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論文内容の要旨

Bones have crucial roles in supporting body as one of the locomotor apparatuses, maintaining mineral homeostasis and protecting vital organs. Bone tissues are classified into two types by visual appearance and location, "cancellous (trabecular) bone" and "cortical bone". Cancellous bone, also known as sponge bone, is found in vertebral body and both ends of long bone like tibia and femur. It has larger surface area but shows weaker strength than cortical bone. It provides structural strength to bone tissue. Cortical bone forms cortex of most bones. It is stronger and stiffer than cancellous bone. The outer shell surrounding the cancellous bone at the end of the joints is also made up of cortical bone.

Bones in adulthood are continuously renewed by old bone resorption by osteoclasts and new bone formation by osteoblasts. This process is called "bone remodeling". Bone remodeling is important for bone homeostasis, and is essential for maintenance of healthy bones. Disruption of ideal balance in bone remodeling causes abnormal bone mass such as osteoporosis and osteopetrosis. A series of longitudinal studies of human populations have showed that bone mineral density (BMD) is correlated with physical strength of bone, and a most important risk factor for bone fracture.

However, human studies also indicated that BMD at any time points is well correlated with peak (highest) bone mass in the early growth phase of bone. Furthermore, these studies have also revealed that more than half of variation in the early growth phase of bone, which occurs over a short time period during puberty, is determined predominantly by genetic factors.

Recent advance in X-ray micro-computed tomography (micro-CT) has enabled to analyze three-dimensional (3D) structure of the cancellous bones without tissue destruction. Such studies have indicated that the risk of bone fracture in human elders increases as BMD decreases, but the biomechanical competence (physical strength) of bone and risk factor of bone fracture are dependent not only on BMD as the absolute amount of bone mass, but also on the 3D microstructure of cancellous bone. This fact suggests that we need to pay more attention to bone microstructure for identifying genetic risk factors for bone fracture. Recent studies also showed that there is a very good correlation between the bone microstructural measure obtained from the X-ray micro-CT and that obtained from conventional histological 2D sections. In particular, excellent correlation was found between bone volume fraction (BV/TV), which is the measure of cancellous bone volume per total volume surrounded by cortical bone, and the data of the histological 2D sections. Subsequent studies of microstructure of mouse cancellous bone by X-ray micro-CT have also showed that cancellous bone loss is observed even in the early growth phase of bone, and occurs continuously until the later adulthood stage, consistent with facts that cancellous bone is highly sensitive to change of bone metabolism, and is easily fractured in human elders.

Forward genetics approaches to explore polygenic factors responsible for bone homeostasis can be efficiently pursued in animal models. Usage of the animal models enables to control genome heterogeneity and environmental factors, and improves the power to identify heritable regulation of bone homeostasis. Variations in genome sequences and bone phenotypes among different inbred strains of mice are amenable to genetic analyses of polygenic complex traits of bone phenotypes via the quantitative trait loci (QTL) analysis. In fact, several studies with inbred mouse strains have revealed QTLs that affect microstructure of mouse cancellous bone. However, none of causative genes for the QTLs has been identified so far.

In this study, I focused on genetic factors responsible for variation in microstructure of mouse cancellous bone at the earlier growth phase of 6 to 10 weeks of age, because the early growth phase is critical for bone density and microstructure at any time in the later adulthood stage. The genes that regulate bone formation during embryonic development have been intensively studied by reverse genetics approaches with knockout mouse strains, and the genes involved in bone homeostasis at the later adulthood stage have been extensively studied by genome wide association study for human populations. By contrast, there is only limited information for genes acting for forming bone microstructure at the earlier and growing phase of bone. This is another reason that I focused on the early growth phase of mouse bone. For genetic analyses in this study, I used two strains, a laboratory inbred strain C57BL/6J (hereafter abbreviated B6) and Japanese wild mouse (*Mus musculus molossinus*)-derived MSM/Ms.

These two strains are genetically very distant one another, and more than ten million SNPs have been identified between these two strains, and the SNP data are now fully available. As a consequence of its genome divergence from B6 and other laboratory strains, MSM/Ms appeared to have unique complex traits that are never observed in standard laboratory strains. Another great advantage to use the B6 and MSM/Ms strains is that a full set of consomic strains, in which every B6 chromosome is replaced by counterpart of MSM/Ms, is now established and available for exploring QTLs.

At the beginning of this study, the X-ray micro-CT phenotyping of microstructure of cancellous bone of tibia at 10 weeks of age showed that MSM/Ms has far smaller value of bone volume fraction (BV/TV) than that of B6. This finding prompted me to pursue systematic phenotype screening of the same trait for the full set of B6-MSM/Ms consomic strains. As a result, I found that among all consomic strains B6-Chr15MSM carrying MSM/Ms-derive chromosome 15 (Chr15) shows the smallest cancellous bone volume fraction, indicating that mouse Chr15 harbors QTLs affecting bone microstructure, which is likely relevant to bone physical strength. Next, in order to further dissect genetic factors into sub-regions of Chr15, I generated nested sub-consomic strains that harbor sub-divided fragments of MSM/Ms Chr15. Bone

phenotyping of these strains revealed that at least four chromosomal blocks of Chr15 genetically control the trait of cancellous bone microstructure. I named these blocks, Block 1 to 4, each of which contains at least one QTL affecting microstructure of cancellous bone. None of these four QTLs has been reported by other groups' previous studies.

