CHAPTER IV
DISCUSSION

This study demonstrated that *Cspg2*$^{\Delta 3/\Delta 3}$ fibroblasts whose versican lacks the A subdomain of the G1 domain exhibit a lower proliferation rate within 20 passages, and by additional passages over 30, acquire anchorage-independent autonomous growth and immortality. When injected into nude mice, the immortal cells form fibrosarcoma with dermal invasion. In the *Cspg2*$^{\Delta 3/\Delta 3}$ fibroblast culture, the disorganized HA-matrix by decreased versican deposition enhances CD44-mediated signal transduction, leading to constitutively activate ERK1/2 and elevated levels of p53 expression, which may account for their senescence. When they exhibit rapid proliferation, both phosphorylation of ERK1/2 and p53 expression are almost abrogated, which may account for the autonomous cell growth. This study illustrates that altered matrix structure involving versican and HA causes the premature senescence and autonomous cell growth under the mechanisms as depicted in figure 4.1.

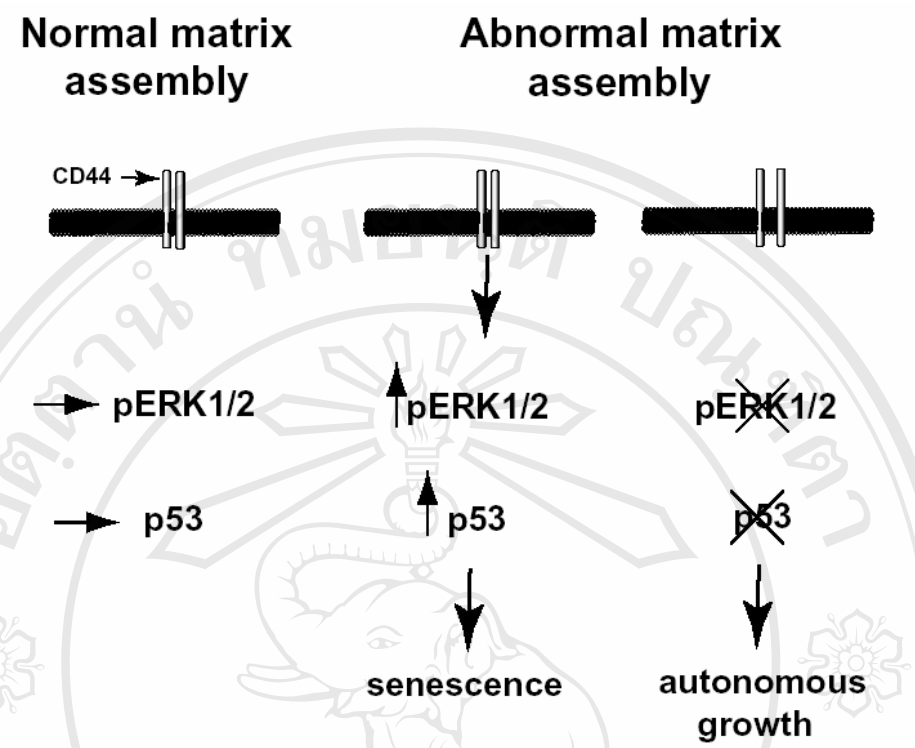

Figure 4.1 A schematic diagram showing the mechanism underlying acquisition of the senescence and the immortality in the *Cspg2*$^{\Delta 3/\Delta 3}$ fibroblasts.

Premature senescence caused by decreased versican deposition in the ECM

The level of versican mRNA and that of chondroitin sulfate in the senescent *Cspg2*Δ3/Δ3 fibroblasts were substantially decreased, presumably due to both a decreased transcription level caused by insertion of *neo*$_r$ gene into exon 3, and a lack of the A subdomain which enhances interaction of versican with HA (Matsumoto *et al.*, 2003). The decreased expression of the mutant versican with reduced HA-binding affinity in the *Cspg2*$^{\Delta 3/\Delta 3}$ fibroblasts possibly affects HA deposition. In WT fibroblast culture, HA exhibited a network structure together with versican, whereas in the *Cspg2*$^{\Delta 3/\Delta 3}$ fibroblast culture did not show such network structure. Although the

