Do not overwork: Cellular communication network factor 3 for life in cartilage

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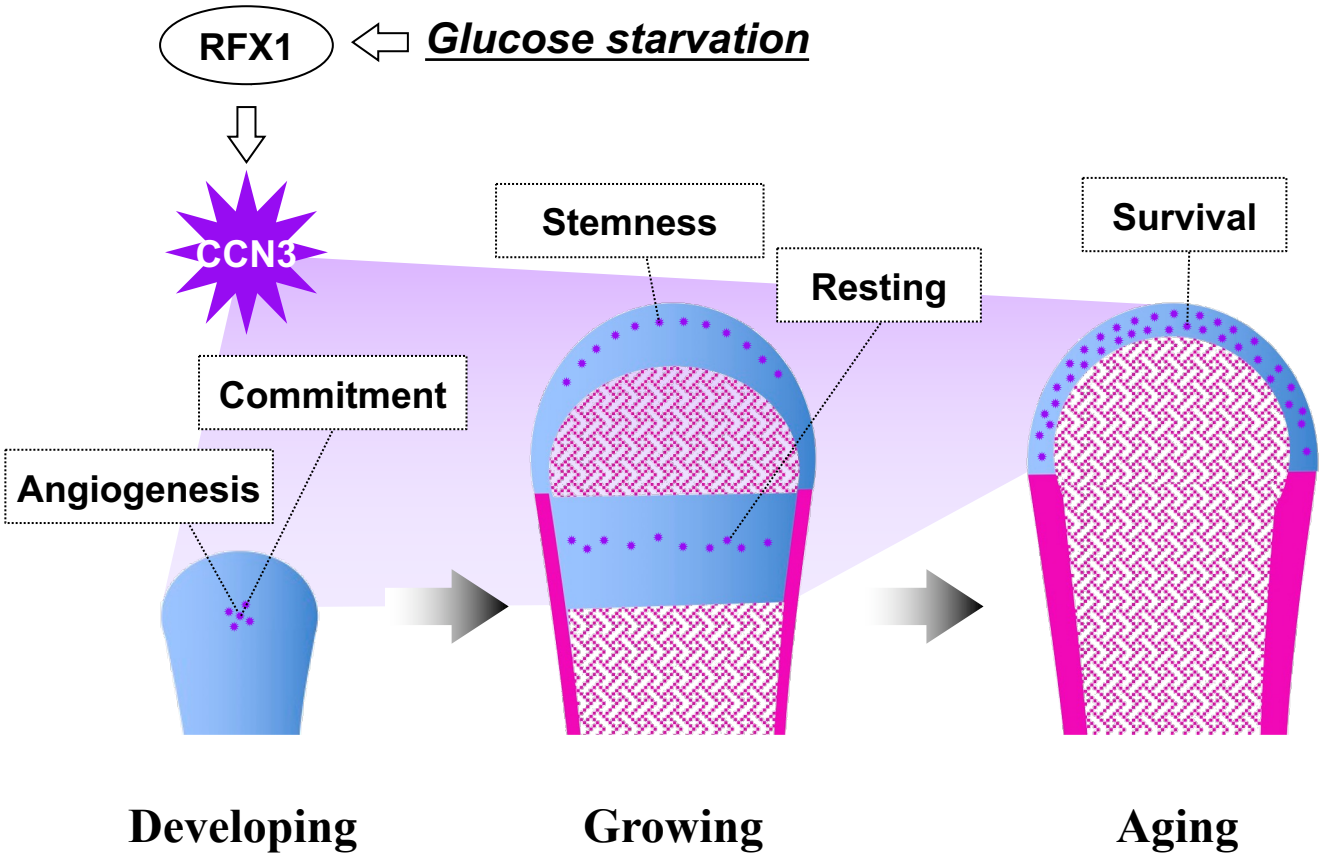

Graphical Abstract. Kubota et al.

