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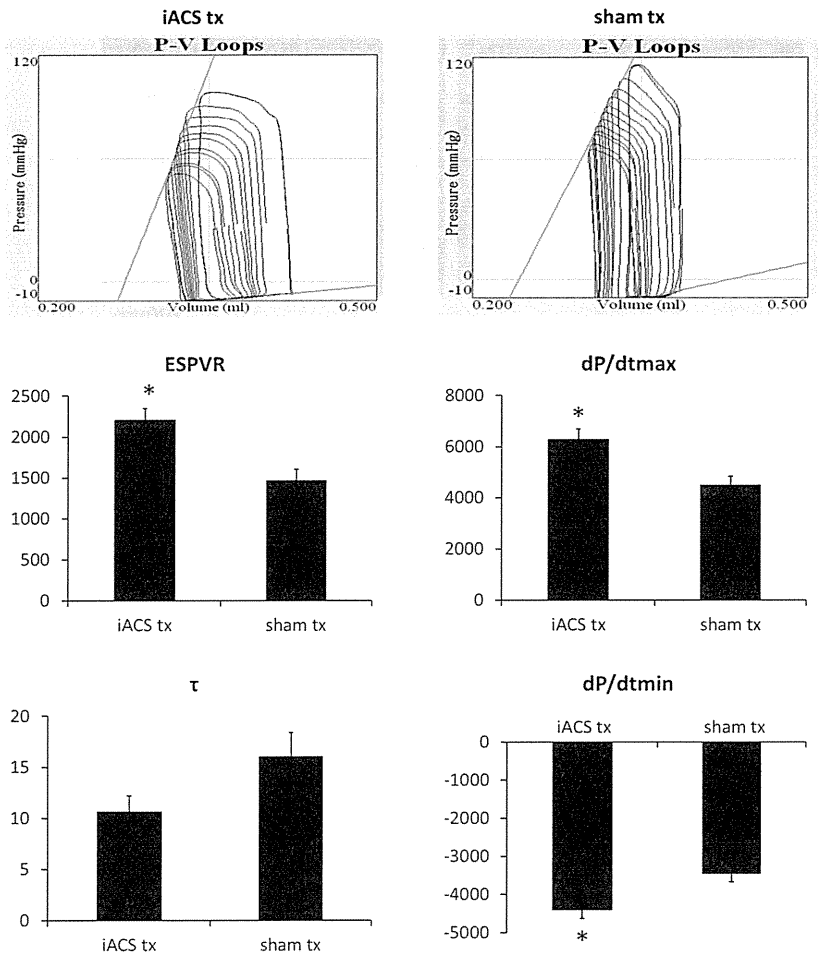

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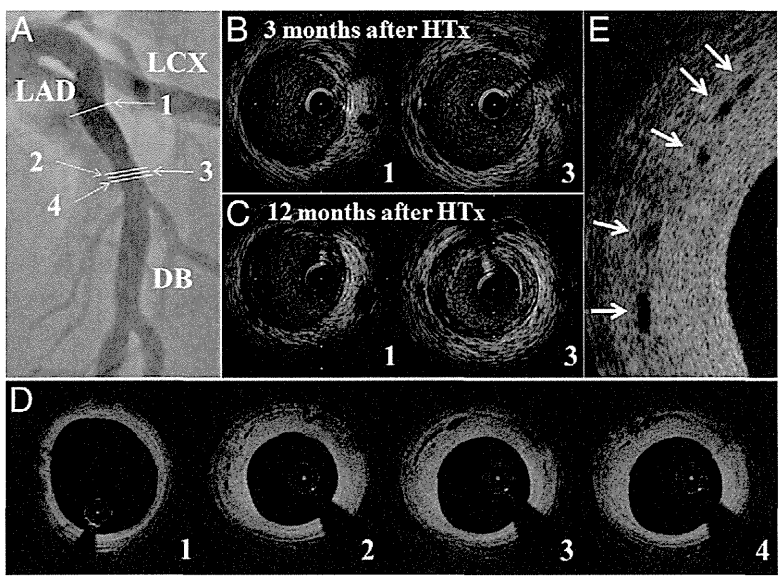
IMAGES IN CARDIOLOGY

Cardiac Allograft Vasculopathy Progression Associated With Intraplaque Neovascularization

Yasuhiro Ichibori, MD,* Daisaku Nakatani, MD, PHD,* Yasushi Sakata, MD, PHD,* Kouichi Tachibana, MD, PHD,* Takashi Akasaka, MD, PHD,† Shunsuke Saito, MD, PHD,‡ Norihide Fukushima, MD, PHD,‡ Yoshiki Sawa, MD, PHD,‡ Shinsuke Nanto, MD, PHD,* Issei Komuro, MD, PHD*

Osaka and Wakayama, Japan



From the *Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Osaka, Japan; †Department of Cardiovascular Medicine, Wakayama Medical University, Wakayama, Japan; and the ‡Department of Cardiovascular Surgery, Osaka University Graduate School of Medicine, Osaka, Japan.
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A 52-year-old man underwent scheduled cardiac catheterization for assessment of cardiac allograft vasculopathy (CAV) at 3 and 12 months after heart transplantation (HTx). Intravascular ultrasound showed only mild CAV in the left anterior descending coronary artery (LAD) at 3 months (**B**, Online Video 1). However, at 12 months, CAV progression was detected, predominantly in the proximal LAD (**A and C3**, Online Video 2). Corresponding optical coherence tomography images revealed the presence of many no-signal tubuloluminal structures within the fibrous plaque in serial frames. Parts of those structures were found to be connected to the vessel lumen, indicating neovascularization (**D2, D3, D4, and E [arrows]**, Online Video 3). No neovascularization was observed in lesions without CAV progression **(C1 and D1)**. To our best knowledge, this is the first reported case demonstrating CAV progression associated with intraplaque neovascularization in vivo. Intraplaque neovascularization could play an important role in CAV progression. DB = diagonal branch; LCX = left circumflex coronary artery.

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REVIEW

Cardioprotection From Ischemia/Reperfusion Injury
– Basic and Translational Research –

Tetsuo Minamino, MD, PhD

Because ischemic heart diseases (IHDs) are a major cause of mortality and heart failure, novel therapeutic approaches are expected to improve the clinical outcomes of patients with IHDs such as acute myocardial infarction and ischemic heart failure. Brief episodes of nonlethal ischemia and reperfusion before sustained ischemia or at the onset of reperfusion can reduce ischemia-reperfusion injury. These ischemic conditioning phenomena are termed "ischemic preconditioning" and "ischemic postconditioning", respectively. Furthermore, brief episodes of nonlethal ischemia and reperfusion applied to the organ or tissue distal to the heart reduce myocardial infarct size, known as "remote ischemic conditioning". The cardioprotection afforded by these ischemic conditionings can be used to treat patients with acute myocardial infarction or cardiac operations. Extensive research has determined that autacoids (eg, adenosine, bradykinin opioid) and cytokines, their respective receptors, kinase signaling pathways and mitochondrial modulation are involved in ischemic conditioning. Modification of these factors by pharmacological agents mimics the cardioprotection by ischemic conditioning and provides a novel therapeutic intervention for IHDs. Here, the potential mechanisms of ischemic conditioning and its "proof-of-concept" translational studies are reviewed. In the near future, large, multicenter, randomized, placebo-controlled, clinical trials will be required to determine whether pharmacological and ischemic conditioning can improve the clinical outcomes of patients with IHDs. (*Circ J* 2012; **76:** 1074–1082)

Key Words: Pharmacological conditioning; Postconditioning; Preconditioning; Proof-of-concept clinical studies; Remote conditioning

Despite the recent advances in therapies, ischemic heart diseases (IHDs) are a major cause of mortality and heart failure in western countries and Japan.[1,2] Thus, developing novel drugs or interventions to improve the clinical outcomes of patients with IHDs is a world-wide unmet medical need. Because myocardial infarct size is recognized as a determinant of acute and long-term prognosis in patients with acute myocardial infarction (AMI),[3] reducing the size of the infarct is a therapeutic goal. Early reperfusion can prevent the myocardial damage due to ischemia and reduce infarct size.[4] This concept was quickly introduced for patients with AMI by the use of primary percutaneous coronary intervention (PCI) and thrombolytic therapy.[5] Although reperfusion can salvage myocardium after sustained ischemia, the reperfusion itself paradoxically induces myocardial injury named "reperfusion injury", which attenuates the benefits of myocardial reperfusion[6,7] (**Figure 1**).

Over 20 years ago, Murry et al first demonstrated that brief episodes of nonlethal ischemia and reperfusion before sustained ischemia reduce myocardial infarct size, and it was termed "ischemic preconditioning".[8] The infarct-size limiting effects of ischemic preconditioning have been consistently confirmed in many species and different models of ischemia-reperfusion (IR) injury. Brief episodes of nonlethal IR at the onset of reperfusion also reduce myocardial infarct size, known as "ischemic postconditioning.[9] The therapeutic goal of ischemic postconditioning is to attenuate "reperfusion injury" (**Figure 1**). After these landmark studies, extensive basic investigation has elucidated the underlying mechanisms of ischemic conditioning and led to their translation into the clinical setting by pharmacological agents.[10] Here, I will review the potential mechanisms of ischemic conditioning and the "proof-of-concept" translational studies.