Cspg2$^{\Delta 3/\Delta 3}$ fibroblast culture showed substantially decreased staining for HA, the deposition level of HA was decreased only to ~85%. Thus versican may function to assemble HA chains to a network as well as to maintain the HA level in the ECM. The G3 domain of versican and aggrecan binds various ECM molecules including fibulins (Olin *et al.,* 2001), known to form dimers that act as cross-linkers between different HA-aggrecan complexes. They may similarly form HA-versican complexes, and facilitate assembly of HA chains to fibrils. Decreased versican deposition in *Cspg2*$^{\Delta 3/\Delta 3}$ fibroblast culture may abrogate formation of the complex that facilitates the HA-assembly. Recent studies have revealed that HA can present in many structural forms, exhibiting specific functions (Hascall *et al.,* 2004). For example, when cultured colon smooth muscle cells are treated with a viral mimetic, poly I: C, they exhibit HA with a cable structure, which has presumably formed from coalescence of smaller HA strands. Monocytes recognize and bind the HA cable structure but not the HA patches (Majors *et al.,* 2003). Furthermore, Zhuo *et al.*(Zhuo *et al.,* 2006) recently demonstrated that serum-derived HA associated protein (SHAP) potentiates CD44-mediated leukocyte adhesion to the HA substratum, suggesting that SHAP organizes the HA structure. These results may provide another line of evidence that HA in an organized structure regulate cell behavior.

Treatment of *Cspg2*$^{\Delta 3/\Delta 3}$ fibroblasts with an anti-CD44 antibody that blocks HA-CD44 interaction substantially decreased phosphorylation of ERK1/2. CD44 is known to activate ERK1/2 in some cell culture systems. Treatment of proximal tubular cells with HA transduces a signal via CD44, activates ERK1/2, and promotes cell migration (Ito *et al.,* 2004). Heparan sulfate-modified CD44 binds hepatocyte growth factor/scatter factor (HGF/SF) and promotes receptor tyrosine kinase (RTK)-mediated signal transduction (van der Voort *et al.,* 1999). In this experiment,

treatment of the *Cspg2*$^{\Delta 3/\Delta 3}$ fibroblasts with a MEK1 inhibitor PD98059 attenuated the phosphorylation of ERK1/2 to a certain extent, whereas treatment with EGF did not attain further enhancement of the phosphorylation. These results indicate that full activation of ERK1/2 is achieved by the signal induced from the altered ECM, and that the activation can be regulated by the receptor tyrosine kinase (RTK). Recent studies have revealed that a CD44v6 variant form physically interacts with RTKs and activates them (Cheng *et al.,* 2006). The link between CD44 and RTKs remains to be studied. Oncogenic Ras is known to induce the premature senescence in primary fibroblasts (Serrano *et al.,* 1997). Although several Ras-dependent signaling pathways have been identified, constitutively active Ras-Raf-MEK-ERK1/2 cascade, or even active MEK is sufficient for the senescence. Cellular senescence is accompanied by upregulated expression of p53, p16, and p21, and accumulation of senescence associated β-galactosidase (SA-β-gal). The *Cspg2*$^{\Delta 3/\Delta 3}$ fibroblasts showed a high phosphorylation level of ERK1/2, which is indicative of constitutively active MEK. In addition, they exhibited upregulated p53 expression and positive staining for β-galactosidase. All these observations suggest that the premature senescence in the *Cspg2*$^{\Delta 3/\Delta 3}$ fibroblasts is due to constitutively active ERK1/2. HA is known to exhibit different effects on cell behavior, dependent on its sizes and the condition whether it is incorporated in the extracellular matrix or not. For example, endogenous HA transduces the signal via interaction with CD44 and that the HA oligosaccharides competitively inhibit their interaction and downregulate the signal (Camenisch *et al.,* 2000; Tsatas *et al.,* 2002). In this study, It was shown that hyaluronidase treatment increased phosphoERK1/2 levels in WT fibroblasts, which was partially inhibited by blocking the interaction of HA with CD44 using a specific anti-CD44 antibody. In addition, treatment with HA fragments substantially elevated the phosphoERK1/2

levels. These observations strongly suggest that free HA chains rather than those incorporated in the matrix transduce the CD44-mediated signal, leading to phosphorylation of ERK1/2. Interestingly, HA with the size of ~1,000 kDa increased the phosphoERK1/2 levels in a dose-dependent manner, whereas HA with the size of ~104 kDa did highest at the concentration of 5 μg/ml. Other factors may be involved in the accessibility, local concentration, and the optimal signal transduction of HA.

