibers were prepared from 3 isolated hearts. B, Ca^{2+} sensitivity (pCa50) of force-pCa relationships in skinned cardiac muscle fibers at 2, 5, and 10 months (M) of age. *P<0.05. C, Maximum force-generating capabilities. Fibers (n=9 to 11) were prepared from 3 isolated hearts of each group. D, The NFAT–luciferase reporter activity (RLU μg^{-1}) in the hearts at 5 months of age. n=4 in each group. *P<0.05. E, Western blot analyses in the hearts. Arrows indicate the calpain cleaved forms of talin and caspase-12. The graph indicates relative protein levels of cleaved talin or caspase-12 and ratio of cleaved forms to total proteins. n=4 in each group. *P<0.05.

tivity.[7] We examined myofilament Ca^{2+} sensitivity in mActin-Tg mice. The force-pCa relationship was shifted rightward in mActin-Tg mice compared with wild-type littermates (Figure 4A). The pCa value at half-maximal force generation (pCa50, an index of Ca^{2+} sensitivity) was significantly lower in mActin-Tg mice (Figure 4B), suggesting that skinned cardiac muscle fibers prepared from mActin-Tg mice show a decrease in Ca^{2+} sensitivity of force generation. The degree was the same between 2 and 10 months of age (Figure 4B), suggesting that the reduction in Ca^{2+} sensitivity is not a result of cardiac dysfunction. Despite the reduced Ca^{2+} sensitivity, there was no significant difference in maximum force-generating capabilities between wild-type littermates and mActin-Tg mice (Figure 4C). The decrease in myofilament Ca^{2+} sensitivity is expected to influence intracellular Ca^{2+} handling in cardiomyocytes of mActin-Tg mice. To clarify whether intracellular Ca^{2+} levels in cardiomyocytes are changed in mActin-Tg mice, we examined the activity of Ca^{2+}-dependent enzymes such as calcineurin and calpain. We generated double-transgenic mice by crossing mActin-Tg mice and the transgenic mice carrying a luciferase reporter driven by a cluster of NFAT binding sites, which is activated by calcineurin-dependent NFAT proteins.[15] The NFAT–luciferase reporter activity was higher in mActin-Tg mice than in wild-type littermates at 5 months of age (Table IV in the online-only Data Supplement and Figure 4D). Furthermore, the ratio of the calpain-induced cleaved forms of talin and caspase-12 to total proteins was significantly increased in mActin-Tg mice compared with wild-type littermates (Figure 4E). We next examined Ca^{2+} transients in cardiomyocytes using fluo-3AM (Figure IVA in the online-only Data Supplement). Although the time to peak amplitude of Ca^{2+} was significantly slower in mActin-Tg mice than in wild-type littermates (Figure IVB in the online-only Data Supplement), there was no significant difference in peak amplitude between wild-type littermates and mActin-Tg mice at 2 and 10 months of age (Figure IVC in the online-only Data Supplement). The expression levels of SERCA2a, but not Na^+/Ca^{2+} exchanger, were decreased in mActin-Tg mice (Figure III in the online-only Data Supplement).

CaMKIIδ Is Activated in mActin-Tg Mice

It has been reported that among Ca^{2+}-dependent proteins, expression of CaMKIIδ is increased in human DCM hearts[24] and that overexpression of CaMKIIδ induces heart failure in mice.[25,26] We thus examined the expression and phosphorylation of CaMKIIδ and phosphorylation of its target protein, phospholamban (Thr17). The protein levels of total (both CaMKIIδB and CaMKIIδC) and phosphorylated CaMKIIδ and of phosphorylated phospholamban (Thr17) were increased in mActin-Tg mice compared with wild-type littermates (Figure 5A and Figure VA in the online-only Data Supplement), suggesting that CaMKIIδ is activated in mActin-Tg mice. The protein levels of phosphorylated phospholamban (Ser16), which is activated by protein kinase A, were also increased in mActin-Tg mice (Figure 5A).

Because it has been reported that the sympathetic nervous system is activated in failing hearts and that β-adrenergic

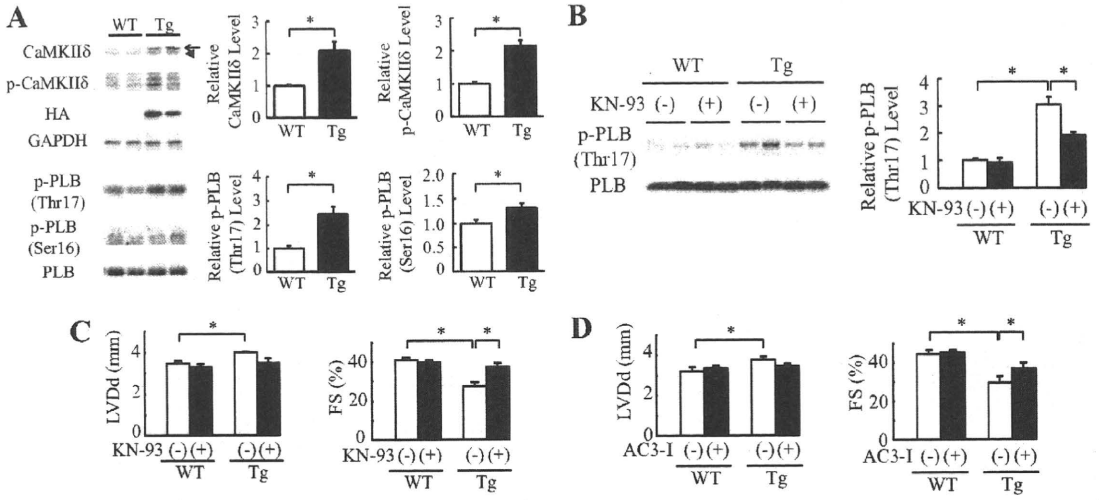

Figure 5. CaMKIIδ is activated in mActin-Tg mice. A, Western blot analyses in the hearts of wild-type littermates (WT) or mActin-Tg (Tg) mice at 5 months of age. The graph indicates relative protein levels of total and phosphorylated CaMKIIδ (p- CaMKIIδ) or phosphorylated phospholamban (p-PLB). Arrow and arrowhead indicate CaMKIIδB and CaMKIIδC, respectively. n=6 in each group. *P<0.05. B, Western blot analyses in the hearts at 5 months of age. The graph indicates relative protein levels of p-PLB (Thr17). n=4 in each group. *P<0.05. C and D, Echocardiographic analyses at 5 months of age. WT/KN-93(−), n=11; WT/KN-93(+), n=7; Tg/KN-93(−), n=8; Tg/KN-93(+), n=6; WT/AC3-I(−), n=8; WT/AC3-I(+), n=18; Tg/AC3-I(−), n=10; Tg/AC3-I(+), n=14. KN indicates KN-93; LVDd, left ventricular end-diastolic dimension; and FS, fractional shortening. *P<0.05.

receptor signal activates CaMKIIδ,[27] we treated mActin-Tg mice with the β-blocker bisoprolol to clarify the relationship between β-adrenergic receptor signal and activation of CaMKIIδ. The treatment with bisoprolol ameliorated cardiac dysfunction of mActin-Tg mice, and there was no significant difference in cardiac function between wild-type littermates and mActin Tg mice with bisoprolol treatment (Figure VB in the online-only Data Supplement). Furthermore, the increase in CaMKIIδ levels in mActin-Tg mice was prevented by bisoprolol treatment (Figure VC in the online-only Data Supplement), suggesting that the activation of CaMKIIδ in mActin-Tg mice might be due to activation of β-adrenergic receptor signaling.