Abstract

Cellular communication network factor (CCN) 3, which is one of the founding members of the CCN family, displays diverse functions. However, this protein generally represses the proliferation of a variety of cells. Along with skeletal development, CCN3 is produced in cartilaginous anlagen, growth plate cartilage and epiphysial cartilage. Interestingly, CCN3 is drastically induced in the growth plates of mice lacking CCN2, which promotes endochondral ossification. Notably, chondrocytes in these mutant mice with elevated CCN3 production also suffer from impaired glycolysis and energy metabolism, suggesting a critical role of CCN3 in cartilage metabolism. Recently, *CCN3* was found to be strongly induced by impaired glycolysis, and in our study, we located an enhancer that mediated *CCN3* regulation via starvation. Subsequent investigations specified regulatory factor binding to the X-box 1 (RFX1) as a transcription factor mediating this *CCN3* regulation. Impaired glycolysis is a serious problem, resulting in an energy shortage in cartilage without vasculature. CCN3 produced under such starved conditions restricts energy consumption by repressing cell proliferation, leading chondrocytes to quiescence and survival. This *CCN3* regulatory system is indicated to play an important role in articular cartilage maintenance, as well as in skeletal development. Furthermore, CCN3 continues to regulate cartilage metabolism even during the aging process, probably utilizing this regulatory system. Altogether, CCN3 seems to prevent "overwork" by chondrocytes to ensure their sustainable life in cartilage by sensing energy metabolism. Similar roles are suspected to exist in relation to systemic metabolism, since CCN3 is found in the bloodstream.

***Keywords*:** CCN family · CCN3 · cartilage · chondrocytes · energy metabolism

CCN2 and CCN3: Yin and yang in the cartilage

The cellular communication network factor (CCN) family is composed of six cysteine-rich proteins in mammals (Bork, 1993; Perbal, 2001; Brigstock et al., 2003; Perbal et al., 2018). All of its members share common structural characteristics, represented by the retention of three (CCN5) or four (the others) conserved modules and diverse functionalities that are highly dependent upon the biochemical microenvironment (Perbal, 2004; Leask and Abraham, 2006; Kubota and Takigawa, 2013; Lau, 2016). Among these members, CCN2 and CCN3 are particularly well-known as two of the three founding members, and thus have been extensively investigated (Bradham et al., 1991; Kubota and Takigawa, 2015; Joliot et al., 1992; Takigawa, 2018). Interestingly, despite their structural similarities, research conducted over three decades revealed that CCN2 and CCN3 played the roles of "yang" and "yin" in a variety of biological situations (Leask, 2009; Riser et al., 2010; Kubota et al., 2022a). Yin and yang are principles or entities that create and maintain a world by means of their counteracting forces, in a process which is based on mutual regulation with profound interconnections. With regard to molecular function, CCN2 has been shown to basically promote the proliferation of various kinds of cells (Shimo et al., 1999; Kothapalli and Grotendorst, 2000; Nakanishi et al., 2000; Nishida et al., 2000; Grotendorst and Duncan, 2005; Asano et al., 2005; Pandey et al., 2009; Xie et al., 2010; Kubota et al., 2015a), whereas CCN3 contrarily represses this proliferation (Gupta et al. 2001; Bleau et al. 2007; Janune et al., 2011a; Janune et al., 2011b ; Janune et al., 2017). In general, in cases where CCN2 plays a critical role, CCN3 is absent, and vice versa, which suggests their genetic interaction (Kubota et al., 2022a). Such yin-yang behaviors of CCN2 and CCN3 have been observed in both physiological and pathological processes. One typical example of the