Ischemic Preconditioning

Ischemic preconditioning confers different forms of cardioprotection and can reduce infarct size, lethal arrhythmia and contractile dysfunction.[11–13] Originally, Murry et al hypothesized that ATP preservation during ischemia is the major cardioprotective mechanism underlying ischemic preconditioning, but this hypothesis is not sufficient to explain its cardioprotection.[14] Currently, the major effects of ischemic preconditioning are assumed to prevent cell death due to reperfusion injury. Different factors such as autacoids (eg, adenosine, bradykinin, opioids), their respective receptors, kinase signaling

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Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Suita, Japan
Mailing address: Tetsuo Minamino, MD, PhD, Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita 565-0871, Japan. E-mail: minamino@cardiology.med.osaka-u.ac.jp
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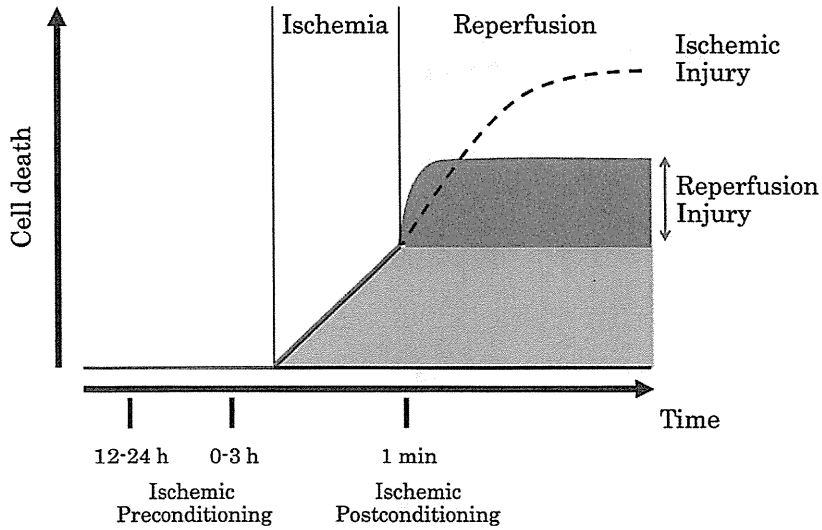

Figure 1. Myocardial ischemia/reperfusion (IR) injury and ischemic conditioning. Myocardial IR injury consists of 2 phases. During ischemia, cell death within the myocardial risk areas increases as the duration of ischemia extends if the ischemic myocardium is not reperfused (broken black curve). Although reperfusion can salvage myocardium after sustained ischemia, the reperfusion itself paradoxically induces myocardial injury during the early stage of reperfusion (red curve). "Early" and "delayed" ischemic preconditioning may reduce both myocardial ischemia and reperfusion injury. Ischemic postconditioning or pharmacological conditioning reduces the reperfusion injury (blue curve). (Adapted from Garcia-Dorado and Piper[7] with permission.)

pathways and mitochondria modulation are implicated in the cardioprotective effects of ischemic preconditioning (**Figure 2**). Nonlethal ischemia results in the production of endogenous autacoids such as adenosine, opioids, bradykinin. These autacoids initiate numerous signaling pathways that activate protein kinases through their respective receptors (**Figure 2**). These cardioprotective signaling pathways, including extracellular-regulated kinase (ERK)1/2, phosphatidylinositol 3 kinase (PI3K)/Akt, protein kinase C and protein kinase G, lead to the inactivation of mitochondrial glycogen synthase kinase-3β (GSK-3β). The inactivation of GSK-3β inhibits the opening of the mitochondrial permeability transition pore (mPTP), which plays a crucial role in myocardial necrosis.[15,16] Reactive oxygen species (ROS) production in mitochondria, where the mitochondrial ATP-dependent potassium channels play an essential role, is also involved in the cardioprotective mechanisms of ischemic preconditioning.[17] Although these findings are consistently observed in experimental models, applying ischemic preconditioning in the clinical setting is restricted to scheduled cardiac operation and elective PCI.[18] A meta-analysis showed that ischemic preconditioning may provide additional myocardial protection over cardioplegia alone.[19] However, cardiovascular surgeons do not like to repeatedly clamp and unclamp the aorta in patients with advanced atherosclerosis.

The cardioprotective effects of ischemic preconditioning disappear 2–3 h after the onset of the preconditioning insult, but reappear 24 h later. This phenomenon is recognized as "delayed" ischemic preconditioning.[20,21] A major difference in the cardioprotective mechanisms of early and delayed preconditioning is that early ischemic preconditioning results in the modification or turnover/translocation of existing molecules,[15,22] whereas delayed ischemic preconditioning is exerted by newly synthesized cardioprotective proteins. The triggers and mediators of early and delayed ischemic preconditioning are largely common and lead to the activation of transcriptional factors (**Figure 2**). They transcribe the de novo synthesized proteins involved in delayed ischemic preconditioning, including manganese superoxide dismutase, heat stress proteins and inducible nitric oxide synthase.[21] A potential clinical example of delayed ischemic preconditioning is "pre-infarct angina" by which patients who have suffered from repeated episodes of angina can preserve postischemic left ventricular function.[23] However, the clinical application of delayed ischemic preconditioning has not been fully investigated.

Ischemic Postconditioning

In 2003, Zhao et al demonstrated that brief episodes of coronary occlusion and reperfusion at the onset of reperfusion following 60 min of coronary occlusion reduced myocardial infarct size by 40% in canine hearts.[9] The protocols for ischemic postconditioning have been extensively investigated and the cardioprotective effects afforded by ischemic postconditioning have been confirmed in many species, including humans.[24,25] At the same time, the existence of reperfusion injury is strongly supported by the cardioprotection afforded by the intervention during reperfusion. One proposed mechanism through which ischemic postconditioning attenuates reperfusion injury is the prevention of rapid changes in intracellular pH and robust ROS generation. In the ischemic/reperfused myocardium, the ionic environment dramatically changes. Within a few minutes of myocardial ischemia, the interstitial and intracellular pH values rapidly decrease due to the accumulation of protons. Upon reperfusion, these interstitial protons are promptly washed out and intracellular low pH is corrected through the sarcolemmal Na^+/H^+ exchanger, which results in a massive Na^+ influx.[26] Intracellular Na^+ accumulation stimulates the passive, inverted action of the sarcolemmal Na^+/Ca^{2+} exchanger and in turn allows intracellular Ca^{2+} overload, which causes myocardial cell death or myocardial contractile dysfunction.[27,28] Therefore, the rapid normalization of intracellular pH enhances myocardial damage in the early stage of reperfusion and a gradual correction of low intracellular pH by acidic reperfusion would be cardioprotective through inhibition of the opening of mPTP,[29] preventing the activation of Ca^{2+}-dependent protease[30] and reducing the gap junction communication involved in spreading cell death.[31] The cardioprotective effects of ischemic postconditioning are associated with the maintenance of low intracellular pH during reperfusion and are comparable to the effects of acidic reperfusion.[32] Furthermore, during the early stage of reperfusion, there is robust ROS production in vascular endothelium, cardiomyocytes and mito-

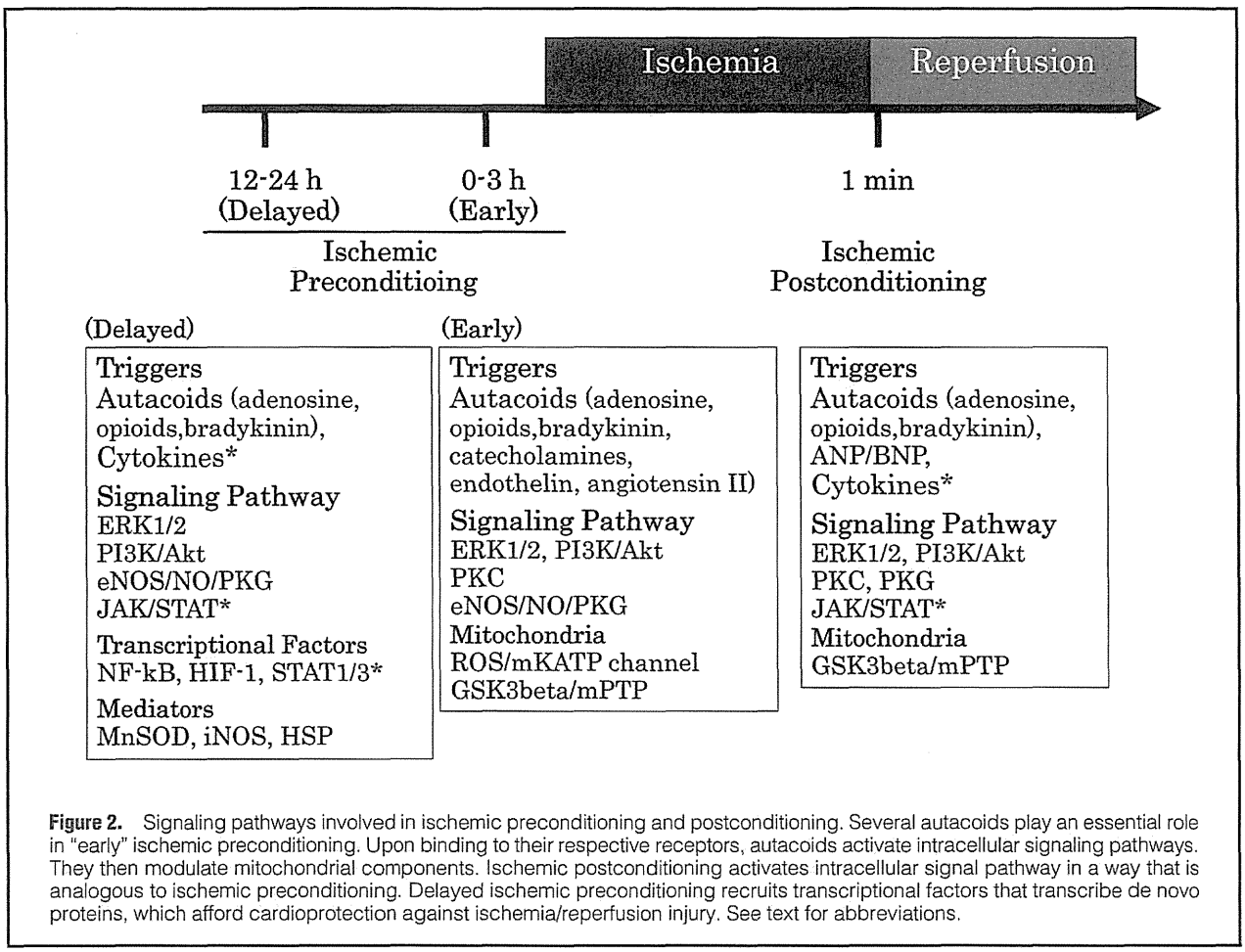

Figure 2. Signaling pathways involved in ischemic preconditioning and postconditioning. Several autacoids play an essential role in "early" ischemic preconditioning. Upon binding to their respective receptors, autacoids activate intracellular signaling pathways. They then modulate mitochondrial components. Ischemic postconditioning activates intracellular signal pathway in a way that is analogous to ischemic preconditioning. Delayed ischemic preconditioning recruits transcriptional factors that transcribe de novo proteins, which afford cardioprotection against ischemia/reperfusion injury. See text for abbreviations.