To test whether activation of CaMKIIδ induces cardiac dysfunction, we first treated mActin-Tg mice with KN-93, a CaMKII inhibitor. Levels of both phosphorylated phospholamban (Thr17) and phospholamban (Ser16) were decreased by KN-93 treatment in mActin-Tg mice (Figure 5B and Figure VD in the online-only Data Supplement). Echocardiography revealed that KN-93 treatment prevented left ventricular dilatation and preserved cardiac function in mActin-Tg mice (Figure 5C). On the other hand, KN-92, an inactive derivative of KN-93, did not show any effects (Figure VE in the online-only Data Supplement). To confirm the role of CaMKIIδ in mActin-Tg mice, we crossed mActin-Tg mice and AC3-I mice, which expressed the CaMKII-inhibitory peptide AC3-I in the heart [mActin(+)/AC3-I(+)-DTg].[16] Echocardiography revealed that fractional shortening was better in mActin(+)/AC3-I(+)-DTg mice than in mActin(+)/AC3-I(−)-Tg mice (Figure 5D), suggesting that the activation of CaMKIIδ in the DCM mouse model induces left ventricular dilatation and contractile dysfunction.

We next examined the relation between CaMKIIδ activation and p53. The increase in p53 was attenuated by treatment with KN-93 or overexpression of AC3-I (Figure 6A and Figure VIA in the online-only Data Supplement). Furthermore, KN-93 treatment inhibited the increase in Bax expression and TUNEL-positive cardiomyocytes (Figure 6A and 6B). It has been reported that CaMKIIδC, but not CaMKIIδB, induces cardiomyocyte death.[27–29] To clarify the mechanism of how CaMKIIδ increases protein levels of p53 and which

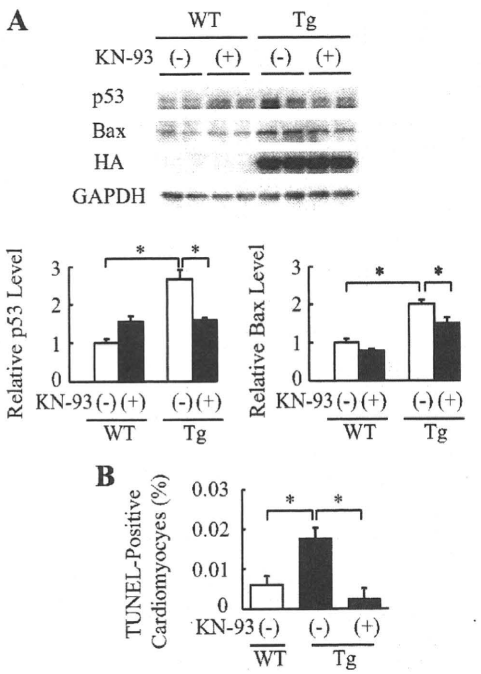

Figure 6. CaMKIIδ regulates expression of p53 in cardiomyocytes. A, Western blot analyses in the hearts of wild-type littermates (WT) or mActin-Tg (Tg) mice. The graph indicates relative protein levels of p53 or Bax. n=4 in each group. *P<0.05. B, Quantitative analyses of TUNEL-positive cardiomyocytes. n=5 in each group. *P<0.05.

CaMKIIδ, δB or δC, plays an important role in apoptosis of cardiomyocytes, we transfected constitutively active forms of CaMKIIδ (caCaMKIIδ) into cardiomyocytes. Only caCaMKIIδC, not caCaMKIIδB, increased protein levels of p53 (Figure VIB in the online-only Data Supplement). Furthermore, p53 protein levels in caCaMKIIδC-transfected cardiomyocytes did not increase with MG132 treatment compared with MOCK-treated cardiomyocytes (Figure VIC in the online-only Data Supplement). These results suggest that activation of CaMKIIδC increases apoptotic cardiomyocytes at least in part via stabilization of p53 in the DCM mouse model.

Discussion

In the present study, we established a novel mouse model of DCM to clarify the mechanisms of how mutant genes lead to DCM (Table II in the online-only Data Supplement and Figure 1). The mice expressing cardiac α-actin R312H mutant in the heart, which has been reported to cause DCM in humans,[5] showed dilatation and dysfunction of left ventricle with an increase in ANP messenger RNA levels, which is consistent with human heart failure (Figure 1A and 1E and Table II and Figure III in the online-only Data Supplement). Higher heart rate and hyperphosphorylated phospholamban (Ser16) (Table II in the online-only Data Supplement and Figure 5A) suggest the activation of the sympathetic nervous system to compensate for reduced cardiac systolic function, resulting in an increase in spontaneous Ca^{2+} sparks and Ca^{2+} waves (Table III in the online-only Data Supplement). Myofilament Ca^{2+} sensitivity was decreased in mActin-Tg mice even at 2 months of age (Figure 4B), when cardiac phenotypes such as left ventricular dilatation and cardiac fibrosis were not recognized (Table II in the online-only Data Supplement and Figure 1). These results suggest that the decrease in myofilament Ca^{2+} sensitivity is a primary cause of, not a secondary result from, cardiac dysfunction. Because these phenotypes were quite similar to those of human DCM, mActin-Tg mice are useful for examining the underlying mechanisms of how gene mutations lead to DCM.

There was no significant difference in the peak amplitude of Ca^{2+} transients between wild-type littermates and mActin-Tg mice (Figure IVC in the online-only Data Supplement), suggesting that global Ca^{2+} levels underlying each contractile cycle do not differ between the 2 groups. It has been reported that the peak amplitude of Ca^{2+} transients, which is associated with decreased Ca^{2+} sensitivity and systolic dysfunction, is higher in another mouse model of DCM, [7] suggesting that Ca^{2+} transients are augmented to compensate for decreased myofilament Ca^{2+} sensitivity in this model. In mActin-Tg mice, despite the preserved Ca^{2+} transients (Figure IVC in the online-only Data Supplement), global cardiac function was gradually impaired (Table II in the online-only Data Supplement). Local Ca^{2+} concentration has been reported to be important for the activation of Ca^{2+}-dependent enzymes such as calcineurin, calpain, and CaMKII in cardiomyocytes.[30] The activation of these molecules in mActin-Tg mice (Figures 4D, 4E, and 5A) might be attributed to an increase in local Ca^{2+} levels. It still remains to be determined whether local Ca^{2+} levels are really increased and, if so, how the decrease in Ca^{2+} sensitivity increases local Ca^{2+} levels.