former processes is skeletal development. Cartilage is the tissue that develops and grows the mammalian skeleton, which is known as temporary cartilage, as well as a permanent component of tissues that require flexible behavior (Kubota et al., 2022a; Kubota et al., 2022b). CCN2, the yang in this case, encourages the chondrocytes to proliferate in articular, auricular and growth-plate cartilage (Nakanishi et al., 2000; Nishida et al., 2003; Fujisawa et al., 2008), whereas the proliferation of articular and growth-plate chondrocytes is repressed by the yin, CCN3 (Kawaki et al., 2008; Janune et al., 2017). Also in the growth plate, where bone growth is conducted through an endochondral ossification process, the production of these two CCN family members is performed by distinct populations of chondrocytes. In this process, chondrocytes start differentiation from the resting stage, followed by the serial steps of the proliferative, pre-hypertrophic and terminal hypertrophic stages (Kubota et al, 2022b). CCN2 is predominantly produced by mature chondrocytes in the hypertrophic zone (Nakanishi et al., 2000, Takigawa, 2013) to support the growth of the bone from the inside, whereas CCN3 is mainly produced by resting chondrocytes to maintain their quiescence (Kawaki et al., 2008; Kubota et al., 2021). CCN3 also appears in hypertrophic chondrocytes at the terminal stage to cease the proliferation (Perbal, 2001), leading to the replacement of the cartilaginous extracellular matrix (ECM) with calcified bone after their programmed cell death. As such, the emergence and molecular action of CCN2 and CCN3 in a yin-yang manner are crucial in our skeletal development.

Metabolic regulation of *CCN2* and *CCN3* in cartilage

The positive role of CCN2 in skeletal development has been firmly established through a number of studies. CCN2 not only encourages the proliferation of

chondrocytes, but also promotes all of the steps involved in endochondral ossification, acting on osteoblasts as well as chondrocytes in the growth plate (Nakanishi et al., 2000; Nishida et al., 2000). Such "yang" functions of CCN2 are well represented by the regeneration potential of CCNs and their derivatives and the phenotype of *Ccn2*-null mice (Nishida et al., 2004; Abd El Kader et al., 2004). Indeed, *Ccn2*-null mice are lethal upon delivery due to their severe skeletal deformity caused by impaired endochondral ossification (Ivkovic et al., 2003; Kawaki et al., 2008). Through the characterization of *Ccn2*-null chondrocytes from the rib cages of those mice, it was revealed that CCN2 and CCN3 are subject to tight regulation by energy metabolism. Metabolomic analysis of *Ccn2*-null chondrocytes revealed that the cellular ATP level was drastically decreased (Maeda-Uematsu et al., 2014; Kubota et al., 2015b). According to subsequent investigations, glycolysis was impaired, with repressed expression of the genes encoding glycolytic enzymes, whereas no change was observed in the mitochondrial membrane potential (Maeda-Uematsu et al., 2014; Murase et al., 2016). Interestingly, a prominent elevation in the *Ccn3* mRNA level accompanied the impaired glycolysis in *Ccn2*-null chondrocytes, suggesting a relationship between CCN3 gene expression and glycolytic activity (Kawaki et al., 2008; Maeda-Uematsu et al., 2014). In order to clarify this point, the effect of impaired glycolysis on CCN3 gene expression in chondrocytes was evaluated *in vitro* with human cells. Inhibition of glycolysis by two distinct compounds, monoiodoacetate (MIA) and sodium fluoride (NaF), resulted in a dose-dependent induction of CCN3 (Akashi et al., 2018; Mizukawa et al., 2021). Notably, *CCN2* was contrarily repressed under the same conditions (Akashi et al., 2018; Akashi et al., 2020). The simultaneous repression of *CCN2* and induction of *CCN3* was reproduced by the impaired glycolysis caused by glucose depletion for 48 h in the same cells (Akashi et al.,

2020; Mizukawa et al., 2021). *CCN5* was also induced by MIA but was not by NaF. All of the other CCN family members were unaffected, indicating that CCN2 and CCN3 are key members, the properties of which are tightly linked to energy metabolism in the cartilage (Akashi et al., 2018; Mizukawa et al., 2021). Notably, the repression of CCN2 by oligomycin was also observed, which arrested aerobic ATP production in mitochondria, whereas CCN3 was not affected at all (Akashi et al., 2018). Therefore, CCN2 could be under the control of the cellular ATP level, rather than the glycolytic activity therein. The negative regulation of CCN3 by glycolysis was found to be active also in human breast cancer cell lines, suggesting that this regulatory system is widely utilized among different types of cells (Mizukawa et al., 2021). Nevertheless, since the cartilage lacks vasculature, glycolysis is its predominant metabolic pathway for obtaining ATP (Hollander and Zeng, 2019). The regulation of CCN3 by glycolysis should be the most efficient in cartilaginous tissues, among the other tissue types where CCN3 is required.

