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Klotho as a therapeutic target during the development of renal fibrosis

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【Running title】 Klotho and renal fibrosis

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【Summary】

Systemic symptoms such as the ectopic calcification, atrophy of skin and muscle, and impaired sexual function observed in chronic kidney diseases (CKD) have been reported to coincide with those observed in geriatric symptoms. Regarding the kidney, clinical/pathological characteristics in CKD patients also coincide with those in the aging kidney. These findings suggest common mechanisms in the development of both CKD and aging. Our investigation of aging factors associated with renal fibrosis in IgA nephropathy patients revealed a significant correlation between accumulation of cells with arrested cell cycle and decreased expression of Klotho protein. Because cell cycle arrest has a protective effect on organs in the acute phase, the proposed therapeutic target against the aging process is to maintain expression of Klotho protein. In addition, transforming growth factor (TGF)-β1 is recognized to play a central role in the development of renal fibrosis. However, TGF-β1 has also been reported to decrease expression of Klotho protein. In this report, we provide an interpretation of our new treatment strategy which involves controlling histone methylation.

[Introduction]

Chronic kidney disease (CKD) is estimated to have a global prevalence of 8% to 16%, and it is recognized as the third most common disease in Japan after hypertension and diabetes mellitus. Various primary diseases can cause CKD, but advancing age can also cause impairment of renal function, and increases the risk of developing acute kidney injury (AKI) and renal carcinoma (1). Even in healthy individuals, it is known that renal function decreases with age while the incidence of AKI and renal carcinoma increases, showing that CKD patients and the elderly have clinical characteristics in common. Renal fibrosis is a pathological finding common to all renal disease, and one of the typical pathological characteristics of aged kidneys is renal fibrosis, suggesting these conditions may share a common pathogenesis on the molecular level.

Many factors have already been associated with aging, but basic research has tied the following from among those that increase or decrease with age, to the development of AKI and carcinomas, and renal fibrosis: DNA damage induced by oxidative stress, accumulation of cells in cell cycle arrest, and decreases in Klotho, an anti-aging protein (2).

[DNA damage due to oxidative stress]

Many organisms require oxygen to survive and maintain their life activities. However, some forms of oxygen are extremely unstable and change into reactive oxygen species which readily react with many substances. Reactive oxygen species cause peroxidation of lipids, protein degeneration, enzyme inactivation and DNA damage, and they are implicated in the progression of various diseases. 8-hydroxydeoxyguanosine (8-OHdG) is formed by hydroxylation of the 8 position in deoxyguanosine, one of the base

components of DNA. This compound is used as a marker of DNA damage due to oxidative stress. With aging, 8-OHdG expression is enhanced, particularly in the kidneys and liver, suggesting that the kidneys are one of the organs easily affected by oxidative stress due to aging. In addition, the use of antioxidants has been shown effective in inhibiting 8-OHdG production, and improving renal fibrosis in a unilateral ureteral obstruction model (UUO) (3), while oxidative stress appears to be intricately related to the onset of AKI and cancer (4, 5). However, reactive oxygen species may also play a role in various physiological functions such as the regulation of intracellular communication, metabolism, and immune function. Before instituting any therapeutic interventions, we should first determine the types and amounts of any antioxidants present at the sites of injury, together with reactions taking place at these sites.

[Accumulation of cell-cycle arrested cells]

Most human somatic cells do not express telomerase, the enzyme that repairs telomeres, so with repeated mitosis, telomeres become shorter and smaller and eventually, the cell cycles stop, causing cell aging. Recently, it has been discovered that stress, in addition to shortening telomeres, can induce other forms of cell aging. This type of cell aging is unrelated to mitotic lifespan, and referred to as "stress-induced cell aging." During the acute phase, aging functions as a normal, protective, physiological mechanism that sets in when normal cells are stressed. However, when cells arrested in the chronic phase of the cell cycle accumulate, this not only enhances cellular sensitivity towards the next stressor, but humoral factors are secreted and have been implicated in causing organ dysfunction. In fact, when p16, which induces cell cycle arrest, is knocked-out in mice, acute phase renal fibrosis worsens in a UUO model, but the renal fibrosis observed during

the chronic phase, produced 4 weeks after ischemic reperfusion injury, is attenuated (6,7). Moreover, p16 is known to be a cancer-inhibiting factor, but when fibroblasts in cell cycle arrest are cultured long-term with epithelial cells, these epithelial cells have been reported to become cancerous (8). This suggests that aging fibroblasts may be involved in the development of renal carcinomas in dialysis patients. Similarly, lung and liver fibrosis are known to contribute to the development of cancer, and fibroblasts in cell cycle arrest are known to be involved in this process as well. Cell cycle arrest may thus have organ-protective effects while in the acute phase, but chronically, contributes to renal fibrosis progression, AKI, and cancer development. One of our future goals will be to determine the appropriate timing for a therapeutic intervention.