Table 1. Clinical Trials of Ischemic/Pharmacological Conditioning in Patients With STEMI

	Outcome	Reference
Conditioning		
Postconditioning	Decrease (IS), improved LVEF at 12 months	25, 39
Remote conditioning	Decrease (IS)	86
Pharmacological agents		
Adenosine	Decrease (IS)	44
Atrial natriuretic peptide	Decrease (IS), improved LVEF at 6–12 months	49
Cyclosporine A	Decrease (IS), improved LVEF at 6 months	62
Erythropoietin		
High dose	No change (IS, LVEF)	52, 53, 53
Low dose	Improved LVEF at 6 months	55, 56
Nicorandil	No change (IS)	49
Statin	No change (IS)	67
Protein kinase C inhibitor	No change (IS, LVEF)	68

STEMI, ST-elevation myocardial infarction; IS, infarct size; LVEF, left ventricular ejection fraction.

chondria. ROS generation is suppressed in the postconditioned heart.[33,34]

In addition to the effects of ischemic postconditioning on ionic changes and ROS production, ischemic postconditioning activates intracellular signal transduction in a way that is analogous to ischemic preconditioning. Autacoids (eg, adenosine, bradykinin and opioids), natriuretic peptides (atrial and brain natriuretic peptides) and cytokines play a crucial role in postconditioning[35] (**Figure 2**). These autacoids activate a kinase signaling pathway known as the reperfusion injury risk kinases (RISK) pathway, which consists of the PI3K/Akt and ERK1/2 pathways.[36] The activation of RISK pathway inactivates GSK3β, which inhibits mPTP opening at reperfusion. The inhibition of mPTP opening is the final common target

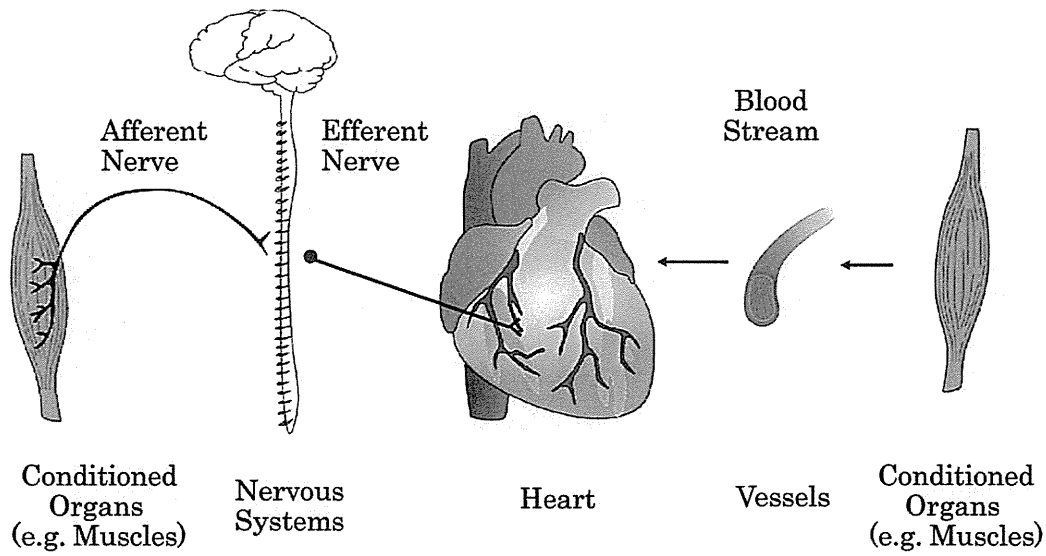

Figure 3. Potential mechanisms of remote ischemic conditioning. See text for details about the neural and humoral hypotheses.

through which the signaling pathways can protect against necrosis.[15] Activation of the JAK-STAT pathway by cytokines has also been implicated in the cardioprotective effects induced by ischemic postconditioning.[37,38] This pathway is named the "survivor activating factor enhancement (SAFE)" pathway; however, it is not fully understood how SAFE pathway is involved in the cardioprotection afforded by ischemic postconditioning. In contrast to ischemic preconditioning, ischemic postconditioning can be easily applied in patients with AMI undergoing primary PCI. A small number of "proof-of-concept" studies have showed that a postconditioning procedure reduced myocardial infarct size[25] and improved left ventricular ejection fraction (LVEF) at 1 year.[39] Prospective and randomized studies are now ongoing to evaluate the infarct-size-limiting effects of ischemic postconditioning in patients with ST-segment elevation myocardial infarction (STEMI) who are undergoing primary PCI.[40,41]

Coronary blood flow must be interrupted in order to apply ischemic postconditioning, which increases the time required for the procedure and could potentially cause atherosclerotic emboli. Pharmacological manipulation of autacoids, their receptors, kinase signaling pathways and modulation of the mPTP opening, all of which are involved in ischemic postconditioning, could be easily utilized to treat patients with AMI undergoing primary PCI (**Table 1**). Adenosine is a representative autacoid that is involved in both ischemic preconditioning and postconditioning, and its administration at the onset of reperfusion provides myocardial protection from IR injury in animal models.[42] The results of a randomized, double-blinded, placebo-controlled multicenter trial of a 3-h adenosine infusion as an adjunct to thrombolytic reperfusion in the treatment of anterior wall STEMI (AMISTAD-II) have been reported.[43,44] Clinical outcomes, including new congestive heart failure, first re-hospitalization for chronic heart failure and death, were not significantly improved with adenosine administration, although infarct size was reduced in response to a high-dose infusion.[44] Post-hoc analysis revealed that adenosine infusion within the first 3.17 h after the onset of anterior wall STEMI enhanced early and late survival, and reduced the composite clinical endpoints of death or chronic heart failure at 6 months.[45] In the J-WIND study, a multicenter, randomized clinical trial was conducted to test the acute effect of either the sarcolemmal KATP channel opener, nicorandil, or the recombinant human atrial natriuretic peptide (ANP), carperitide, as an adjunct to successful PCI.[46,47] The administration of carperitide, but not nicorandil, produced a small but significant 15% reduction in myocardial infarct size and an improvement in LVEF.[48] Experimental studies showed that erythropoietin, a hematopoietic cytokine, reduces myocardial infarct size and prevents cardiac remodeling in the chronic stage.[49,50] In patients with STEMI, the administration of a high dose of erythropoietin did not improve LVEF or reduce infarct size;[51–53] however, the use of erythropoietin was related to fewer major adverse cardiovascular events in 1 study.[52] In contrast, a low dose of erythropoietin appears to be cardioprotective.[54,55] Platelet activation by a high-dose of erythropoietin and the existence of an optimal dose for limiting infarct size will explain the dose-dependent discrepancy of erythropoietin-induced cardioprotection.[56–58] Therefore, a large-scale, double-blinded, placebo-controlled study is being conducted to clarify the effects of a low dose of erythropoietin on cardiac function after 6 months in patients with AMI who received successful PCI in Japan (UMIN000005721). Pharmacological inhibitors of mPTP reduce myocardial infarct size in experimental models.[59,60] Recently, Piot et al demonstrated that the mPTP inhibitor, cyclosporine A, administered as an intravenous bolus immediately before coronary artery reperfusion by PCI, resulted in a 40% reduction in enzyme release and prevented cardiac remodeling.[61,62] The data are promising and large, multicenter, randomized, placebo-controlled, clinical trials are

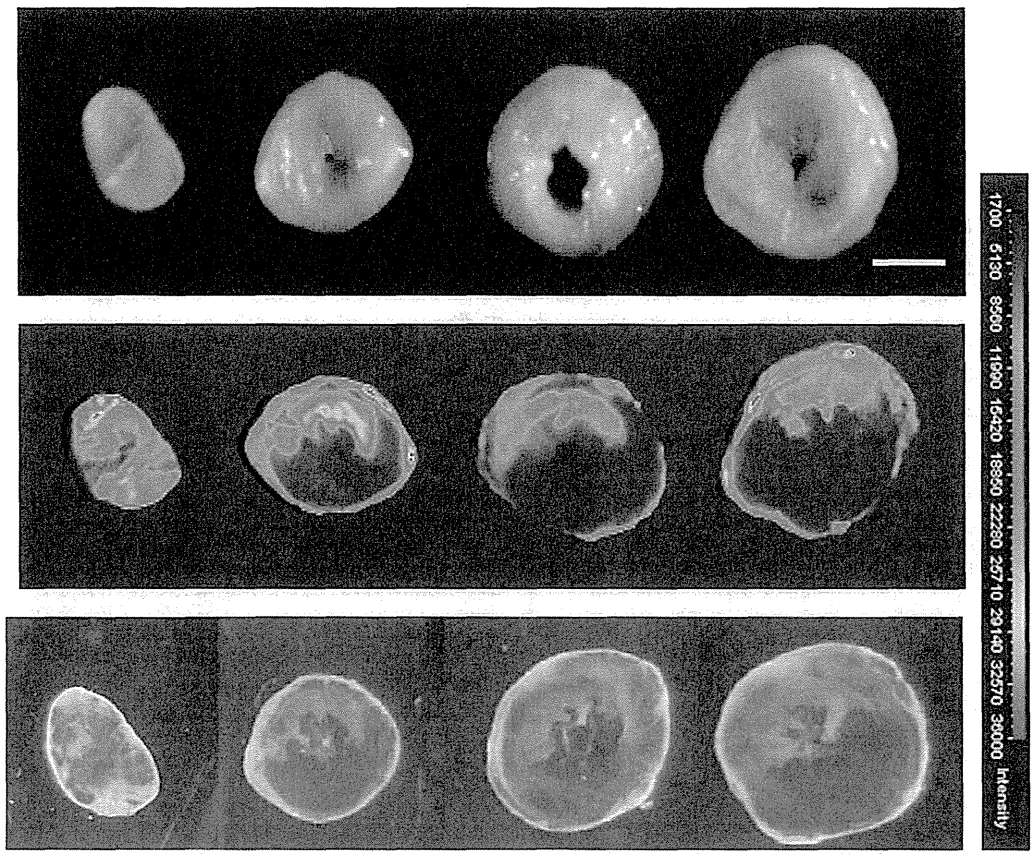

Figure 4. Detection of fluorescent-labeled nano-sized particles in ischemic/reperfused myocardium. Representative photographs at 3 h after myocardial infarction of hearts from rats that received fluorescent-labeled nano-sized liposomes. (**Upper panels**) Short axis cross-section of each heart (bright field). (**Middle panels**) Same sections analyzed under a fluorescence microscope. (**Lower panels**) Same sections stained with triphenyltetrazolium chloride. Note that high fluorescent areas correspond to the infarcted and border areas. Scale bar=5 mm. (Adapted from Takahama and Minamino[90] with permission.)

required to elucidate the improvement in clinical outcomes.