Recent reports have shown that CaMKIIδ plays a crucial role in cardiovascular diseases.[16,31] The transgenic mice that overexpressed CaMKIIδ showed heart failure with systolic dysfunction and left ventricular dilatation.[25,26] In this study, CaMKIIδ was activated in the hearts of mActin-Tg mice (Figure 5A), and inhibition of CaMKIIδ by KN-93 or AC3-I ameliorated cardiac dysfunction in mActin-Tg mice (Figure 5C and 5D), suggesting that CaMKIIδ also plays an important role in gene mutation–induced cardiac dysfunction.

It has been reported that apoptosis of cardiomyocytes is observed in hearts of human DCM[10] and that cardiomyocyte death could cause cardiac dysfunction.[20] However, it remains unclear whether apoptosis of cardiomyocytes causes cardiac dysfunction and how cardiomyocyte apoptosis is induced in hearts of DCM. In this study, there were more apoptotic cardiomyocytes in mActin-Tg mice (Figure 2A), and cardiac function was improved by protecting cardiomyocytes from apoptosis through overexpression of Bcl-2 (Figure 2B). These results suggest that cardiomyocyte apoptosis plays a crucial role in the development of DCM. Several key proapoptotic and antiapoptotic genes have been reported to be positively or negatively regulated by p53, and increased expression of p53 induces left ventricular dilatation and dysfunction in mice deficient in MDM4, an E3 ligase for p53.[23] Furthermore, we have recently demonstrated that p53 is critically involved in pressure overload–induced cardiac dysfunction.[22] The protein levels of p53 were increased in mActin-Tg mice (Figure 3A), and loss of a single p53 allele reduced the number of apoptotic cardiomyocytes (Figure 3D) and improved cardiac function (Figure 3B). These results suggest that p53 is critically involved in induction of cardiomyocyte apoptosis, resulting in left ventricular dysfunction in the mouse model of DCM.

The present study indicates that p53 might be a therapeutic target for DCM. In this study, CaMKIIδ was activated in the hearts of mActin-Tg mice (Figure 5A), and the inhibition of CaMKIIδ attenuated the increase in p53 protein levels (Figure 6A and Figure VIA in the online-only Data Supplement), suggesting that CaMKIIδ regulates protein levels of p53 in the DCM model mice. Although it remains to be determined how CaMKIIδ regulates protein levels of p53, inhibition of CaMKIIδ may become a new therapeutic strategy for DCM patients by reducing p53 protein levels in the heart.

Limitations

This study has a couple limitations. First, we cannot completely rule out the nonspecific effects of overexpression of cardiac α-actin gene with tag because of a lack of transgenic mice that overexpress wild-type cardiac α-actin gene. However, we think the cardiac dysfunction observed in mActin-Tg was due to cardiac expressions of the cardiac α-actin R312H mutant in the heart, not to high-level expressions of the cardiac α-actin protein with tag because of the following reasons: We obtained 3 independent founders of the transgenic mice, and the reduction in cardiac function was well correlated with protein levels of the cardiac α-actin R312H mutant (Figure I in the online-only Data Supplement). An-

other transgenic mouse that expressed cardiac α-actin A331P mutant with an HA tag in the heart did not show cardiac dysfunction (Table I in the online-only Data Supplement), although the protein levels of the mutant in the A331P mutant transgenic mice were almost same as those of the R312H mutant in line 307, which had the highest expression (Figure II in the online-only Data Supplement). Second, we found that CaMKIIδC increases p53 protein levels mainly by its stabilization, but the underlying mechanisms remain to be determined.

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Disclosures

Dr Anderson is named on patents claiming to treat heart failure by CaMKII inhibition and is a cofounder of Allosteros. The other authors report no conflicts.

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CLINICAL PERSPECTIVE

Heart failure is an important cause of morbidity and mortality in many industrial countries, and dilated cardiomyopathy (DCM) is one of its major causes. Molecular genetic studies over the last 2 decades have revealed many mutations of various genes in DCM patients, but the precise mechanisms of how such mutations lead to DCM remain largely unknown partly because of a lack of good animal models of DCM. Here, we established the mouse model of DCM by expressing a mutated cardiac α-actin gene, which has been reported in patients with DCM, in the heart. The transgenic mice showed gradual dilatation and dysfunction of the left ventricle, resulting in death by heart failure. These phenotypes of the transgenic mice were quite similar to those of human DCM. The number of apoptotic cardiomyocytes and protein levels of p53 were increased in the hearts of the DCM mice. Overexpression of Bcl-2, an antiapoptotic factor, or downregulation of p53 decreased the number of apoptotic cardiomyocytes and improved cardiac function. The DCM mice showed activation of CaMKIIδ. The inhibition of CaMKIIδ prevented the increase in p53 and apoptotic cardiomyocytes and ameliorated cardiac function. These results suggest that CaMKIIδ plays a critical role in the development of heart failure in part by accumulation of p53 and induction of cardiomyocyte apoptosis in the DCM mouse model. The inhibition of CaMKIIδ may become a new therapeutic strategy for DCM patients.

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Targeting Wnt Signaling: Can We Safely Eradicate Cancer Stem Cells?

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CCR FOCUS

Targeting Wnt Signaling: Can We Safely Eradicate Cancer Stem Cells?

Fumi Takahashi-Yanaga[1] and Michael Kahn[2,3,4]

Abstract

The Wnt signaling pathways have been conserved throughout evolution and regulate cell proliferation, morphology, motility, and fate during embryonic development. These pathways also play important roles throughout adult life to maintain homeostasis of tissues including skin, blood, intestine, and brain by regulating somatic stem cells and their niches. Aberrant regulation of the Wnt pathway leads to neoplastic proliferation in these same tissues. It has been suggested that Wnt signaling is also involved in the regulation of cancer stem cells (CSC), because there are many similarities in the signaling pathways that regulate normal adult stem cells and CSC. In this Perspective, we have focused on the Wnt/β-catenin signaling pathway, which is the most intensively studied and best characterized Wnt signaling pathway. We provide an overview on the function of the Wnt/β-catenin signaling pathway in CSC, and the possibility of the development of novel therapeutics to target this pathway. *Clin Cancer Res; 16(12); 3153–62.* ©*2010 AACR.*

Wnt/β-catenin Signaling Pathway (Canonical Pathway) and Cancer

Cell signaling cascades activated by Wnt proteins (collectively, the Wnt signaling pathways) have been well conserved throughout evolution. In addition to regulating cellular processes including proliferation, differentiation, motility, and survival and/or apoptosis, the Wnt signaling pathways play key roles in embryonic development and maintenance of homeostasis in mature tissues. Among the described Wnt signaling pathways, [the canonical pathway (Wnt/β-catenin pathway) and the noncanonical pathways (the planar cell polarity pathway, the Wnt/Ca^{2+} pathway, the protein kinase A pathway)], the Wnt/β-catenin signaling pathway is by far the best characterized (1–6).