[Expression of Klotho proteins]

Klotho genes have been identified as genes responsible for spontaneous mutation mice that express a variety of aging symptoms (9). Mice with overexpression of Klotho genes, however, are known to have extended lifespans (10). The Klotho protein is expressed on the cell surface membrane of renal tubules and plays a role as a co-receptor for fibroblast growth factor 23 (FGF 23) which is known as a phosphorus diuretic hormone. Decreased expression of Klotho protein which accompanies decreased renal function, is important as a factor that can cause bone mineral abnormalities related to CKD. Interestingly, Klotho deficient mice are short-lived with a lifespan of about 8 weeks. They also show arterial calcification, ectopic calcification, osteoporosis, growth dysfunction, skin and muscle atrophy, sexual gland dysfunction, and other symptoms that are similar to those seen in patients with chronic renal failure. Since these symptoms can be alleviated through correction of hyperphosphatemia and hypervitaminosis D, the above symptoms

may be considered a result of calcium, phosphorus, and vitamin D metabolism abnormalities.

On the other hand, Klotho protein exists as secretory types into the blood and urinary system. Klotho protein inhibits insulin-like growth factor1 (IGF1) signals, and it has been reported that when expression of insulin receptor substrate (IRS)-1 is decreased in Klotho-deficient mice, not only is there an extension in lifespan, but the aforementioned arteriosclerosis and ectopic calcification also improve (10). Furthermore, Klotho proteins have recently been reported to inhibit intracellular phosphorus uptake, thus inhibiting transformation of vascular smooth muscle (11). When Klotho protein is administered to Klotho-deficient mice, calcification is inhibited without a change in phosphorus concentrations (12), suggesting that secretory Klotho proteins have physiological activity. Until now, administration of Klotho proteins and excessive expression of Klotho genes have shown organ-protective effects, whereas various experimental animal models have been used to prove that mice with heterogeneous deficiencies for Klotho-gene expression have aggravated organ damage. An even more surprising finding is that even in organs besides the kidneys that only express small amounts of Klotho, there are papers showing that Klotho protein expression does result in organ-protective effects (13). In this way, in addition to bone mineral metabolism, maintaining expression of the Klotho protein is vital in terms of its organ-protective activity.

[Target to treat renal aging]

Using renal biopsy tissue from IgA nephropathy, the most common form of chronic glomerulonephritis, we looked at 8-OHdG, p16, and Klotho protein expression and the relationship to renal interstitial fibrosis. Single regression analysis revealed that in

addition to factors shown thus far to be associated with renal fibrosis such as age, body mass index (BMI), systolic blood pressure, and urinary protein content, there is a significant relationship with 8-OHdG, p16, and Klotho protein expression. Multiple regression analysis showed that aging and increased p16 expression, and decreased expression of Klotho protein are independently correlated to renal fibrosis (Table 1) (2). Based on these results, in renal biopsy samples taken from actual clinical practice, the findings strongly suggest molecules related to aging are involved in progressive CKD. As described above, intervention into cell cycle arrest is the result of avoiding stress and therefore, in practice, we must discover how to maintain Klotho protein expression and this will be our treatment target going forward. On the other hand, various stimuli have been reported to cause decreases in Klotho expression. In fact, we experienced a phenomenon where when we intervened in UUO mice resulting in improvement in renal fibrosis, Klotho protein expression in the kidneys did not improve (14). We need to elucidate the mechanism behind decreased Klotho expression and establish a treatment method.

[The role of TGF-β1-Smad2/3 signals in the renal fibrosis process, and issues related to drug discovery]

Renal fibrosis is the characteristic pathological feature common to CKD progress, so it is clear that transforming the growth factor (TGF)-β1-Smad2/3 signal is intricately involved in this process. However, as shown in Table 1, TGF-β1-Smad2/3 signal has anti-inflammatory effects and so long-term inhibition of this signal pathway may induce severe systemic inflammation. As a matter of fact, TGF-β1 knockout mice may die due

to severe inflammation after birth. We therefore need to develop a therapeutic agent that specifically inhibits just the effects of TGF-β1-Smad2/3 signal in promoting fibrosis.