To date, most clinically tested agents that induce cardioprotection, except adenosine and cyclosporine A, have failed to reduce infarct size in the clinical setting[63–65] (**Table 1**). These negative results of "proof-of-concept" studies can be attributed to multiple factors.[66–68] Pharmacological intervention as an adjunct to primary PCI is estimated to be effective for only 25% of AMI patients with an infarct size larger than 20% of the left ventricle and who have adverse symptoms.[68,69] Proper patient selection is required to evaluate the benefit of pharmacological conditioning. In addition to the ischemic risk zone, infarct size is also determined by the duration of ischemia. If the duration of ischemia extends beyond 60 min, the infarct-size limiting effects of ischemic postconditioning are largely attenuated in experimental models.[24] Thus, some proportion of patients in the study may have already been beyond the appropriate time-window within which myocardial salvage can be achieved. Another important point is the timing of drug administration. Reperfusion injuries such as robust ROS production, Ca^{2+} overload and mPTP opening occur within the first few minutes of myocardial reperfusion.[70] In the cyclosporine A study, this compound was administered just before coronary artery reperfusion by PCI, whereas most drugs were administered after successful reperfusion therapy. Finally, we need to consider confounders such as sex and age and comorbidities such as hypercholesterolemia, diabetes and hypertension, which are not present in animal studies as compared with clinical reality.[71] For example, pharmacological postconditioning with cyclosporine A failed to provide cardioprotection in the prediabetic but normoglycemic heart of Zucker obese rats.[72] Erythropoietin fails to exert infarct-size limiting effects in hypertensive hypertrophied hearts.[73] Thus, both appropriate study design and execution are required to translate future novel cardioprotecive agents into the clinical setting.[66,67]

Remote Ischemic Conditioning

Brief episodes of nonlethal ischemia and reperfusion applied to the organ or tissue distal to the heart reduce myocardial infarct size, which is known as "remote ischemic conditioning".[74,75] Transient upper or lower limb ischemia is a simple noninvasive stimulus with important potential clinical applications and high-cost performance. Furthermore, the remote ischemic conditioning procedure can be applied before and during sustained ischemia[76] and at the onset of reperfusion.[77] An experimental study showed that the infarct-size-limiting effects

Table 2. MicroRNAs Involved in Hypertrophy/Failure and Ischemia/Reperfusion

MicroRNA	Hypertrophy/ failure[94]	Ischemia[95]	Ischemic preconditioning[96]	Ischemic postconditioning[97]
miR-1	↓	↓	↑	↑
miR-9	↓	ND or NC	ND or NC	ND or NC
miR-17	ND or NC	↓	ND or NC	ND or NC
miR-21	ND or NC	↓	↑	ND or NC
miR-23	↑	ND or NC	ND or NC	ND or NC
miR-24	ND or NC	↓	↑	ND or NC
miR-26	↓	ND or NC	ND or NC	ND or NC
miR-30	↓	ND or NC	ND or NC	ND or NC
miR-92a	ND or NC	↑	ND or NC	ND or NC
miR-126	ND or NC	↓	ND or NC	ND or NC
miR-133	↓	↓	ND or NC	↑
miR-138	ND or NC	↓	ND or NC	ND or NC
miR-155	ND or NC	↓	ND or NC	ND or NC
miR-199a	↑	↑	ND or NC	ND or NC

NC, not changed; ND, not determined.

of remote conditioning are comparable to the effects of ischemic postconditioning.[78] It remains unclear how remote ischemic conditioning exerts cardioprotection; however, 2 major hypotheses have been proposed (**Figure 3**). The neural hypothesis states that autacoids released from the ischemic remote organ affect the local afferent neural pathway, which in turn, activates the efferent neural pathways to trigger end-organ protection. The humoral hypothesis states that autacoids released from the ischemic remote organ are transported to the end organ, resulting in the activation of kinase signaling pathways in the end organ. Remote ischemic preconditioning is associated with the activation of PI3K/Akt[79] or STAT5[80] in the heart. The clinical application of remote ischemic conditioning was tested in patients undergoing CABG, but the results were inconsistent.[81,82] Multicenter randomized double-blinded controlled clinical trials to clarify the effects of remote conditioning on clinical outcomes and the incidence of atrial fibrillation in patients with CABG are now ongoing.[83,84] Recently, remote ischemic conditioning before hospital admission was shown to increase myocardial salvage measured by myocardial perfusion imaging and have a favorable safety profile in patients with AMI.[85]

Future Directions

Recent advances in nanotechnology open up new possibilities in the development of drug delivery systems (DDS) for the treatment of patients with IHDs. DDS enhance the therapeutic concentrations of the drugs in diseased tissues and reduce the side effects.[86] Nano-sized particles can passively accumulate in tissues where vascular permeability is enhanced.[87] This concept is particularly applicable for developing anti-cancer and anti-inflammatory drugs, because vascular permeability is enhanced in tumors and inflamed tissues.[88,89] In the rat IR model, after the intravenous administration of fluorescence-labeled nano-sized particles, high fluorescent areas corresponded to infarcted and border, but not non-ischemic areas in the rat heart, suggesting that the nano-sized particles specifically accumulated in the myocardial infarct and border, but not in non-ischemic tissue (**Figure 4**).[90] These findings suggest that ischemic/reperfused myocardium has enhanced permeability and that nano-sized liposomes can accumulate there. In a rat IR model, the intravenous administration of nano-sized liposomes containing adenosine, but not free adenosine, at the onset of reperfusion significantly reduced myocardial infarct size and lethal arrhythmia during repefusion.[91] Encapsulated adenosine in nano-sized liposomes enhances the cardioprotective effects of adenosine and attenuates the hypotension induced by the systemic administration of adenosine. Targeting cardioprotective agents to ischemic/reperfused tissues using nano-sized liposomes may maximize the effect of the drug and minimize its side effects.[92,93] Liposomes are a promising DDS for developing new treatments for patients with AMI who have undergone successful PCI.[92]

MicroRNAs have emerged as important regulators of gene expression that affects cardiovascular function.[94] MicroRNAs regulate gene expression through the degradation and translational inhibition of target messenger RNAs. IR stimuli alter the expression of microRNAs.[94] Recent studies revealed that microRNAs are implicated in cardiac pathology including hypertrophy and failure[95] and IR injury[96] (**Table 2**). Therefore, microRNAs are novel promising therapeutic targets for IHDs. Cheng et al demonstrated that ischemic preconditioning up-regulates microRNA 21, which protects the heart against IR injury.[97] Yin et al showed that an injection of microRNAs induced by ischemic preconditioning in the heart exerted cardioprotective effects against IR injury, which is comparable to that induced by the late phase of ischemic preconditioning.[98] With advances in nanotechnology, microRNAs are potentially good candidates for targeting ischemic/reperfused myocardium with nano-sized liposomes.[99]

Perspectives

Basic and translational research examining the therapeutic potential of ischemic conditioning are now actively ongoing. We need to continue to investigate the molecular mechanisms of ischemic conditioning, improve DDS, design study protocols to consider the timing and dose of drug administration and select patients who can benefit from pharmacological intervention. These efforts will lead to solving the unmet medical need for therapeutic drugs and interventions that improve the clinical outcomes of patients with IHD.

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Cell

Complement C1q Activates Canonical Wnt Signaling and Promotes Aging-Related Phenotypes

Atsuhiko T. Naito,[1,3] Tomokazu Sumida,[4] Seitaro Nomura,[4] Mei-Lan Liu,[4] Tomoaki Higo,[1] Akito Nakagawa,[1] Katsuki Okada,[1] Taku Sakai,[1] Akihito Hashimoto,[1] Yurina Hara,[1] Ippei Shimizu,[4] Weidong Zhu,[4] Haruhiro Toko,[4] Akemi Katada,[4] Hiroshi Akazawa,[1,3] Toru Oka,[1,3] Jong-Kook Lee,[1,3] Tohru Minamino,[4] Toshio Nagai,[4] Kenneth Walsh,[5] Akira Kikuchi,[2] Misako Matsumoto,[6] Marina Botto,[7] Ichiro Shiojima,[1,3] and Issei Komuro[1,3,4,*]

[1]Department of Cardiovascular Medicine

[2]Department of Molecular Biology and Biochemistry

Osaka University Graduate School of Medicine, Osaka 565-0871, Japan

[3]Japan Science and Technology Agency, CREST, Tokyo 102-0075, Japan

[4]Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan

[5]Molecular Cardiology, Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, MA 02118, USA

[6]Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Hokkaido 060-8638, Japan

[7]Centre for Complement and Inflammation Research, Department of Medicine, Imperial College London, London SW7 2AZ, UK

*Correspondence: komuro-tky@umin.ac.jp

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SUMMARY

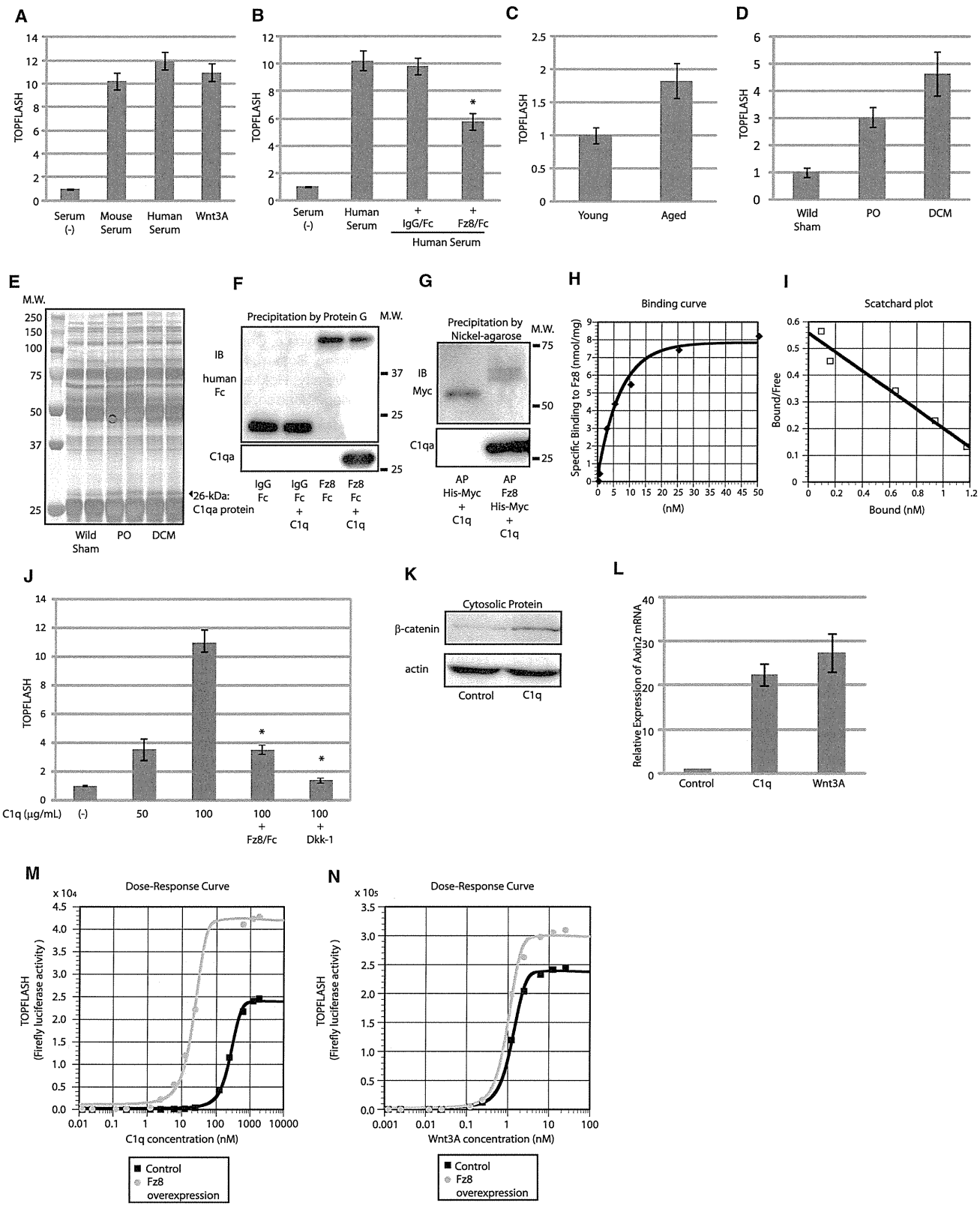
Wnt signaling plays critical roles in development of various organs and pathogenesis of many diseases, and augmented Wnt signaling has recently been implicated in mammalian aging and aging-related phenotypes. We here report that complement C1q activates canonical Wnt signaling and promotes aging-associated decline in tissue regeneration. Serum C1q concentration is increased with aging, and Wnt signaling activity is augmented during aging in the serum and in multiple tissues of wild-type mice, but not in those of C1qa-deficient mice. C1q activates canonical Wnt signaling by binding to Frizzled receptors and subsequently inducing C1s-dependent cleavage of the ectodomain of Wnt coreceptor low-density lipoprotein receptor-related protein 6. Skeletal muscle regeneration in young mice is inhibited by exogenous C1q treatment, whereas aging-associated impairment of muscle regeneration is restored by C1s inhibition or *C1qa* gene disruption. Our findings therefore suggest the unexpected role of complement C1q in Wnt signal transduction and modulation of mammalian aging.