Molecular mechanism of negative *CCN3* regulation by glycolysis

Recent studies clarified that this negative regulation of *CCN3* is carried out at a transcriptional level. Reporter gene analysis with deletion mutants located an enhancer in the *CCN3* proximal promoter region. Among a number of transcription factors that were predicted to bind to the sequence around the enhancer *in silico*, regulatory factor binding to the X-box (RFX) 1 was specified as the most probable transcription factor (Mizukawa et al., 2021; Kubota et al., 2021). As a result of the analysis of the transcription factor chromatin immunoprecipitation-sequencing (ChIP-seq) data that were publicly available, RFX1 was found to actually bound to the enhancer in MCF7 breast cancer cells, in which the system of *CCN3* regulation by glycolysis was proven to

be active. The functional requirement of RFX1 in the induction of *CCN3* via impaired glycolysis was experimentally confirmed via a gene silencing approach (Mizukawa et al., 2021). RFX1 is a typical transcription factor with distinct DNA binding and activation/repression domains (Sugiaman-Trapman et al., 2018). Importantly, this transcription factor itself was also induced by impaired glycolysis, suggesting that RFX1 could be a central regulator of metabolically controlled genes. As such, under starved conditions, RFX1 is induced in chondrocytes for the enhanced production of CCN3, which represses cellular activities that are best represented by cell proliferation. Consequently, chondrocytes are led to quiescence, which is one of the key factors for cellular stemness, cytodifferentiation and survival (Fig. 1).

Skeletal development and aging supported by starvation-induced CCN3

Cartilage is the central tissue that contributes to the development of the vertebrate skeleton, as well as its permanent components. Because of its avascular nature, this *CCN3* regulatory system can be activated in a variety of situations by an insufficient nutrition supply in the cartilage, playing crucial roles in skeletal development. Most of our skeletal parts are initially formed as cartilaginous anlagen without blood vessels and a calcified ECM. At this stage, nutrition and oxygen can be supplied only from the outside of the cartilage, which indicates that cells in the deep zone are subject to conditions of starvation (Fig. 2, left panel). Thus, CCN3 could be induced in the deep zones, far from the surface of the cartilage, utilizing the *CCN3* regulatory system described in the previous section. CCN3, produced on the inside, invites blood vessels into the cartilage anlagen for ossification center formation with its intrinsic angiogenic activity (Lin et al., 2003; Kubota et al., 2007; Henrot et al., 2020). This idea is supported

by the fact that ossification centers are initially formed at the very center of the cartilaginous anlagen and subsequently in the central areas of epiphyses. It should be also noted that CCN3 represses the proliferation and directs the differentiation of epiphysial chondrocytes at this stage towards articular chondrocytes. Specifically, the expression of articular chondrocyte marker genes is induced, whereas that of growth plate chondrocytes is repressed by the application of CCN3 to chondrocytes from developing epiphysial cartilage (Janune et al., 2011a; Janune et al., 2011b). Along with the formation of ossification centers, endochondral ossification starts to develop, causing the bones to grow. The emergence and role of CCN3 in maintaining the quiescence of resting chondrocytes as the "yin" component of the system has already described in the first section. Since the resting zone is far from the bone marrow, the induction of CCN3 in the resting zone may well be performed via glycolysis-driven negative CCN3 regulation (Fig. 2, middle panel). At later stages of skeletal growth, the growth-plate cartilage gradually disappears, leaving permanent articular cartilage at the synovial joints. Around this stage, CCN3 appears to play a critical role in the maintenance of this tissue, which is required for locomotive actions. The overall expression of CCN3 in articular cartilage is not high, but a limited population of chondrocytes located therein do produce CCN3 (Kubota et al., 2021). In the middle zone of the articular cartilage, which is distant from both the joint surface and the bone marrow, CCN3-positive chondrocytes can be distinctly observed, again indicating the involvement of the *CCN3* regulatory system via starvation (Fig. 2, middle panel). Evaluations of the functioning of CCN3 with chondrocytes isolated from adult rat articular cartilage revealed that CCN3 enhances the expression of *Prg4*, which encodes lubricin, a critical protein for cellular stemness retention, as well as a marker of articular chondrocytes in the superficial layer (Janune et