Renal fibrosis involves a process as follows: 1) loss properties of renal fibroblast, renal tubular cells, vascular endothelial cells, 2) transformation to muscle fibroblasts, 3) muscle fibroblast to extracellular matrix protein production, and in all of these, the TGF-β1-Smad2/3 signal is known to play a role. If the effect of TGF-β1 is classified by transcription activity, the loss of cell properties can be classified as decreased transcription activity, while fibroblast property acquisition and extracellular matrix protein production can be classified as enhanced transcription activity. In inflammatory cells, inhibition of IL-2, IL-4, INF-γ expression by TGF-β1-Smad2/3 signal can be classified as inhibited transcription activity, while enhanced IL-10 expression can be classified as increased transcription activity. However, inhibition of transcription activity due to some kind of stimulation cannot be explained by the existing "stimulation--> transcription --> translation" central dogma, and so the transcription activity inhibition mechanism due to TGF-β1 stimulation must be elucidated.

[Treatment of renal fibrosis through histone methyltransferase inhibition]

Epigenetics has become a hot topic as a regulatory system for gene expression that is not dependent on changes in DNA base sequence. Epigenetics involves processes such as methylation of DNA, modification of the histone tail, and microRNA, but while DNA methylation and microRNA negatively regulate genetic expression, histone tail modification involves a process that both up- and down-regulates transcription activity. Among modifications to the histone tail, methylation of the histone tail is regulated by a specific enzyme and so there is a possibility that by regulating this process, it may be

possible to separate these complex effects. As presented in Fig. 2, we succeeded in showing enhanced expression of H3K4 histone methyltransferase SET domain-containing lysine methyltransferase 7/9 (SET7/9) that enhances genetic expression though TGF-β1 stimulation, and H3K9 histone methyltransferase G9a, which inhibits genetic expression. Furthermore, we proved that monomethylation of H3K4 and H3K9 is an important histone modification associated with renal fibrosis (Fig. 2) (15, 16). On the other hand, results have shown that expression of TGF-β1 immunosuppression requires enhanced expression of IL-10 through trimethylation of H3K4, while inhibited expression of IL-2 is due to H3K9 trimethylation, making it possible to distinguish TGF-β1's effects in promoting fibrosis from its immunosuppressive effects.

[Epigenetics involvement in decreased expression of Klotho protein]

Klotho expression is known to decrease when stimulated by inflammatory cytokines, reactive oxygen species, indoxyl sulfate, and TGF-β1, not only in in vivo studies involving aging or renal dysfunction models, but also in in vitro studies. This suggests that not only does Klotho expression decrease with renal tissue damage, but that an epigenetic mechanism may also be involved.

The Klotho gene promoter region forms a GC-rich island. Methylation of this region regulates whether or not the cell expresses Klotho or not. However, it is not clear what regulates the methylation. Interestingly enough, reports say the degree of methylation correlates to the severity of CKD (17). Moreno et al. have shown that inflammatory cytokines cause nuclear factor (NF)-κB-dependent inhibition of Klotho expression that can be inhibited with the use of histone deacetylase inhibitor, suggesting acetylation of the histone tail may be involved in this process (18). Moreover, there are reports saying

that in cancer cells, Klotho expression is regulated by modification of the H3K9 (19). We have actually confirmed the presence of a Klotho gene promoter region in areas regulated by monomethylation of the H3K9 and it is clear that histone modification is involved in maintaining Klotho expression (16). Binding to the 3`untranscribed region of microRNA-339 and microRNA-556, is also reported to be an important mechanism in inhibiting Klotho expression, and we eagerly await elucidation of its role in renal disease (20).

[In conclusion]

We described our research findings as they relate to aging as a new, potential therapeutic target in renal fibrosis. As described above, we believe that aging is a progressive process that occurs as an extension of physiological function much like oxidative stress. Alternatively, it can be considered the result of protective mechanisms against stress, such as the accumulation of cells in cell cycle arrest. In addition, the significance of a decrease in Klotho expression due to various stimuli may be interpreted as a phosphorus retention mechanism against stress. Furthermore, progress in epigenetics research allows us to view disease from a novel perspective as enhanced or depressed transcription activity. It may prove to be a persuasive mechanism to explain the diversity of cytokines. In the future, we hope to see the development of drugs that regulate epigenetics as treatments for renal diseases.

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Table 1. Univariate and multivariate regression analyses of factors associated with

interstitial fibrosis

	univariate regression		multivariate regression		
	r	*P*	β	*P*	95% CI
Age, year	0.62	<0.0001	0.38	0.001	0.07 to 0.26
BMI, kg/m^2	0.27	0.025	-0.038	0.69	-0.41 to 0.27
SBP, mmHg	0.36	0.002	0.086	0.37	-0.04 to 0.11
Urinary protein, g/24 hour	0.36	0.002	0.14	0.17	-0.35 to 2.5
8-OHdG	0.26	0.031	0.025	0.78	-0.40 to 0.53
p16	0.52	<0.0001	0.24	0.016	0.14 to 1.3
Klotho	-0.56	<0.0001	-0.23	0.019	-1.2 to -0.096

BMI, body mass index

SBP, systolic blood pressure

r, correlation coefficient

R^2 values were 0.57 (unadjusted) and 0.523 (adjusted)

β, standardized β coefficient

Figure Legends

Figure 1. Molecular mechanism of TGF-β1-Smad2/3 signal inflammatory cytokine production.