The activity of the Wnt/β-catenin signaling pathway is dependent on the amount of β-catenin in the cytoplasm. Normally, cytoplasmic β-catenin is maintained at a low level through ubiquitin-proteasome-mediated degradation, which is regulated by a multiprotein "destruction" complex containing axin, adenomatous polyposis coli (APC), and glycogen synthase kinase-3β (GSK-3β). Upon binding of Wnt proteins to a receptor complex comprised of Frizzleds/low-density lipoprotein receptor-related protein (Fz/LRP), cytoplasmic dishevelled (Dvl), a protein downstream of the receptor complex is phosphorylated thereby inhibiting GSK-3β, resulting in the accumulation of nonphosphorylated β-catenin in the cytoplasm. Nonphosphorylated β-catenin avoids degradation and translocates into the nucleus. In the nucleus, β-catenin in the classical Wnt signaling cascade forms a complex with members of the T-cell transcription factor (TCF)/lymphoid enhancer-binding factor (LEF) family of transcription factors. To generate a transcriptionally active complex, β-catenin recruits the transcriptional coactivators, cAMP response element-binding protein (CREB)-binding protein (CBP) or its closely related homolog, p300 (7, 8), as well as other components of the basal transcription machinery, leading to the expression of a host of downstream target genes. Secreted Wnt inhibitor factor 1 (WIF1) and secreted Frizzled-related proteins (SFRP), soluble Wnt receptors and competitors of Fz, respectively, are Wnt target genes that function as endogenous inhibitors of the Wnt signaling pathway (Fig. 1; refs. 1–3).

The importance of aberrant Wnt signaling in some types of cancer (colorectal most definitively) is clear. However, aberrant Wnt signaling may play a role in many other types of malignancies even those in which the classical

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mutations associated with the pathway (i.e., APC truncations, β-catenin mutations) are not present. For example, although our knowledge about the role of Wnt signaling in breast cancer is far from complete, its importance and significance has been the subject of numerous reports during the past 5 years (9, 10). In human breast cancer, there are many reports of inactivation of negative regulators of the Wnt signaling pathway. Similarly, there are numerous studies that have documented the amplification or overexpression of positive regulators of components of this pathway. Dvl is amplified and upregulated in 50% of ductal breast cancers (11). Frizzled-related protein 1 (*FRP1/FRZB*), a secreted Wnt inhibitor, located within chromosomal locus 8p11-21, is frequently deleted in human breast cancers. In approximately 80% of malignant breast carcinomas, Frp1 expression is either repressed, or absent, making it one of the most frequent alterations in breast cancers (12). Axin is downregulated in a small percentage of breast cancers (13). *AXIN2*, on chromosome 17q23-q24, exhibits frequent loss of heterozygosity in breast cancers (14). Both are negative regulators of the canonical Wnt signaling pathway. Collectively, loss of heterozygosity (23 to 40%), mutation (6 to 18%), and hypermethylation of the APC gene, have been shown to result in loss of expression in approximately 36 to 50% of breast tumors (15). In mice, it has long been known that misexpression of Wnt-1, -3, or -10 induces mammary adenocarcinomas (16). The APC^{Min} mouse has also been shown to exhibit an enhanced incidence (~10%) of spontaneous mammary cancer and a greatly increased susceptibility (90%) to carcinogen-induced mammary cancer (17, 18).

WNT Signaling in Stem Cells and Cancer Stem Cells

Although most would agree that Wnt signaling is important in stem cell biology, there is no consensus on whether Wnt signaling is important for proliferation and maintenance of potency (pluri- or multipotency; for example, see refs. 19–21) or differentiation of stem and/or progenitor cells (22, 23). Wnt/β-catenin signaling has been shown to maintain pluripotency in ES cells (20) and is critical for the expansion of neural progenitors, thereby increasing brain size (24). However, Wnt/β-catenin signaling is also required for neural differentiation of embryonic stem cells (25), fate decision in neural crest stem cells (26), and Wnt3a has been reported to promote differentiation into the neuronal and astrocytic lineages by inhibiting neural stem cell maintenance (27). Clearly, Wnt/β-catenin signaling also plays a critical role in lineage decision and/or commitment. These dramatically different outcomes upon activation of the Wnt signaling cascade have fueled enormous controversy about the role of Wnt signaling in the maintenance of potency and induction of differentiation.

The similarities between normal adult stem cells and cancer stem cells (CSC; ref. 28), suggest that the signaling pathways (e.g., Wnt, Hedgehog, and Notch) involved in regulating somatic stem cell maintenance are also involved in the regulation of CSC (29, 30). Aberrant regulation of these same pathways leads to neoplastic proliferation in the same tissues (31, 32). Interestingly, progression of chronic myelogenous leukemia from chronic phase to blast crisis and imatinib resistance was correlated with increased nuclear β-catenin levels, a hallmark of increased Wnt/β-catenin transcription (33). Recent studies have revealed that multidrug resistance genes, including MDR-1, ABCG2, ABCA3, and BRCP1 are also intrinsically expressed in stem and/or progenitor cells from multiple adult tissues and that they may contribute to the side population (SP) phenotype of malignant cells (34–37). Wnt/β-catenin signaling seems to play an important role in *ABCB1*/MDR-1 transcription. This observation was initially based upon the increased expression of MDR-1 associated with intestinal crypt cells, which carry a defective *APC* tumor suppressor gene in both the Min mouse and FAP patients (38, 39). Putative TCF binding elements were also identified in the *ABCB1* promoter (−1,813 to −275 bp; ref. 38). Canonical Wnt signaling is believed to play an important role in the maintenance of hematopoietic progenitors and also in the lineage commitment of progenitors during hematopoiesis. Expression of *survivin*, which is a Wnt/CBP/β-catenin-regulated gene (40), is important during hematopoiesis (41), and it is prominently upregulated in $CD34^+$ hematopoietic stem and/or progenitor cells upon growth factor treatment. *Survivin*-deficient hematopoietic progenitors show defects in erythroid and megakaryocytic formation (42). Recently, continued expression of *survivin* upon differentiation has been associated with teratoma formation by hES cells (43). However, it is worth noting that β-catenin-deficient (44) and even β,γ-double-deficient (45) mice maintain apparently normal hematopoiesis through the Wnt signaling cascade (46), pointing to yet uncharacterized catenin-like molecule(s) that can compensate for the loss of both β and γ-catenin.