INTRODUCTION

Wnts constitute a large family of secreted proteins that elicit evolutionarily conserved intracellular signaling and affect diverse cellular responses during development. Wnt signaling also plays critical roles in various physiological and pathological processes in adult organisms, including stem cell self-renewal/differentiation, degenerative diseases, and carcinogenesis (Blanpain et al., 2007; Clevers, 2006; Logan and Nusse, 2004). The β-catenin-dependent canonical Wnt pathway is the most understood signaling cascade initiated by Wnt proteins. Upon Wnt stimulation, cytosolic β-catenin is stabilized and translocates to the nucleus, where it binds to T cell factor/Lymphoid enhancer factor (Tcf/Lef) and induces Tcf/Lef-dependent transcription (Logan and Nusse, 2004). This canonical Wnt signaling is mediated by two types of cell surface receptors, the Frizzled (Fz) family of serpentine proteins and the single-transmembrane protein low-density lipoprotein receptor-related protein 5/6 (LRP5/6) (Angers and Moon, 2009; MacDonald et al., 2009).

Recent studies have revealed a role of Wnt signaling in the regulation of mammalian aging. Wnt/β-catenin signaling is augmented in a mouse model of accelerated aging (Liu et al., 2007), and inhibition of canonical Wnt signaling reverses the aging-associated impairment of skeletal muscle regeneration (Brack et al., 2007). Moreover, this age-related activation of Wnt signaling was attributed to the substance(s) in the serum that binds to the extracellular cysteine-rich domain (CRD) of Fz (Brack et al., 2007). However, because Wnt proteins tightly bind to the cell surface and/or extracellular matrix and are thought to act in a short-range manner (Kikuchi et al., 2007; White et al., 2007), the substance(s) in the serum that activates Wnt signaling was assumed to be distinct from classical Wnt proteins.

Here, we show that complement C1q is an activator of Wnt signaling. C1q activates canonical Wnt signaling by binding to Fz receptors and subsequently inducing C1s-dependent cleavage of the ectodomain of LRP6. Serum C1q concentration and the expression of C1q in various tissues are increased with aging, which are associated with increased Wnt signaling activity in serum and in multiple tissues during aging. We further demonstrate that activation of Wnt signaling by C1q accounts for the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C1q
Wnt3A
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Dose-Response Curve
TOPFLASH (Firefly luciferase activity)
C1q concentration (nM)
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Fz8 overexpression
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Fz8 overexpression

impaired regenerative capacity of skeletal muscle in aged mice. These results suggest that C1q activates Wnt signaling and modulates mammalian aging-related phenotypes.

RESULTS

Complement C1q Is a Fz-Binding Protein in the Serum

Consistent with a previous report (Brack et al., 2007), mouse and human serum activated canonical Wnt signaling, as assessed by the TOPFLASH reporter gene assay that reflects Tcf/Lef-dependent transcription (Figure 1A). Human serum-induced activation of Wnt signaling was partly suppressed by a Fz8 CRD-IgG/Fc fusion protein (Fz8/Fc), but not by IgG/Fc (Figure 1B), and serum from aged mice showed higher TOPFLASH activity than serum from young mice (Figure 1C). We also found that the serum obtained from two different mouse models of heart failure more potently increased TOPFLASH activity compared with serum from aged mice (Figure 1D). We therefore hypothesized that the serum of mice with heart failure contains the Wnt activator more abundantly than that of aged mice, and we used the former as a starting material to isolate the Wnt activator in the serum. Precipitation of Fz8/Fc-binding proteins followed by SDS-PAGE identified a 26 kDa protein that was upregulated in the serum from mice with heart failure (Figure 1E). Mass spectrometric analysis revealed that this 26 kDa protein was complement C1qa, which is a major constituent of complement C1q.

C1q is composed of 18 polypeptides: 6 C1qa, 6 C1qb, and 6 C1qc chains, each encoded by 3 individual genes. Although C1q is known to bind to Fc portion of aggregated immunoglobulins, purified C1q was precipitated by Fz8/Fc and a Fz8 CRD-alkaline phosphatase (AP) fusion protein, but not by IgG/Fc or AP protein in a pull-down assay (Figures 1F and 1G and Figures S1A and S1B available online), indicating that C1q binds to CRD of Fz8. C1q also bound to CRD of other Fz receptors such as Fz1, 2, 4, and 7 (Figure S1C).

Complement C1q Is an Activator of Canonical Wnt Signaling

We next investigated whether C1q is a specific ligand for Fz receptors. A binding assay demonstrated that the interaction between C1q and Fz8 CRD was specific and saturable (Figure 1H). A Scatchard plot analysis revealed that C1q has a single binding site for Fz8 CRD, with a binding affinity comparable to that of Wnt3A (Kd_{C1q}: 2.8 nM, Kd_{Wnt3A}: 1.25 nM) (Figures 1I, S1D, and S1E). A heterologous competition assay revealed that C1q and Wnt compete with each other for the binding to Fz8 CRD (Figure S1F). Purified C1q dose dependently increased TOPFLASH activity (Figure 1J), stabilized cytosolic β-catenin (Figure 1K), and increased the expression of *Axin2*, a well-established target gene of canonical Wnt signaling (Figure 1L). C1q-induced TOPFLASH activity was inhibited by Fz8/Fc or Dkk1 (Figure 1J). These results strongly suggest that C1q is a Fz-binding protein that activates canonical Wnt signaling.

Despite the similar binding affinity to Fz receptor, dose-response curves of C1q and Wnt3A on TOPFLASH activity revealed that the EC_{50} of C1q on activation of Wnt signaling (259 nM) was 200-fold higher than that of Wnt3A (1.27 nM) (Figures 1M and 1N). Based on the mode of C1q activation by immunoglobulins or SIGN-R1 (Duncan and Winter, 1988; Kang et al., 2006; Schumaker et al., 1986), in which the binding of multiple or aggregated immunoglobulins or SIGN-R1 to C1q initiates C1q activation, we hypothesized that increasing the amount of Fz receptors may promote C1q-induced activation of Wnt signaling. Indeed, overexpression of Fz8 decreased the EC_{50} of C1q by 13-fold (259 nM to 22.8 nM), whereas the EC_{50} of Wnt3A was less affected (1.27 nM to 0.852 nM) (Figures 1M and 1N). These results suggest that the mode of Wnt signaling activation by C1q is distinct from that by Wnt3A and is affected by the cellular context, including the density of Fz receptors.

C1q Mediates Serum-Induced Activation of Wnt Signaling In Vitro and Maintains Basal Wnt Signaling Activity in Multiple Tissues In Vivo

We assessed whether serum-induced activation of Wnt signaling is attributable to C1q. C1q-depleted serum or serum treated with Fz8/Fc showed lower TOPFLASH activity compared with normal serum and C3- or C5-depleted serum, and addition of Fz8/Fc to C1q-depleted serum did not further reduce TOPFLASH activity (Figure 2A). Likewise, serum from C1qa-deficient mice showed lower TOPFLASH activity compared with serum from wild-type

Figure 1. Complement C1q Binds to Fz and Activates Wnt Signaling

(A–D) TOPFLASH assay. Mouse and human serum (10%) and Wnt3A protein (10 ng/ml) activated canonical Wnt signaling to the same degree (A). Activation of Wnt signaling by human serum was suppressed by Fz8/Fc (500 ng/ml). *$p < 0.05$ versus human serum (B). Serum-induced Wnt signaling activity was higher in aged mice (C) and in mice with heart failure (D). Data are presented as mean ±SD. PO, mice with pressure overload; DCM, mice with dilated cardiomyopathy.

(E) Silver staining of SDS-PAGE gel. Serum obtained from control mice and mice with heart failure were incubated with Fz8/Fc and precipitated by protein G. SDS-PAGE of the precipitates revealed that the amount of a protein of ~26 kDa (arrowhead) was increased in the serum from mice with heart failure. PO, mice with pressure overload; DCM, mice with dilated cardiomyopathy.

(F and G) Pull-down assay. C1q was precipitated by Fz8/Fc, but not by IgG/Fc (F). C1q was precipitated by Fz8 CRD-AP, but not by AP (G).

(H and I) Binding kinetics of C1q to Fz8 CRD. A binding curve (H) and a Scatchard plot (I) are shown.

(J) TOPFLASH assay. C1q dose dependently activated canonical Wnt signaling, which was blocked by Fz8/Fc (20 μg/ml) or Dkk-1 (20 ng/ml). Data are presented as mean ±SD. *$p < 0.01$ versus of C1q (100 μg/ml).

(K) β-catenin stabilization assay. β-catenin stabilization assay was performed in HEK293 cells 1 hr after C1q stimulation (200 μg/ml).

(L) *Axin2* mRNA levels. C1q (100 μg/ml) and Wnt3A (10 ng/ml) activate canonical Wnt signaling to the same degree as assessed by *Axin2* mRNA induction in HEK293 cells. *Axin2* mRNA was assessed 24 hr after stimulation. Data are presented as mean ±SD.

(M and N) Dose-response curves of C1q and Wnt3A on TOPFLASH activity. Fz8 overexpression induced marked leftward shift of the response curve of C1q-induced TOPFLASH activity (M) but had minimal effects on that of Wnt3A-induced TOPFLASH activity (N).

See also Figure S1.