It is of interest to note that the MSM/Ms alleles at the QTLs in the Block 2, 3 and 4 decrease the bone volume fraction (BV/TV) relative to B6 as were expected, but the MSM/Ms allele at the QTL in Block 1 rather increases the BV/TV value relative to B6. Thus, the analysis with the sub-consomic strains revealed marked complexity of genetic architecture to control cancellous bone microstructure in mouse, and demonstrated that the analysis with consomic and sub-consomic strains has strong power to detect each of numerous QTLs, even if its phenotypic effect is modest.

Finally, I paid special attention to one short sub-block, named Sub-block 1-1, included in Block 1, the borders of which are defined by difference in the MSM/Ms-derived fragments of two sub-consomic strains. I intensively explored candidates of the causative gene for the QTL in Sub-block 1-1, because no known gene to be involved in bone regulation and homeostasis is assigned to this interval. I identified eight genes as candidates. In particular, four of them, *Ankrd33b, Ropn1l, March6 and Fam173b*, are good candidates from the aspects of expression pattern difference and amino acid change between B6 and MSM/Ms. Thus, this study provides indispensable clues for understanding genetic architecture underlying bone regulation and homeostasis of mouse, and for searching genetic factors responsible for osteoporosis in human.

博士論文の審査結果の要旨

私たちの体を支える主な骨組織は、海綿骨（cancellous bone）と皮質骨（cortical bone）からなる。海綿骨の３次元的構造は、骨密度（BMD）とともに骨の強度に重要であると考えられている。片岡君は、マウスMSM/Ms系統においては、C57BL/6J系統と比べ、X線マイクロ・コンピュータートモグラフィーを行なうことにより測定できる、海綿骨の３次元的構造の指標であるBone volume fraction（BV/TV）（骨体積と全体積の比）が、顕著に小さな値を示すことを見出し、これに興味をもった。そこで、この表現型について染色体マッピングを行い、BV/TVの値を変化させる原因となる染色体、さらには原因となる遺伝子を同定することを目的として研究を開始した。

片岡君は、C57BL/6J系統の遺伝的背景にMSM/Ms系統の１対の染色体を導入したとコンソミック系統の表現型を解析し、BV/TVを小さくする因子が、MSM/Ms由来の15番染色体上にあることをつきとめた。さらに片岡君は、かけあわせによりサブコンソミック系統を多数作製し、SNPマーカーを調べることにより、サブコンソミック系統がもつMSM/Ms由来の染色体領域を同定し、それら系統の表現型解析を行なった。その結果、MSM/Ms由来の15番染色体上のBV/TVに影響を与える領域を4カ所発見し、それらをブロック１ー４と名付けた。興味深いことにMSM/Ms由来のブロック２ー４はBV/TVを減少させることに寄与するが、ブロック１はBV/TVを増加させる方向に寄与することを見出した。

片岡君は、このブロック１が示した興味深い表現型についてさらに解析を進めた。サブコンソミック系統の表現型を詳細に調べたところ、約3.5Mbのブロック１の１部（ブロック１−１と命名）がBV/TVを増加させることを発見した。片岡君は、このブロック１−１内に、蛋白質をコードする遺伝子を8個見出した。それらはいずれもこれまでに十分解析がなされていない、機能未知遺伝子であった。

片岡君は、骨組織から抽出したRNAを用いてRT-PCRを行い、それらのうち7個が骨組織で発現していることをつきとめた。さらにC57BL/6J系統、MSM/Ms系統及びコンソミック系統を用いた解析により、MSM/Ms由来のブロック１−１をもつ個体においては、これらのうち3個（*Ropn1l, March6, Fam173b*）の発現が減少し、1個（*Ankrd33b*）の発現が増加していることを明らかにした。また、*in silico*解析により4個の遺伝子（*Ankrd33b*、*Ropn1l*、*Cmbl*、*Fam173b*）が非同義置換変異をもつことを明らかにした。特に*Ankrd33b*における置換は機能に重要であると考えられるアンキリンリピート内に存在した。進化的な比較解析により、この置換は実験用マウス系統とその近縁亜種で生じたものと考えられた。このことは機能との相関が予想され興味深い。このように、BV/TVに影響を与える有力候補として*Ankrd33b*遺伝子を同定することに成功した。

片岡君は、粘り強くサブコンソミック系統の作製と解析を行い、興味深い表現型を特定の遺伝子座、さらには遺伝子にまで結びつけるという、優れた研究成果をあげることができた。したがって、本論文は、博士号授与の要件を満たすと審査員全員一致で判断した。