Interestingly, many of the cell surface markers (including LGR5/GPR49, CD44, CD24, and Epcam) that have been used to identify and isolate putative tumor stem cell populations in a variety of tissues are direct Wnt targets. The role of the Wnt signaling cascade, particularly in CSC in other malignancies, which do not carry classical activating mutations in the Wnt pathway, is becoming more apparent. For example, multiple myeloma is quite responsive to a wide array of therapeutic protocols including conventional cytotoxics, corticosteroids, radiation therapy, and an increasing number of targeted chemotherapeutic agents, e.g., the proteasome inhibitor bortezomib. Despite this, few if any patients are "cured" using these approaches and relapse remains a critical issue. The majority of multiple myeloma infiltrates phenotypically resemble normal terminally differentiated plasma cells with the ability to produce monoclonal immunoglobin. That the majority of myeloma plasma cells are quiescent, particularly at diagnosis, led to the investigation of a restricted "stem cell" population critical for tumor growth. Thirty years ago, Salmon and Hamburger showed the ability of

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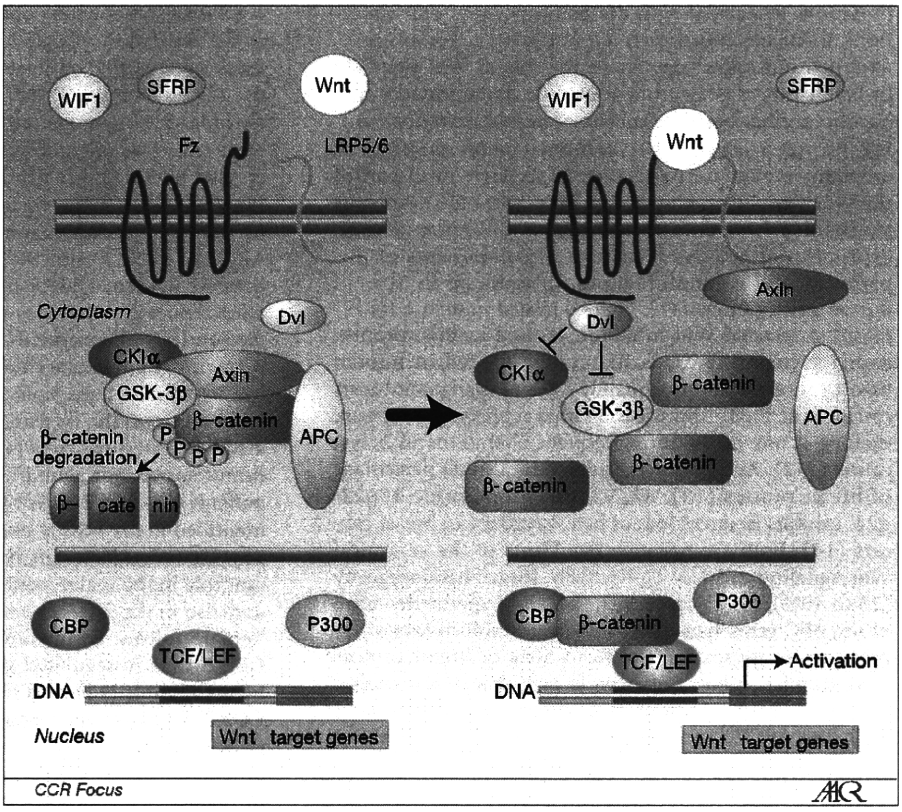

Fig. 1. Canonical Wnt signaling pathway. Protein complex formed by axin, APC, GSK-3β, and CK1α (named β-catenin destruction complex) binds to β-catenin in the absence of Wnt, resulting in the degradation of β-catenin by 26S proteasome system. WIF1 and sSFRPs can work as endogenous inhibitors of the Wnt signaling pathway (left). When Wnt binds to the receptor Fz and co-receptor LRP5/6, these receptors mediate signals into cells and inhibit the function of β-catenin destruction complex. Accumulated-β-catenin in the cytoplasm translocates to nucleus. In the nucleus, β-catenin activates the transcription of target genes together with transcription factors (TCF/LEF) and other transcriptional cofactors, such as CBP (right). Adapted with permission from Takahashi-Yanaga et al. (117). CK1α, casein kinase 1α; sSFRP, secreted Frizzled-related proteins.

~90% of tumor samples from multiple myeloma patients to form colonies and that clonogenic growth occurred at a frequency of between 1 in 100 to 100,000 cells. Importantly, as with other CSC populations, multiple myeloma CSCs have been found to be relatively resistant to existing chemotherapies. Moreover, multiple myeloma stem cells display high expression of multidrug resistance transporters, intracellular detoxification enzymes, and relative quiescence, similar to both other CSC populations as well as normal stem cell populations.

In the Wnt signaling pathway, a change in coactivators (CBP versus p300) interacting with β-catenin (or catenin-like molecules in the absence of β-catenin; ref. 47), and more generally the basal transcriptional apparatus (48), may be very important for a cell deciding to either maintain its level of potency (be that embryonic or somatic stem cell), or to go on to differentiate and lose a level of potency. According to the model developed by Kahn and colleagues, CBP/β-catenin-mediated transcription is essential for stem and/or progenitor cell maintenance and proliferation, whereas a switch to p300/β-catenin-mediated transcription (e.g., increasing the expression of c-jun, fra-1, etc.) is the critical step to initiate differentiation and a decrease in cellular potency. Although a subset of the gene expression cassette that is regulated by the CBP/β-catenin arm is critical for the maintenance of potency and proliferation (e.g., Oct4, survivin, etc.), other genes that are regulated in this manner (e.g., hNkd and axin2) are in fact negative regulators of the CBP/β-catenin arm of the cascade (Fig. 2; refs. 49, 50). Assuming potency and activation of the CBP/β-catenin arm is the default pathway, at some point, in order for development to proceed, one must stop proliferation, exit cell cycle, and initiate the process of differentiation (51, 52). This process is critical for both normal development and tissue homeostasis. Furthermore, the inability to properly initiate and complete differentiation of somatic stem and/or progenitor cells may be the underlying malfunction in essentially all cancers; this, in essence, is another way of restating the CSC hypothesis. Therefore, we would propose that a wide range of mutations (some of which are cell type or tissue specific; e.g., *bcr/abl, K-Ras, Her2*, etc.) can lead to aberrant regulation of the underlying equilibrium between catenin/CBP and catenin/p300; i.e., between proliferation and maintenance of potency and the initiation of differentiation (Fig. 3). Thereby aberrant increase of the CBP/catenin interaction at the expense of the p300/catenin interaction could increase the number of symmetric divisions at the expense of asymmetric divisions. Recent evidence suggests that asymmetric division may function as a tumor suppressive mechanism (53).

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Wnt/β-catenin Signaling as a Therapeutic Target

Accumulating evidence suggests that the Wnt/β-catenin signaling pathway is often involved in oncogenesis and cancer development. Given the fact that multiple mutations can lead to the nuclear translocation of β-catenin, there is a clear need for drugs that attenuate the nuclear transcriptional functions of β-catenin (54, 55). Inhibitors of the Wnt/β-catenin signaling pathway can be grouped into two classes, i.e., small-molecule inhibitors and biologic inhibitors. Small-molecule inhibitors include existing drugs such as nonsteroidal anti-inflammatory drugs (NSAID) and molecular-targeted agents such as the CBP/β-catenin antagonist ICG-001. Biologic inhibitors include antibodies, RNA interference (RNAi), and recombinant proteins. The majority of these inhibitors are in the preclinical stage of development, although at least one has entered clinical trial (also please see Table 1). Below we will briefly summarize development of inhibitors to date.