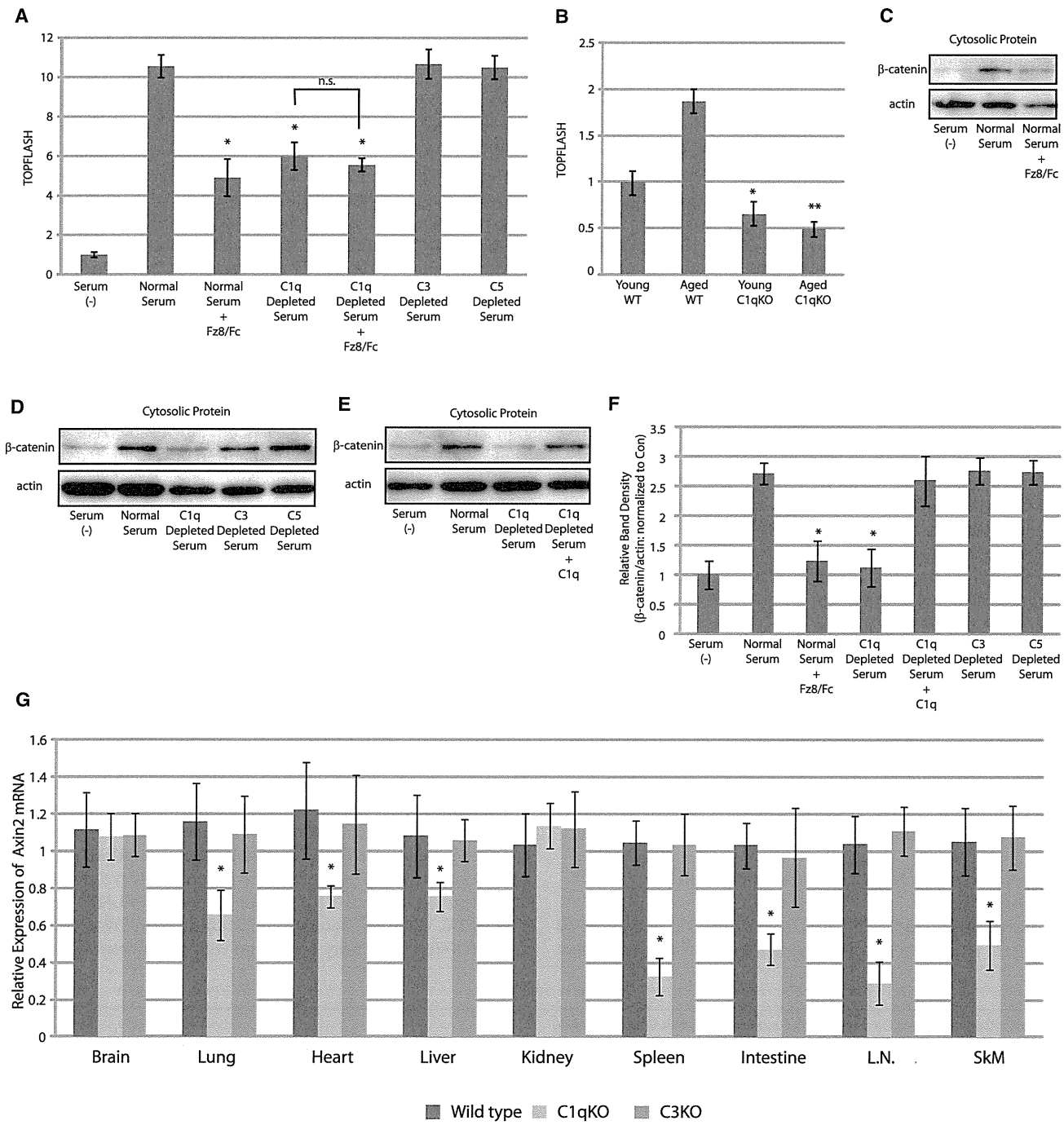

Figure 2. C1q Mediates Serum-Induced Activation of Wnt Signaling In Vitro and Is Required for Basal Wnt Signaling Activity In Vivo

(A) TOPFLASH assay. Wnt signaling activation by serum was partially blocked by Fz8/Fc (10 μg/ml) or C1q depletion, but not by C3 or C5 depletion. Combination of Fz8/Fc and C1q depletion did not further decrease TOPFLASH activity. Data are presented as mean ±SD. *$p < 0.01$ versus normal serum.

(B) TOPFLASH assay. In wild-type (WT) mice, serum from aged mice showed higher TOPFLASH activity than serum from young mice. Serum from young C1qa-deficient mice showed lower TOPFLASH activity compared with serum from young WT mice, and the elevation of TOPFLASH activity during aging was not observed in C1qa-deficient mice. Data are presented as mean ±SD. *$p < 0.01$ versus serum obtained from young WT mice. **$p < 0.01$ versus aged serum obtained from WT mice.

(C–F) β-catenin stabilization assay. Human serum activated Wnt signaling, which was abolished by Fz8/Fc (10 μg/ml) (C). Wnt signaling activation by serum was also abolished by C1q depletion, but not by C3 or C5 depletion (D). Reduced Wnt signaling activation by C1q depletion was fully restored by C1q (10 μg/ml) application (E). The results were quantified by measuring the relative amount of β-catenin over actin (F). Data are presented as mean ±SD. *$p < 0.05$ versus normal human serum ($n = 5$).

or C3-deficient mice at the age of 3 months (Figure S2). Moreover, augmentation of serum TOPFLASH activity by aging was not observed in C1qa-deficient mice (Figure 2B). Thus, C1q mediates serum-induced activation of Wnt signaling and accounts for increased Wnt signal activation by serum from aged mice.

We also assessed the activation of Wnt signaling by analyzing cytosolic β-catenin level at 1 hr after the treatment with serum because TOPFLASH assay is performed at relatively later time points after serum stimulation and therefore may be affected by other factors that indirectly modulate Tcf/Lef-dependent transcription. Indeed, unlike TOPFLASH assay, serum-induced activation of Wnt signaling as assessed by β-catenin stabilization was almost completely blunted by Fz8/Fc or C1q depletion, but not by C3 or C5 depletion, which was fully recovered by the addition of C1q (Figures 2C–2F). These results further support the notion that C1q is responsible for serum-induced activation of canonical Wnt signaling.

We further investigated whether activation of Wnt signaling by C1q is physiologically relevant in vivo. Real-time PCR analysis revealed that expression levels of *Axin2* gene were decreased in various tissues of C1qa-deficient mice, but not in those of C3-deficient mice, most notably in spleen, intestine, lymph nodes, and skeletal muscle (Figure 2G). This result suggests that basal activity of canonical Wnt signaling is at least in part dependent on C1q and underscores the physiological relevance of C1q-induced Wnt signaling activation in vivo.

C1q Mediates Augmented Wnt Signaling Activity Associated with Aging

We next examined whether C1q mediates augmented Wnt signaling activity during aging. ELISA and western blot analysis revealed that serum C1q concentration was increased with aging (Figures 3A and 3B). It was previously reported that cells of the monocyte/macrophage lineage are the major source of serum C1q (Petry et al., 2001). Indeed, expression levels of C1q in peritoneal macrophages were higher in 1-year-old and 2-year-old mice than in young mice (2-months-old) (Figure 3C), consistent with the observation that serum C1q levels were upregulated at these ages (Figures 3A and 3B). Expression levels of C1q were upregulated in various tissues of 2-year-old mice (Figure 3D), suggesting that upregulation of C1q in macrophages causes an initial increase in serum C1q levels and that C1q produced in other tissues at later stages may contribute to a further increase in serum C1q levels.

We also assessed whether C1q is responsible for age-associated augmentation of Wnt signaling activity. An age-associated increase in *Axin2* mRNA was observed in various tissues of wild-type mice. On the other hand, there was no significant difference in *Axin2* mRNA levels between young and aged C1qa-deficient mice in all tissues examined (Figure 3E). Thus, C1q is responsible for augmented Wnt signaling activity in multiple tissues of aged animals.

C1q Activates Canonical Wnt Signaling by Inducing C1s-Dependent Cleavage of the Extracellular Domain of LRP6

The complement system is one of the major components of the mammalian immune responses and plays a pivotal role in innate immunity (Walport, 2001). The classical complement pathway is triggered by C1, which is composed of C1q and two proenzymes, C1r and C1s. Conventionally, C1q binds to aggregated immunoglobulins, which leads to conformational change and subsequent activation of C1q (Duncan and Winter, 1988; Schumaker et al., 1986). Upon C1q activation, C1r undergoes autoactivation and, in turn, cleaves and activates C1s. C1s then cleaves C2 and C4 to instigate following activation steps of the complement system. We therefore tested whether C1r/C1s is involved in C1q-induced activation of Wnt signaling. Consistent with the observation that purified C1q activates Wnt signaling in a serum-free condition (where no exogenous C1r/C1s is thought to exist) (Figures 1J–1L), western blot analysis revealed that both C1r and C1s are expressed in the target cells and secreted into the culture media (Figure 4A). Knockdown of C1r/C1s by siRNAs totally blunted C1q-induced cytosolic β-catenin stabilization and TOPFLASH activation (Figures 4B and 4C). Likewise, addition of C1 inhibitor (C1-INH), an endogenous inhibitor of C1r and C1s, or a neutralizing antibody against C1s (M241) (Matsumoto and Nagaki, 1986) strongly inhibited C1q-induced activation of Wnt signaling (Figure 4D). To test whether C1s is activated upon C1q-Fz interaction, we treated NIH 3T3 cells with C1q and C4 in a serum-free condition. C4 is a target of C1s, and its cleaved product, C4b, covalently binds to the cellular surface after cleavage. We found that overexpression of Fz8 pronouncedly enhanced C4b deposition on the cellular surface (Figures 4E and 4F). These results suggest that endogenous C1r and C1s are activated upon C1q-Fz binding and that C1q-induced activation of Wnt signaling requires protease activity of C1s.

In addition to C2 and C4, C1s has been reported to cleave other cell surface proteins such as major histocompatibility complex (MHC) class I molecules (Eriksson and Nissen, 1990). Because deletion of the extracellular domain of LRP6 results in constitutive activation of canonical Wnt signaling (Liu et al., 2003; Mao et al., 2001), we tested whether LRP6 is the target of C1s. Treatment of LRP6 extracellular domain-IgG/Fc fusion protein with active C1s resulted in the appearance of two major cleaved products (Figure 4G), and N-terminal amino acid sequencing revealed that LRP6 was cleaved between Arg792 and Ala793 in the third β-propeller domain. The C1s cleavage site of LRP6 was conserved in various species, and similar sequences were also found in the third β-propeller domain of LRP5 (Figure 4H). The C1s cleavage site of LRP6 is adjacent to the Dkk1-binding site (Ahn et al., 2011; Chen et al., 2011). However, the inhibitory effect of Dkk1 on C1q-induced Wnt activation (Figure 1J) does not appear to be due to the direct inhibition of LRP6 cleavage because Dkk-1 did not have major impact on in vitro cleavage of LRP6 by C1s (data not shown).