In inflammatory cells, TGF-β1-Smad2/3 signals inhibit expression of inflammatory cytokines such as IL-2, IL-4, and INF-γ, and are involved in a pathway related to enhanced expression of IL-10, a cytokine that inhibits inflammation.

Figure 2. How Histone Methylation Affects Renal Fibrosis

Unilateral Ureter Obstruction (UUO) mice and TGF-β1-induced histone methylated enzyme SET7/9 and G9a cause H3K4 monomethylation (H3K4me1) and M3K9 monomethylation (H3K9me1). H3K4me1 produces extracellular matrix, while H3K9me1 is involved in loss properties of renal cells, and involved in decreasing expression of the Klotho protein.
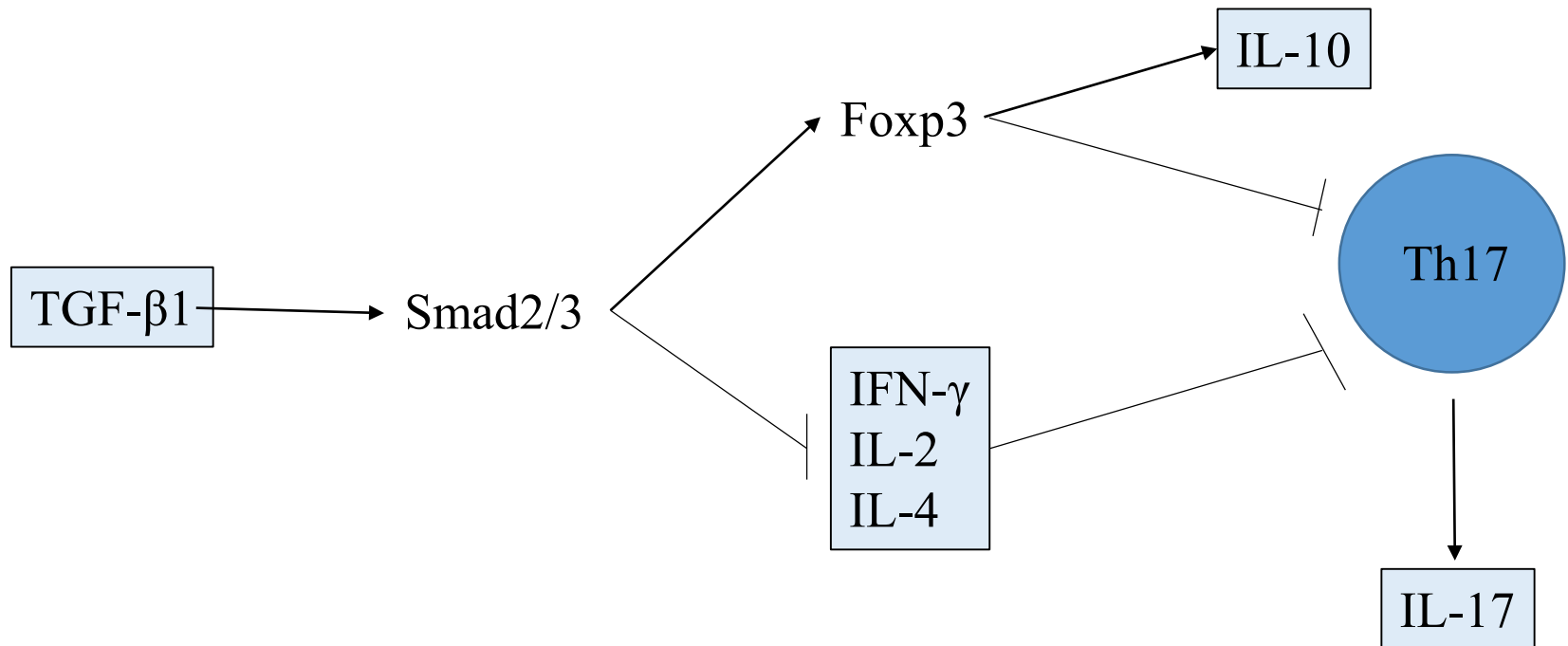
Figure 1.

Figure 2.