al., 2017). Together with the repressive effect of CCN3 on these cells, CCN3 produced by chondrocytes in the middle layer is engaged in the maintenance of the quiescence and stemness of articular chondrocytes. Aging follows the development and growth of any tissue in any individual. Articular cartilage is no exception, and CCN3 plays a role in this process. Investigations with both mice and human patients have indicated that *CCN3* expression is correlated with age, and the artificial induction of cellular senescence in rat chondrocytic cells provoked the induction of CCN3 (Kuwahara et al., 2020). Moreover, the exogenous addition of CCN3 to these cells in culture induced *Cdkn1a* and *Trp53*, representing cell cycle arrest leading to senescence (Kuwahara et al., 2020). The age-dependent increase in CCN3 has also been suggested to occur due to the decreased nutrition supply that accompanies aging. It is probable that CCN3 assists the survival of chondrocytes under limited nutrition in a sustainable manner by restricting their cellular activities (Fig. 2, right panel). In the presence of CCN3, chondrocytes may not be regenerative, as CCN3 represses the cell proliferation and production of CCN2, a cartilage regenerator (Kubota et al., 2022a). This situation could be tolerable for the articular cartilage in aged people, which have less of a locomotive load, and in which cartilage damage is minimal. However, in young people, vigorous locomotive movement occasionally causes minor damage to articular cartilage, which requires a regenerative response. Therefore, excess CCN3 in articular cartilage can be harmful in the young. Consistently with this assumption, cartilage-specific overexpression of CCN3 in young mice displayed osteoarthritis (OA)-like histological findings (Kuwahara et al., 2020). OA is a major locomotive disorder in the elderly, which affects quality of life by impairing physical activities. Similarly, for elderly people with high levels of physical activity, the age-related increase in CCN3 in articular chondrocytes could be a

risk factor (Hirose et al., 2022). The role of CCN3 in OA development is still controversial (Huong et al., 2019; Hirose et al., 2022).

Do not overwork: A systemic message?

As described above, CCN3 plays the role of a local metabolic regulator in the cartilage, which is consistent with its molecular nature as a matricellular protein. However, such functional properties that promote cellular quiescence and stemness can be found in various tissues (van Royen et al., 2008), including the normal hematopoietic system (Gupta et al., 2007; Gupta et al., 2020) and malignancies (Gupta et al., 2001; Benini et al., 2005), suggesting that it has a systemic role. In this context, it should be noted that CCN3 is present in the blood stream at a level comparable to that of a peptide hormone (Li et al., 2019). Therefore, CCN3 may act as an endocrine factor that prevents a number of different cells from overworking. Generally, hormones are produced by particular glands and distributed via the cardiovascular system. We suspect that the adrenal gland could be the major producer of CCN3 as a hormone, since we found that CCN3 is exclusively expressed in the adrenal gland (Kocialkowski et al., 2001; Kubota, 2023). This adrenal-gland-specific gene expression pattern is comparable to that of CYP21A2, which produces cortisol and aldosterone in the adrenal cortex. These findings, taken together, suggest that CCN3 has the properties of an adrenal hormone. Nevertheless, although the expression level in each cell is much lower, adipose tissues do produce significant levels of CCN3 (Twigg, 2018). Considering the total volumes of these tissues, it is not clear which are the major tissues that supply serum CCN3. Further investigation is required to clarify the origin, systemic behavior and biological function of CCN3 in the bloodstream.

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Conflict of interest

The authors have no conflict of interests to declare.