Existing drugs and natural compounds

A number of existing drugs and natural compounds have been identified as inhibitors and/or modulators of Wnt/β-catenin signaling pathway (reviewed in ref. 56). We will briefly discuss them below.

Nonsteroidal anti-inflammatory drugs. NSAIDs, such as aspirin and sulindac, inhibit the activity of cyclooxygenase (COX), a key enzyme in the arachidonic acid cascade. A number of experimental and epidemiological studies in humans suggested that aspirin and other NSAIDs show chemopreventive effects mainly against colon cancer (57–63), and inhibition of the Wnt/β-catenin signaling pathway is one of their potential mechanisms of action (64, 65). For instance, increased COX-generated PGE_2 suppresses β-catenin degradation, resulting in activation of Wnt/β-catenin signaling. Therefore, suppression of elevated COX activity in cancer cells is likely to be an important factor for the anticancer activity of NSAIDs. However, treatment of colon cancer cell lines with celecoxib, a COX-2 selective inhibitor, was shown to inhibit Wnt/β-catenin signaling by inducing the degradation of TCFs, and this effect was independent of COX-2 (66–69).

At present, celecoxib is the only NSAID approved by the US Food and Drug Administration (FDA) for the treatment of familial adenomatous polyposis.

Vitamins. Retinoids, which are synthesized from vitamin A in the body, are used in some forms of cancer therapy (notably acute promyeloctyic leukemia) and also chemoprevention. An active form of vitamin D, 1α,25-dihydroxyvitamin D3, and its synthetic derivatives have shown chemopreventive effects in animal models of colorectal and breast cancers. Although the mechanism by which vitamins inhibit Wnt/β-catenin signaling pathway is not fully understood, it is suggested that activated nuclear receptors for vitamins interact with β-catenin and compete with TCFs (70, 71). Recently it has also been suggested that both vitamin A and D might induce Wnt/β-catenin inhibitory proteins, e.g., Disabled-2 (Dab2) by retinoic

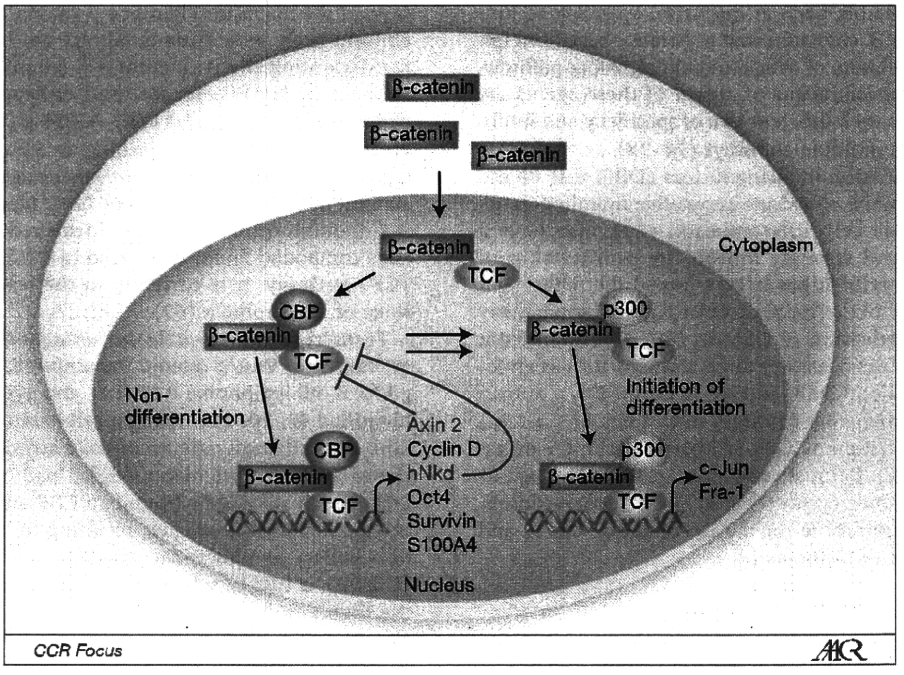

Fig. 2. A negative feedback normally turns off the CBP/β-catenin arm of the pathway and initiates differentiation via the p300/β-catenin arm.

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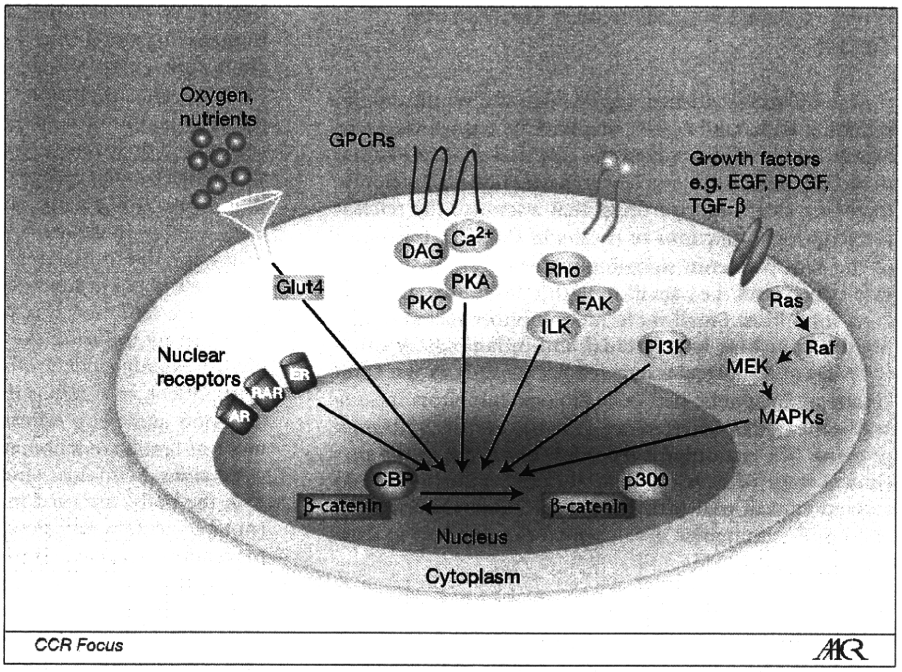

Fig. 3. The ultimate decision for a cell to retain potency or initiate differentiation is dependent upon numerous inputs, some of which are presented here, e.g., concentration of different growth factors, cytokines, and hormones, and the subsequent activation of different signal transduction complexes and kinase cascades, a wide array of mutations, adhesion to substratum. In the end those multiple pathways must be integrated and funneled down into a simple decision point, i.e., a yes-no binary decision. We believe that Wnt signaling and the equilibrium between CBP-mediated and p300-mediated transcription play a central role in integrating these signals.

acids and Dickkopf-1 and -4 (Dkk-1 and Dkk-4) by vitamin D (72, 73).