Previous studies have demonstrated effects of versican domains on cell behavior via EGFR. The G3 domain of versican enhances proliferation of NIH3T3 fibroblasts via EGF-like motifs (Zhang *et al.,* 1998). The G3 domain without the EGF-like motifs enhances EGFR/β1-integrin association and attenuates EGFR phosphorylation in U87 astrocytoma cells, thus impairing the autonomous growth of the cells (Wu *et al.,* 2004). The V1 variant enhances proliferation of NIH3T3 cells by upregulating EGFR expression, whereas the V2 variant exhibits an opposite activity (Sheng *et al.*, 2005). Our immunoblot analysis revealed similar expression levels of β1-integrin in WT and the *Cspg2*$^{\Delta 3/\Delta 3}$ fibroblasts. The phosphorylation level of EGFR was the same in both cell types. These observations, which contrast with the previous data, suggest that versican G3 domain is unlikely to directly affect proliferation in the *Cspg2*$^{\Delta 3/\Delta 3}$ fibroblast cultures.

Acquisition of anchorage-independent autonomous growth

Previous studies have shown that, whereas MEK expression in normal fibroblasts induces the cellular senescence, the expression in p53-null fibroblasts causes transformation (Lin *et al.,* 1998). Suppression of p53 expression in senescent mouse embryonic fibroblasts leads to rapid cell cycle reentry (Dirac and Bernards, 2003). These data indicate that decreased p53 expression is sufficient for alteration of cellular behavior from permanent arrest to uncontrolled mitogenesis. p53 expression observed in the senescent *Cspg2*$^{\Delta 3/\Delta 3}$ fibroblasts was almost abrogated by additional several passages, when they obtained the immortality. Thus, depletion of p53 expression presumably caused the autonomous growth. The phosphorylation of ERK1/2 in the immortal cells was almost abrogated, and was not restored by EGF treatment, indicating that the autonomous growth and the immortality are independent of the Ras-Raf-MEK-ERK1/2 cascade.

In general, immortalized cells can be spontaneously obtained by a clonal selection using the 3T3 protocol. Recent studies demonstrated that embryonic fibroblasts with hypomorphic mutation of breast-cancer-associated gene 1 (Brca1) exhibit the premature senescence, dependent on p53 expression and that the immortalized fibroblasts are obtained after clonal expansion (Cao *et al.,* 2003). These data, showing that only a certain population of the cells acquires the immortality, contrast to our observations that most of the *Cspg2*$^{\Delta 3/\Delta 3}$ fibroblasts begin to proliferate almost simultaneously. Although the mechanism of the depletion of p53 expression is unclear, it is unlikely that DNA damage by serial passages of culture caused p53 genetic mutations. By constitutively active ERK1/2, the cells with the cell cycle arrest may obtain a negative feedback loop and attempt to recover proliferating activity by downregulating p53 expression. Abrogation of p53 alone is

not sufficient for acquisition of the autonomous cell proliferation in vivo, as p53-null mice are viable and fertile with no apparent phenotype. Interestingly, cell lines from these mice are more easily established than from WT mice (Lowe *et al.,* 1994, Tsukada *et al.,* 1993, Kamiya *et al.,* 2002). Thus, autonomous cell growth may require both loss of p53 function and depletion of the extracellular matrix. This study may provide evidence of an important role of the ECM in inhibition of tumorigenesis.

Previous studies have demonstrated involvement of HA in tumor growth (Itano *et al.,* 2002; Simpson *et al.,* 2002; Toole *et al.,* 2002). Soluble CD44 that competitively inhibits HA-interaction of CD44 on the cell surface induces cell cycle arrest in cancer cells (Peterson *et al.,* 2000). A mutant versican exerts a dominant-negative effect on astrocytoma cell proliferation. In contrast to these results, the immortal fibroblasts have much less HA and versican in the ECM, and exhibit rapid growth. Interestingly, ERK1/2 phosphorylation and p53 expression were no more observed. In addition, exogenously added versican in the culture medium did not affect the proliferation. These observations suggest that the immortal cells have escaped regulation by the ECM.

Further Investigation

The results from this study clearly demonstrate pivotal roles of the ECM structure involving versican and HA in acquisition of the premature senescence and the anchorage-independent autonomous growth. Knowledge from this study can be used to explain how the extracellular matrix regulates cellular behaviors. In inflammation and wound repair processes, fibroblasts migrate, proliferate, and then cease to grow during reorganization of the tissue. This study apparently contributes a clue to regulation of inflammation and repair by manipulating the ECM molecules.

Experimental wound healing models in conditional knockout mice of versican or hyaluronan synthase genes would facilitate understanding the roles of versican and HA in these processes. Besides, the immortal fibroblasts appear independent of the extracellular microenvironment. The mechanism of abrogation of p53 expression in *Cspg2*$^{\Delta 3/\Delta 3}$ fibroblasts by numerous passages remains to be elucidated.