(G) Expression levels of *Axin2* mRNA in various tissues of 3-month-old wild-type (n = 8), C1qa-deficient (n = 8), and C3-deficient (n = 4) mice. Expression levels of *Axin2* gene expression were lower in various tissues of C1qa-deficient mice, but not in those of C3-deficient mice. Data are presented as mean ±SD. *$p < 0.05$ compared with wild-type mice. L.N., lymph node; SkM, skeletal muscle.
See also Figure S2.

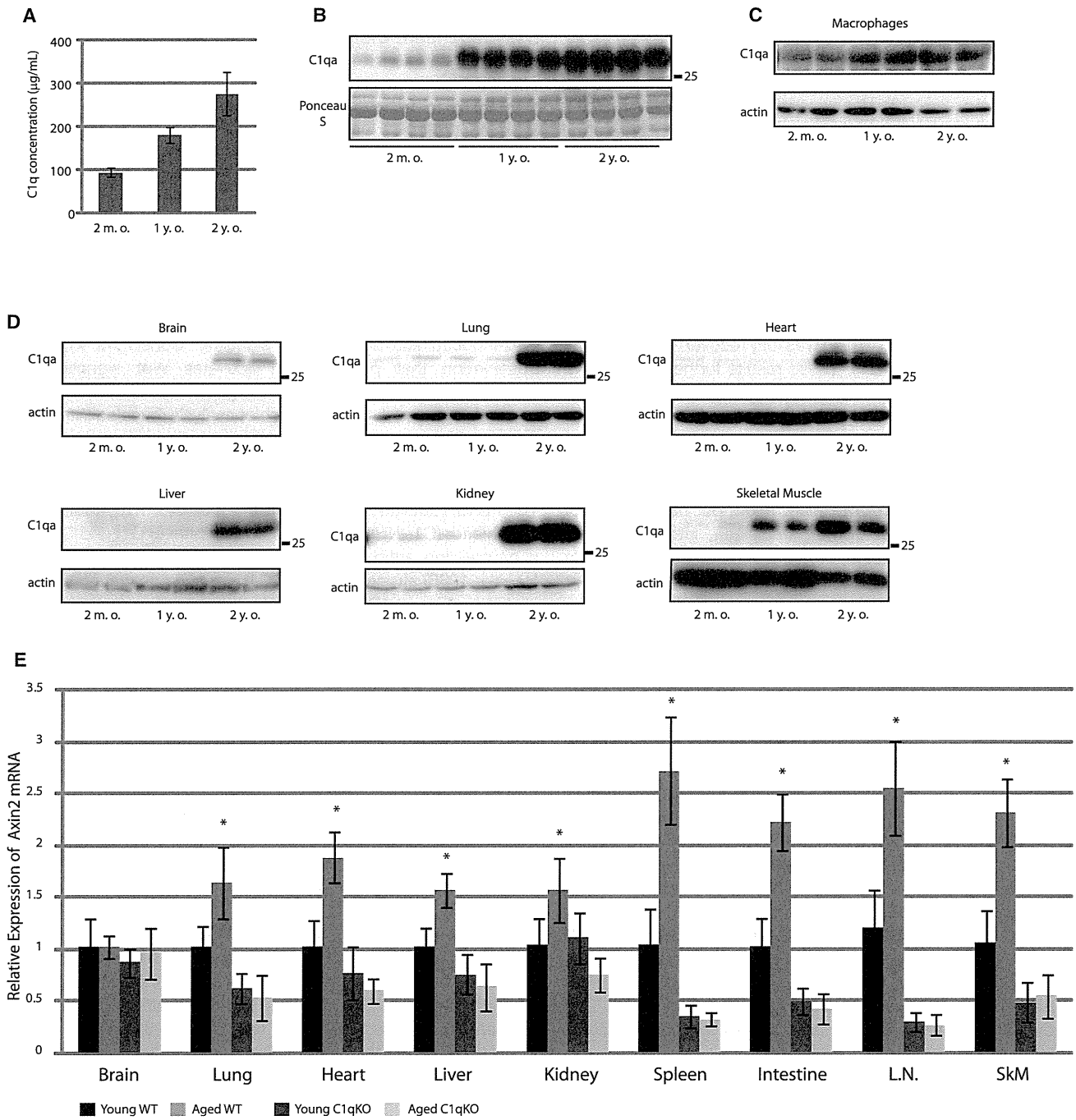

Figure 3. C1q Mediates Augmented Wnt Signaling Associated with Aging

(A and B) Serum C1q concentration of mice at different ages was assessed by ELISA (A) and western blot (B). Serum C1q concentration was increased with aging. Data in (A) are presented as mean ±SD.

(C and D) Western blot analysis of C1q in peritoneal macrophages (C) and in various tissues (D) derived from wild-type mice at different ages. C1q expression in macrophages and skeletal muscle was increased at 1 year of age, whereas a robust increase in C1q expression in other tissues was observed at 2 years of age.

(E) Expression levels of *Axin2* mRNA in various tissues from young (3 months old) and aged (2 years old) wild-type (young, n = 8; aged, n = 4) and C1qa-deficient mice (young, n = 8; aged, n = 3). *Axin2* gene expression was increased with aging in multiple tissues of wild-type mice (WT), but not in those of C1qa-deficient mice (C1qKO). L.N., lymph node; SkM, skeletal muscle. Data are presented as mean ±SD. *$p < 0.05$ compared with young wild-type mice.

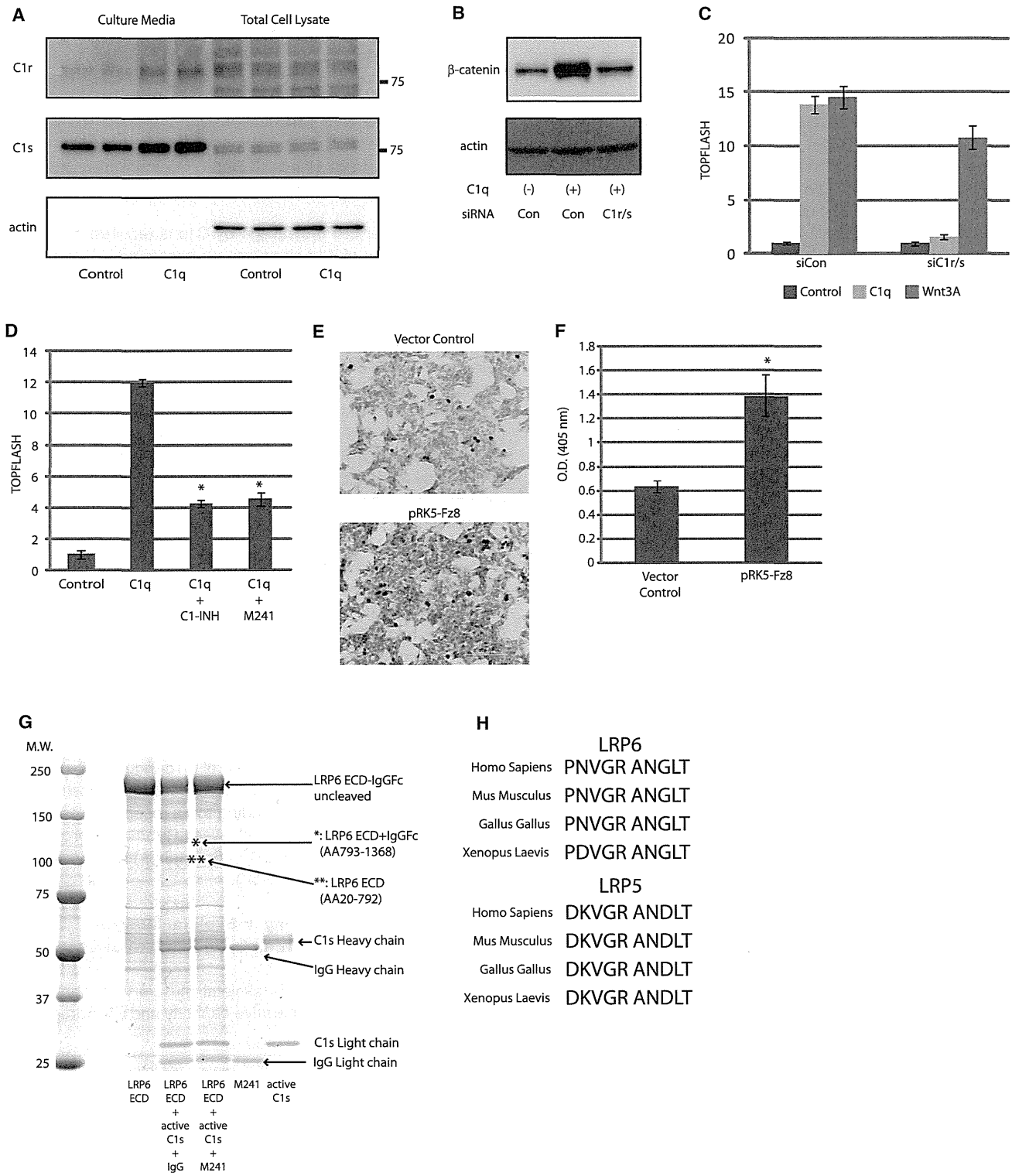

Figure 4. C1q-Induced Activation of Wnt Signaling Is Dependent on Protease Activity of C1s

(A) HepG2 cells were cultured and stimulated with or without C1q (100 μg/ml) in a serum-free condition for 24 hr. Culture media and total cell lysate were analyzed by western blotting. Both C1r and C1s protein were observed in the culture media under serum-free condition.

(B) β-catenin stabilization assay. HepG2 cells transfected with control siRNA (Con) responded to C1q (100 μg/ml), but those transfected with siRNAs against C1r and C1s (C1r/s) did not.

We also assessed whether C1q induces cleavage of endogenous LRP6 in HepG2 cells. C1q-induced activation of Wnt signaling was associated with the appearance of cleaved N-terminal fragment of LRP6 (~100 kDa) in culture media, which was detected by an antibody raised against extracellular portion of LRP6 (LRP6 ECD Ab), but not by an antibody against LRP6 intracellular domain (LRP6 ICD Ab) (Figure 5A). When cells were treated with C1q in the presence of a lysosomal inhibitor Chloroquine, LRP6 ICD Ab detected a protein compatible in size with the C-terminal cleaved fragment of LRP6 (~140 kDa) in the membrane/organelle fraction (Figure 5B). Notably, there was no apparent change in the expression levels of full-length LRP6 by C1q treatment, and this band was not observed in the absence of Chloroquine or when the cells were treated with Wnt3A (Figure 5B). Thus, a relatively small fraction of LRP6 is cleaved by C1s following C1q treatment, and the resultant C-terminal fragment of LRP6 produced by C1s cleavage appears to be subjected to lysosomal degradation.