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Legends to figures

Fig. 1. Metabolic regulation of CCN3 and its biological role in cells. Under conditions of starvation, regulatory factor binding to the X box (RFX1) gene expression is induced in chondrocytes. RFX1 enhances the production of CCN3 by binding directly to an enhancer in the proximal promoter region of the CCN3 gene. The anti-proliferative effect of CCN3 enables cell survival by reducing energy consumption, while maintaining the quiescence and stemness of chondrocytes.

Fig. 2. Biological outcomes conferred by CCN3 in the cartilage. CCN3 molecules (purple asterisks) are produced in the areas with nutrition shortages throughout the development, growth and aging processes of cartilage. During development, CCN3 commits epiphysial chondrocytes to articular chondrocytes, while inviting blood vessels into the cartilage for secondary ossification center formation (left panel). CCN3 in the resting zone of the growth plate and the middle zone of the articular cartilage support the stemness of surrounding chondrocytes, suppressing their cell growth (middle panel). As the process of aging advances, CCN production is enhanced to enable the survival of chondrocytes under conditions of reduced nutrition by economizing energy (right panel).

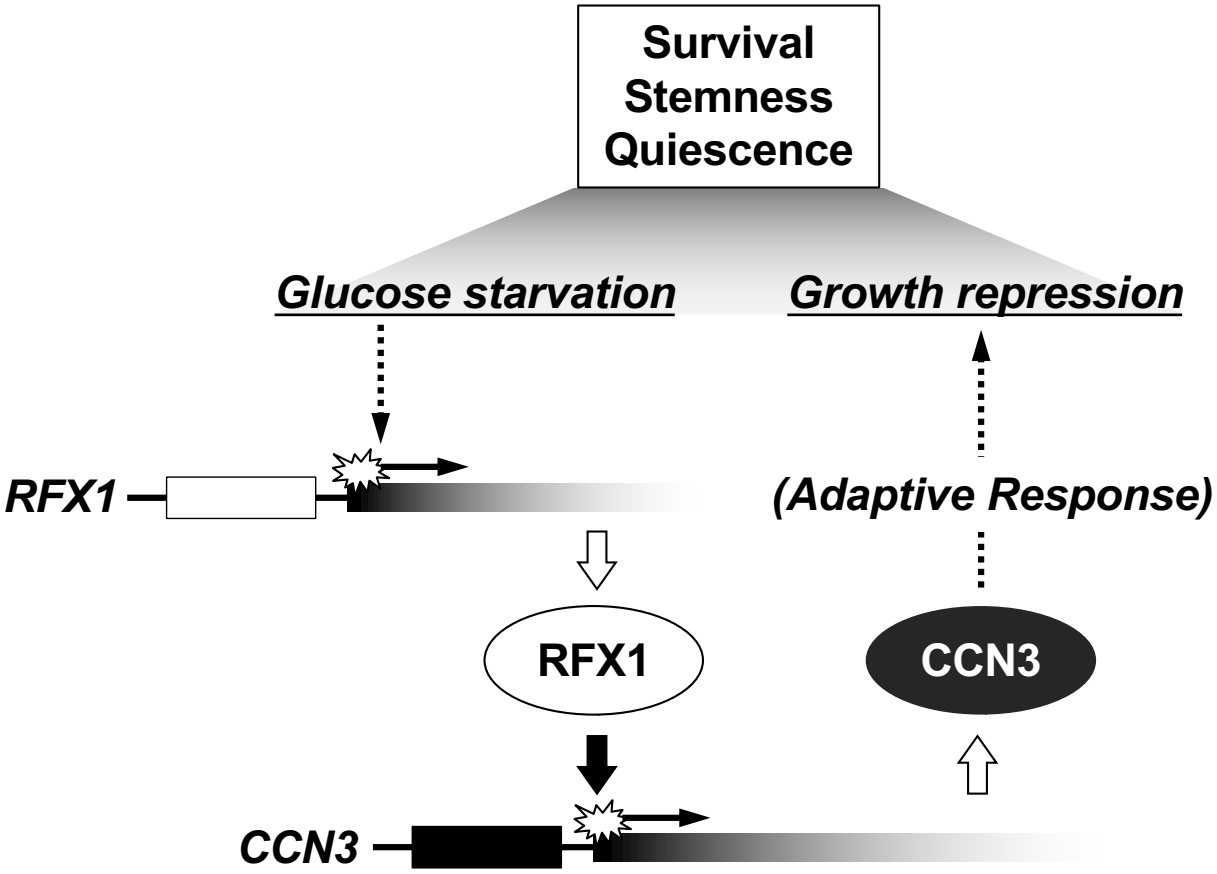

Figure 1. Kubota et al.

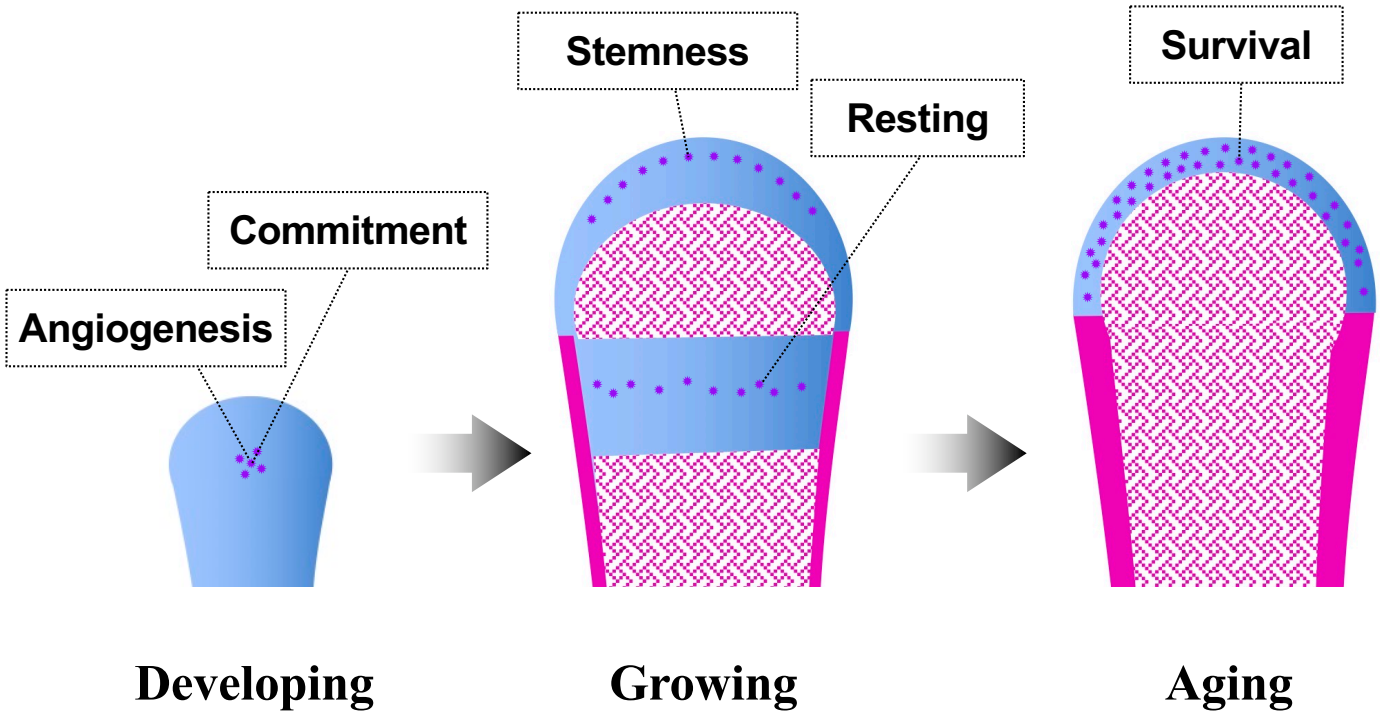

Figure 2. Kubota et al.