Polyphenols. Polyphenols are a group of chemicals found in plants, characterized by the presence of more than one phenol unit or building block per molecule. Several polyphenols, such as quercetin, epigallocatechin-3-gallate (EGCG), curcumin, and resveratrol have been implicated as inhibitors of Wnt/β-catenin signaling pathway, although the mechanisms of action of these agents are not clear due to their inherent lack of specificity and inhibitory effects on multiple pathways (74–78).

The differentiation-inducing factors (DIF), first identified in *Dictyostelium discoideum* as putative morphogens required for stalk cell differentiation (79), also have a phenol unit and strongly inhibit the proliferation of human cancer cells. It has been reported that DIF-1 and DIF-3 inhibit the Wnt/β-catenin signaling pathway through the activation of GSK-3β (80–83). Apart from β-catenin, GSK-3β has many target proteins such as glycogen synthase, Tau, CREB, and AP-1. Cyclin D1, a known oncogene, is also one of target molecules of GSK-3β, and phosphorylation by GSK-3β triggers cyclin D1 degradation (84, 85). Because activators of GSK-3β, such as DIFs, could reduce cyclin D1 mRNA and protein levels, they may be applicable for the treatment of cancer and other proliferative disorders (86).

Small-molecule inhibitors identified via high-throughput screen

The molecularly targeted agents reported to date can be classified into four groups, i.e., β-catenin/TCF-antagonists, transcriptional co-activator modulators, PDZ domain of Dvl binders, and other mechanism-based inhibitors.

β-catenin/TCF interaction antagonists. A high-throughput screen of 6,000 natural and 45,000 synthetic compounds identified 8 natural product low molecular-weight antagonists of the interaction between β-catenin and TCF4 (87). Unfortunately these compounds are not highly selectivity for disrupting the β-catenin/TCF complex as they also interact with APC. Trosset and colleagues identified the synthetic compound PNU 74654 by structure-based virtual ligand (*in silico*) screening as a β-catenin/TCF antagonist (88). However, to our knowledge, the biological activity of PNU 74654 has not been reported. Recently, 2,4-diamino-quinazoline was identified by screening a large compound library and found to inhibit the β-catenin/TCF4 pathway. It is currently in the lead optimization stage of development (89).

Transcriptional co-activator antagonists. Several co-activators for Wnt/β-catenin transcription, including CBP, p300, B-cell lymphoma 9 (BCL9), and pygopus have been identified (7, 90, 91). Using a cell-based reporter screen and a small molecule secondary structure-templated chemical library, we identified the lead compound ICG-001, which selectively bound to CBP and prevented its interaction with β-catenin, resulting in the suppression of a subset of Wnt/β-catenin-driven gene expression. ICG-001 is highly selective and does not interact with the highly homologous co-activator p300. As described above, the switch from β-catenin/CBP to β-catenin/p300 controls fundamental stem and/or progenitor cell switching points, i.e., the initiation of a differentiative

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program with a more limited proliferative capacity (92, 93).

Targeting the PDZ domain of Dvl. Dvl is an essential protein in the Wnt signaling pathway that transduces extracellular Wnt signals to downstream components. Dvl uses its PDZ domain, a common protein-protein interaction motif that recognizes short peptide motifs, to bind to the COOH-terminal region of the Wnt receptor Fz. Three compounds (NSC668036, FJ9, and 3289-8625), which have the ability to block Wnt signaling *in vivo*, were identified through *in silico* screening and nuclear magnetic resonance spectroscopy (94–96). These studies showed that this method can provide a useful tool for developing small-molecule inhibitors of the Wnt pathway.

Alternative mechanism-based inhibitors. In 2009, two additional reports describing Wnt/β-catenin inhibitors were published. Chen and colleagues described several small molecule inhibitors of Wnt response (IWR), which stabilize the protein Axin, and another group termed Wnt production inhibitors (IWP), which inhibit Porcupine, an essential protein for Wnt secretion (97). A recent report described the Wnt inhibitor XAV939, which induces the stabilization of Axin by inhibiting the poly-ADP-ribosylating enzymes Tankyrase1 and 2 (98). These inhibitors successfully inhibited Wnt-mediated cellular responses, indicating that stabilization of Axin could be a new target for the inhibition of the Wnt/β-catenin cascade.

Biologic inhibitors

Therapeutic monoclonal antibodies against Wnt-1 and Wnt-2 have been developed and shown to inhibit Wnt signaling and suppress tumor growth *in vivo* (99–101). Similarly, small interfering RNA (siRNA) against Wnt-1 and/or Wnt-2 had potential therapeutic utility in cancer cell lines. Furthermore, therapeutic proteins, i.e., WIF1 and SFRPs, are presently being developed and tested in preclinical tumor models (102, 103).

Perspectives on Therapeutic Intervention

Drugs that target aberrant activation of the Wnt signaling cascade have enormous potential as novel cancer therapeutics. Furthermore, because of the importance of Wnt signaling in stem and/or progenitor populations, they may offer the ability to eliminate normally drug resistant CSCs, which are thought to be associated with relapse and metastasis. However, this enthusiasm needs to be tempered by the stark reality that the Wnt signaling cascade is also

Table 1. Summary of inhibitors against Wnt signaling pathway

Inhibitors	Subcategory	Therapeutic	Pathway target	Development stage
Small molecules				
Existing drugs and natural compounds	NSAIDs	Aspirin	β-catenin	Clinical
		Sulindac,	β-catenin	Clinical
		Celecoxib	TCF	Clinical
		Etc.		
	Vitamins	retinoids	β-catenin	Clinical
		1α25,-dihydroxy-Vitamin D3	β-catenin	Clinical
	Polyphenols	Querucetin	Unknown	Preclinical
		EGCG	Unknown	Preclinical
		Curcumin	Unknown	Preclinical
		Resveratrol	Unknown	Phase II
		DIF	GSK-3β	Preclinical
		Etc.		
Molecular targeted drugs		PNU 74654	β-catenin/TCF	Discovery
		2,4-diamino-quinazoline	β-catenin/TCF	Preclinical
		ICG-001-related analogs	CBP	Phase I (2010)
		NSC668036	Dvl	Discovery
		FJ9	Dvl	Discovery
		3289-8625	Dvl	Discovery
		IWR	Axin	Discovery
		IWP	Porcupine	Discovery
		XAV939	Tankyrase 1 & 2	Discovery
Biologics				
		Antibodies	Wnt proteins	Preclinical
		Recombinant proteins	WIF1 and SFRPs	Preclinical
		RNA interference	Wnt proteins	Preclinical