We next tested whether serum induces cleavage of LRP6 in a C1q-dependent manner. HepG2 cells were transfected with N-terminally myc-tagged LRP6 and treated with serum. Western blot analysis following immunoprecipitation with anti-myc antibody revealed that the cleaved product of LRP6 was detected in the culture media following treatment with normal serum, but not with C1q-depleted serum (Figure 5C). The ability to cleave LRP6 was fully recovered after restoring C1q to C1q-depleted serum (Figure 5C). The N-terminal fragment of endogenous LRP6 was also detected in the serum from wild-type mice, but not in C1qa-deficient mice, and the concentration of LRP6 C-terminal cleaved fragment was increased by ~2-fold in aged mice compared with young mice (Figures 5D and 5E). These observations indicate that both serum-induced LRP6 cleavage in vitro and an age-dependent increase in LRP6 cleavage in vivo occur in a C1q-dependent manner.

To examine whether LRP6 cleavage by C1s is sufficient for Wnt signaling activation by C1q, we generated a LRP6 deletion mutant that lacks amino acids 21–792 (Del-LRP6). Transfection of Del-LRP6 increased Wnt signaling activity by 47-fold compared with wild-type LRP6 (WT-LRP6) (Figure 5F), suggesting that cleavage of LRP6 between Arg792 and Ala793 is sufficient for activation of canonical Wnt signaling. As phosphorylation of the intracellular region of LRP5/6 is a hallmark of LRP5/6 activation (Tamai et al., 2004; Zeng et al., 2005), we investigated the phosphorylation status of LRP6 after C1q stimulation. When the cells were treated with C1q together with Chloroquine for 3 hr, phosphorylation of cleaved LRP6 C-terminal fragment (~140 kDa) was detected (Figure S3A). Of note, we found that phosphorylation of full-length LRP6 was also increased following C1q treatment (Figure S3A). Moreover, transfected Del-LRP6 was strongly phosphorylated even in the absence of Wnt3A stimulation (Figure S3B) and induced the phosphorylation of simultaneously transfected full-length WT-LRP6 (Figure S3C). These results suggest that a relatively small amount of cleaved LRP5/6 fragment may amplify Wnt signaling by inducing the phosphorylation of uncleaved LRP5/6.

To test whether LRP6 cleavage by C1s is required for C1q-induced activation of Wnt signaling, we generated a C1s-resistant LRP6 mutant in which Arg792 and Ala793 were substituted to glycines (Mt-LRP6). Overexpression of WT-LRP6 or Mt-LRP6 induced an ~7-fold increase in TOPFLASH activity (Figure S3D). Although WT-LRP6-transfected cells and Mt-LRP6-transfected cells responded to Wnt3A treatment similarly, C1q treatment strongly enhanced TOPFLASH activity (~18-fold) in WT-LRP6-transfected cells but only marginally in Mt-LRP6-transfected cells (~1.7-fold) (Figures 5G, 5H, and S3D). This slight increase in C1q-induced TOPFLASH activity in Mt-LRP6-transfected cells presumably reflects the activation of Wnt signaling mediated by cleavage of endogenous LRP6. These results suggest the requirement of LRP6 cleavage in C1q-induced activation of Wnt signaling.

We next tested the requirement of C1r, C1s, LRP5/6, and Fz receptors in C1q-induced LRP6 cleavage and subsequent activation of Wnt signaling by siRNA-mediated knockdown of C1r, C1s, LRP5, and LRP6 (Figure S3E) or by overexpression of Shisa protein to reduce cell surface Fz receptors (Yamamoto et al., 2005; Zeng et al., 2008). The amount of C-terminal (LRP6 ICD) and N-terminal (LRP6 ECD) cleaved forms of LRP6 following C1q treatment was dramatically decreased by C1r/C1s knockdown, LRP5/6 knockdown, or Shisa overexpression (Figure 5I), which was associated with inhibition of C1q-induced β-catenin stabilization and TOPFLASH activation (Figure 5J). These results collectively suggest that C1q binding to Fz receptors results in the activation of C1r/C1s, which cleaves LRP5/6 and produces N-terminal truncated form of LRP5/6, leading to activation of canonical Wnt signaling (Figure 5K).

C1q Activates Wnt Signaling in Skeletal Muscle and Exhibits Differential Effects on Satellite Cells and Fibroblasts

Activation of Wnt signaling in skeletal muscle was shown to mediate a decrease in regenerative capacity and an increase in

(C) TOPFLASH assay. HEK293 cells transfected with control siRNA (siCon) responded to both C1q (100 μg/ml) and Wnt3A (10 ng/ml), but those transfected with siRNAs against C1r and C1s (siC1r/s) responded to Wnt3A, but not to C1q. Data are presented as mean ±SD.

(D) TOPFLASH assay. Activation of Wnt signaling by C1q (100 μg/ml) was inhibited by an endogenous C1-inhibitor (C1-INH: 100 μg/ml) or by a neutralizing antibody against C1s (M241: 100 μg/ml). Data are presented as mean ±SD. *$p < 0.01$ versus C1q alone.

(E and F) C4 cleavage assay. C4b deposition on the cell surface was assessed by immunostaining (E) or ELISA (F). C4b deposition was increased after Fz8 overexpression. Data are presented as mean ±SD. *$p < 0.05$ versus control vector ($n = 5$).

(G) Coomassie staining of SDS-PAGE gel. LRP6 extracellular domain (ECD)-IgG/Fc fusion protein (4 μg) was incubated with active-C1s (176 ng) with or without a neutralizing antibody against C1s (M241). Proteins were fractionated by SDS-PAGE and visualized by Coomassie staining. C1s treatment of LRP6 ECD resulted in the appearance of two major bands (indicated by * and **). Amino acid sequencing revealed that * represented LRP6 ECD (amino acids 793–1368) + IgG/Fc, and ** represented LRP6 ECD (amino acids 20–792).

(H) Amino acid sequence alignment of potential C1s cleavage site in the third β-propeller domain of LRP5 and LRP6. C1s cleavage site is predicted to be between arginine (R) and alanine (A). Cleavage site of C1s is highly conserved among species.

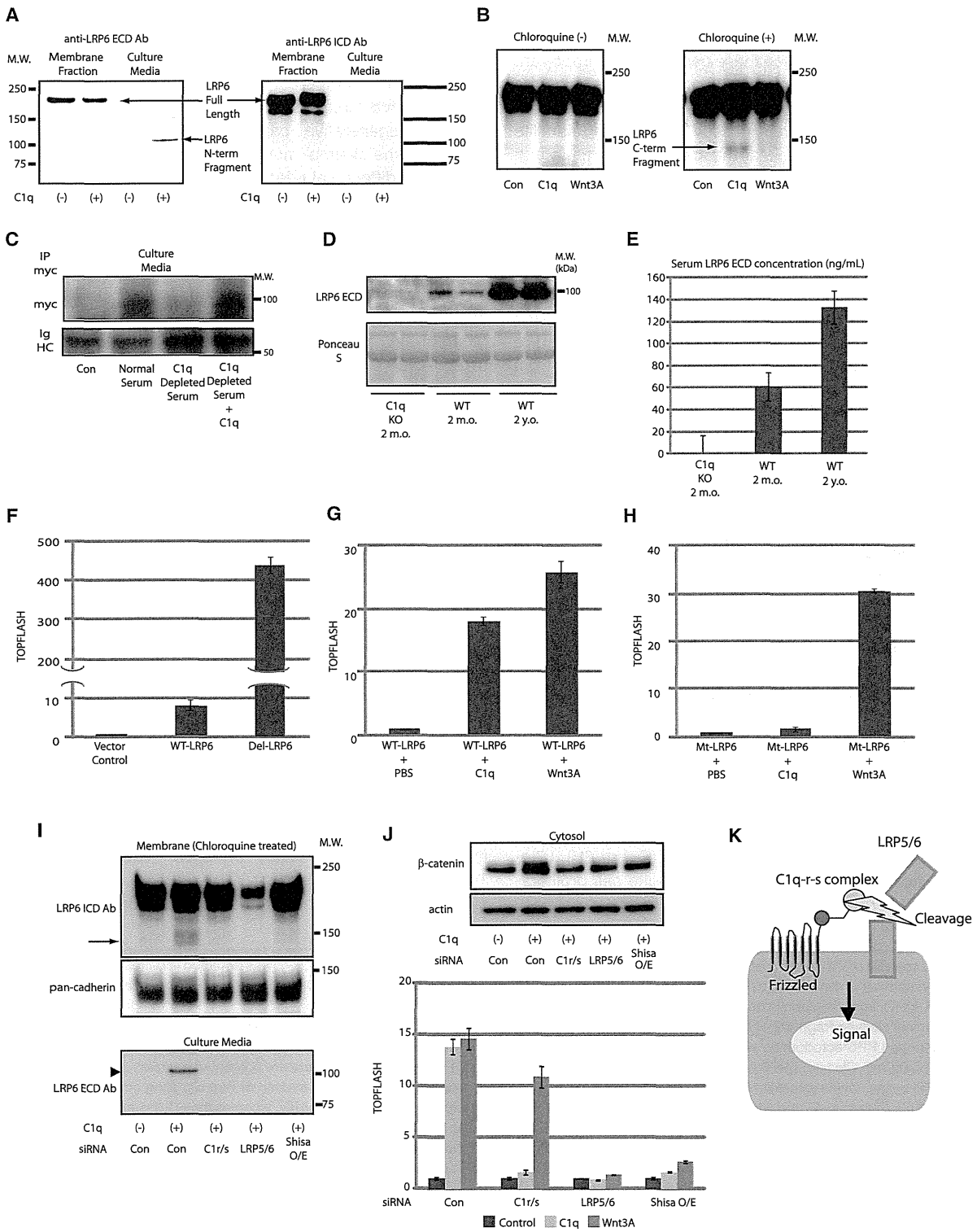

Figure 5. C1q Activates Wnt Signaling by Inducing C1s-Dependent Cleavage of the Extracellular Domain of LRP6

(A) Western blot analysis of LRP6 fragment in the culture media from HepG2 cells treated with C1q (100 μg/ml). N-terminal cleaved fragment of endogenous LRP6 was detected in the culture media. ECD, extracellular domain; ICD, intracellular domain.

(B) Western blot analysis of LRP6 in the membrane/organelle fraction of HepG2 cells treated with C1q (100 μg/ml) or Wnt3A (10 ng/ml). C-terminal cleaved fragment of LRP6 (~140 kDa) was detected by anti-LRP6 ICD Ab only in the cells treated with C1q plus lysosomal inhibitor Chloroquine (50 μM).