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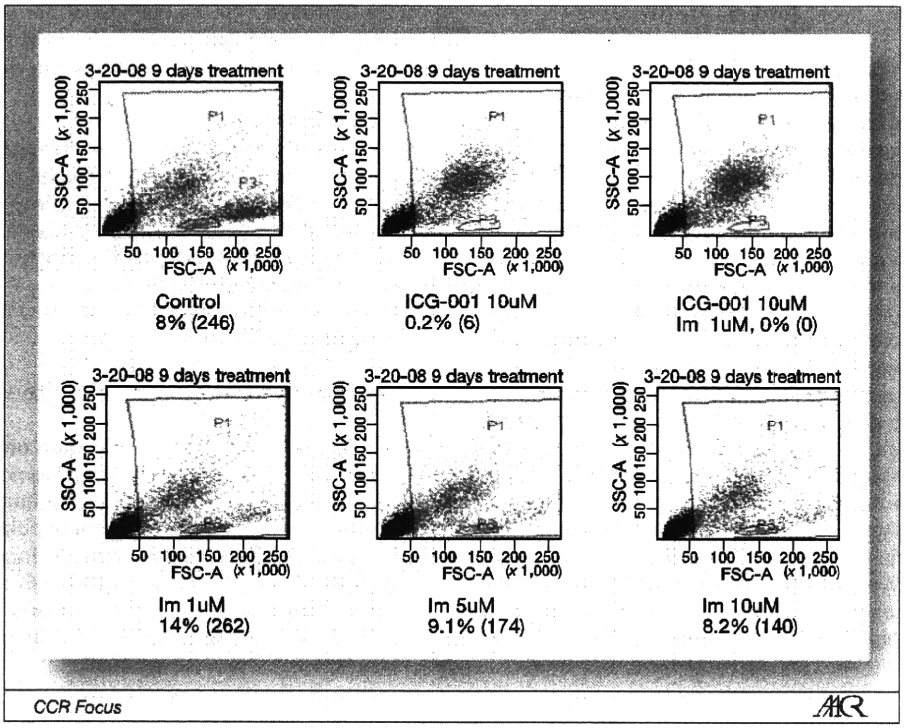

Fig. 4. CBP/catenin antagonism eliminates imatinib-resistant leukemic stem cells. Primary chronic myelogenous leukemia cells were isolated from a leukopheresis sample from a blast crisis patient, who was imatinib naïve. Cells were either treated with imatinib [dimethyl sulfoxide (DMSO) control, Im 1, 5, and 10 μmol/L, ICG-001 10 μmol/L, or Im 1 μmol/L + ICG-001 for 10 days]. The samples were then analyzed by fluorescence-activated cell sorting (FACS). Whereas Im successfully eliminated the blue (P1) population, even increasing concentrations of Im were not effective in eliminating the orange (P3) population. In sharp contrast, ICG-001 effectively eliminated the P3 population, and the combination of ICG-001 + Im effectively eliminated both the P1 and P3 populations. Transplant of 2,000 of the P3 (orange) cells gave rapid engraftment (6 to 12% hCD45 after 2 weeks) in nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) mice and secondary engraftment, whereas 4,000,000 of the P1 (blue) cells gave less than 1% primary engraftment in NOD/SCID mice and no secondary engraftment.

critical in normal somatic stem cell homeostasis and tissue maintenance. As described above, there are a number of drugs and natural compounds that have already been identified to have therapeutic value against cancers associated with aberrant Wnt signaling. However, often because of a lack of specificity, knowledge of their precise target molecule(s) and/or mechanisms by which they inhibit Wnt signaling have not been clarified to date. Identification of these target molecules and determination of the precise mechanism of action of these agents may provide novel targets for the expansion of our anticancer armamentarium. Unfortunately, there is limited evidence that these Wnt inhibitory compounds can target and eliminate the drug-resistant CSC population. Quite recently however, we have been able to show that CBP/catenin antagonists are able to target and eliminate drug-resistant leukemic stem cells both *in vitro* (Fig. 4) and *in vivo* (data are not shown).[5]

Elegant structural studies on the interactions between β-catenin and TCF (104–106), offered *a priori* an attractive mode for inhibition of the Wnt pathway. This high affinity protein-protein interaction has been successfully targeted via a small molecule high-throughput screen, to provide a series of inhibitors of TCF/β-catenin-mediated transcription (88). However, concerns arise about the development of specific inhibitors of this interaction due to the diverse partners besides TCF (e.g., APC and E-cadherin), which also bind to the central Arm repeats of β-catenin (107). Small molecule high-throughput screens have also provided interesting lead compounds (Disheveled PDZ domain and tankyrase inhibitors, etc.) for further development and may provide additional drug leads and novel targets in the future.

The functions of the coactivators CBP and p300 have been described as redundant in several studies (108), and their expression pattern during mouse development is almost identical (109). However, it is becoming increasingly clear that these highly homologous co-activators are not redundant under physiological conditions, and are responsible for distinct transcriptional programs (110–113). The selectivity of ICG-001 is at first quite surprising, given the fact that it interacts with CBP, a co-activator protein used by an extremely wide array of transcription factors (108).

[5] Y. Zhao and M. Kahn, manuscript in preparation.

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However, ICG-001 (MW 548) blocks only a very small percentage of the CBP surface and the compound binds only to CBP, but not the highly homologous co-activator p300.

The critical region of interaction of ICG-001 was mapped to the NH_2-terminus of CBP (amino acids 1 to 111). This region of CBP also interacts with the C-terminal transactivation domain of β-catenin reside (93). Interestingly, this region of CBP also contains binding sites for the retinoic acid (RA) receptor, RXR/RAR, and Vitamin D receptor VDR (108). Therefore, the mechanism of ICG-001, retinoids, and vitamin D may coincide in this regard as they all can antagonize the CBP/β-catenin interaction.

A more potent, specific CBP/β-catenin antagonist, PRI-724, is in development and set to enter phase I clinical trials in 2010. These clinical investigations should allow us to begin to address the question of whether we can safely target CSCs by modulating Wnt signaling.

In regard to recent publications about an increase in Wnt signaling with aging (114, 115) and the importance of stem cell homeostasis in aging and disease, it is interesting to speculate about whether a progressive imbalance in this co-activator equilibrium is associated with the aging process more generally. This observation would coincide with the fact that with many types of cancer, risk increases substantially with aging.

Finally, recent evidence correlated the loss of the β-catenin/E-cadherin interaction in immortalized breast epithelium with both the epithelial-mesenchymal transition and a CSC-like phenotype (116). Although activation of the Wnt signaling cascade was not formally shown, the dramatic increase in the CD44hi, CD24lo population is arguably a mark of activated Wnt signaling. Therefore, it is interesting to speculate that loss of the cytoskeletal-adherens junction role of β-catenin with a concomitant increase in the transcriptional role of β-catenin, may be a hallmark of the epithelial-mesenchymal transition, CSC-enhanced drug resistance, and metastatic capacity in a wide array of malignancies.

Disclosure of Potential Conflicts of Interest

M. Kahn, consultant, commercial research grant, ownership interest (including patents), board member and scientific advisory board member, Prism Biolab; ownership interest (including patents), University of Southern California. F. Takahashi-Yanaga disclosed no potential conflicts of interest.

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