称号及び氏名	博士（応用生命科学）　ISLAM, ZOHIRUL
学位授与の日付	平成３１年２月２８日
論　文　名	Expression and functional role of the orphan G protein-coupled receptor *Gpr137b* in osteoclast differentiation and macrophage polarization （破骨細胞分化およびマクロファージ極性化における リガンド未同定Ｇ蛋白質共役型受容体 *Gpr137b* の発現と機能）
論文審査委員	主査　乾　隆 副査　山地　亮一 副査　小森　雅之 副査　石橋　宰

論文要旨

General Introduction

G-protein-coupled receptors (GPCRs) compose the largest receptor gene family with more than 800 members in the human genome and play pivotal roles in a variety of physiological and pathological processes. Therefore, this large family of transmembrane receptors has attracted great interest as possible therapeutic targets. However, endogenous ligands of more than 100 GPCRs, which are called “orphan GPCRs”, remain unknown. GPCRs can be broadly classified into two groups, *i.e.*, odorant/sensory and non-odorant GPCRs. Odorant/sensory GPCRs are confined to particular cell types and conduct organismal behaviors such as feeding and mating. Conversely, non-odorant GPCRs comprise about 1% genes in the genome and widely expressed in the body but usually at low levels. Despite the low abundance, GPCRs are highly enriched or tightly restricted to a particular cell types, however, have an enormous potential to shed light on the unique traits of these cells.

Moreover, non-odorant GPCRs act in response to diverse endogenous ligands and are

involved in various physiological processes in the host, including hematopoiesis, neurotransmission, immune function, etc. *Gpr137b* is a non-odorant orphan GPCR conserved among mammals. *Gpr137b* was first identified in the kidneys, and later shown to be expressed in other tissues and cell types. However, the biological function of *Gpr137b* is yet to be determined.

Osteoclasts are large multinucleated cells involved in bone resorption and originated from hematopoietic progenitors. Monocyte in peripheral blood and various types of tissue macrophages are also developed from the same progenitor cells. Osteoclasts are formed by the fusion of committed monocytic cells and critical for healthy skeletal development, bone remodeling, and calcium metabolism. In this study, I identified *Gpr137b* as a gene encoding an orphan GPCR predominantly expressed in mature osteoclasts by genome-wide transcriptome analysis (RNA-seq).

Chapter 1: *Gpr137b*-gene knockout by the CRISPR/Cas9 genome editing technique

Since *Gpr137b* was demonstrated to be highly expressed in RAW264 cells and markedly increased during osteoclastic differentiation, Thus, I predict its important role in osteoclastic differentiation and I generated two *Gpr137b*-KO RAW264 cells using the CRISPR/Cas9 genome editing system. PCR to amplify the genomic region near the gRNA-recognizing sequence yielded products of the same size from both clones, which were demonstrated to have 188-nucleotide frameshift deletions at a region containing the translation start codon (ATG). I also isolated two independent cell clones from RAW264 cells transfected with pSpCas9 (BB)-2A-GFP as the controls. I verified that no mutation was introduced in a genomic region near the PAM sequence of *Gpr137b* in these clones, thus designated them *Gpr137b*-wildtype (WT) clones. Genomic PCR using primers designed to recognize the deleted sequence yielded a product of the expected size from the *Gpr137b*-WT clones and no product from the *Gpr137b*-KO clones. Since a pseudogene that is 97% homologues to the *Gpr137b* gene, *Gpr137b-ps*, exists in mouse, I designed a set of specific primers to amplify *Gpr137b* and *Gpr137b-ps*. The corresponding region of the *Gpr137b-ps* gene in these clones was shown to be intact. Next, I demonstrated that the deleted region of the *Gpr137b* gene was not transcribed in the *Gpr137b*-KO clones by qRT-PCR using primers that recognize this region but not the corresponding region of the *Gpr137b-ps* gene. By contrast, I confirmed that the corresponding region of the *Gpr137b-ps* gene is transcribed in the *Gpr137b*-KO clones using primers designed to specifically recognize this region in *Gpr137b-ps*. Further, I verified the lack of GPR137B protein expression in *Gpr137b*-KO clones by immunostaining using the anti-GPR137B polyclonal antibody generated in house. Fluorescence signals detected at the peripheral region as well as nuclei of the *Gpr137b*-WT cells, possibly representing the localization of GPR137B protein at the cell membrane and nucleus, were absent in the *Gpr137b*-KO cells.

Chapter 2: Role of *Gpr137b* in osteoclast differentiation

At first, I confirmed the predominant *Gpr137b* expression in mouse and human osteoclast cells in compare to the representative mouse and human tissues by qRT-PCR. Additionally, I confirmed that *Gpr137b* exhibits an osteoclast specific expression pattern among bone cells by qRT-PCR. Next, to elucidate the role of *Gpr137b* in osteoclast differentiation, generated *Gpr137b*-WT and -KO clones were then treated with soluble receptor activator of nuclear factor kappa-B ligand (sRANKL) to induce osteoclastic differentiation. Gene expression microarray analysis revealed that a set of genes are differentially regulated through RANKL-induced osteoclast differentiation. These genes include representative osteoclast-specific markers such as *Tartrate-resistant acid phosphatase* (*TRAP*), *Cathepsin K* (*CatK*), *ATPase H+ transporting*, *lysosomal V0 subunit D2* (*Atp6V0d2*) and *Matrix metalloproteinase 9* (*MMP-9*), which were dysregulated in the RANKL-treated *Gpr137b*-KO clones compared to the RANKL-treated *Gpr137b*-WT clones, suggesting that *Gpr137b* is possibly involved in RANKL-induced osteoclastic differentiation. These results were also validated by subsequent quantitative RT-PCR (qRT-PCR) and western blotting. KEGG pathway analysis of the *Gpr137b*-dependant RANKL-induced genes revealed the involvement of *Gpr137b* in osteoclast differentiation. My findings strongly suggest that the pharmaceutical targeting of *Gpr137b* possibly represents a novel and innovative approach to the prevention and management of bone diseases.

Chapter 3: Role of *Gpr137b* in macrophage polarization

Macrophages play a crucial role in host defense, disease pathogenesis and maintenance of tissue homeostasis. In general, macrophages can be classified into M1 (classical) and M2 (alternative) subtypes based on their phenotypes and functions. M1/M2 macrophage polarization is well maintained physiologically, and imbalanced polarization causes various pathological conditions. M1 macrophages, which are typically induced by lipopolysaccharide (LPS) and interferon (IFN)-γ, are activated during acute inflammation to produce proinflammatory cytokines, reactive nitrogens, and oxygen intermediates. By contrast, M2 macrophages are typically activated by interleukin (IL-4), IL-13, and anti-inflammatory cytokines produced by a variety of innate and adaptive immune cells, thus they play a role in post-inflammatory tissue repair and reconstruction. Imbalanced M1/M2 macrophage polarization is associated with various disease conditions. M1 macrophages are characterized by an upregulation of *Nos2*, *Cxcl10*, *Il1a* and *Gbp5*; whereas, a distinctive molecular feature of M2 macrophages is the high-level expression of *arginase-1* (*Arg1*); some C-C motif chemokine ligands such as *Ccl17*, *Ccl22*, and *Ccl4*; and *Cd206* (alternatively designated *Mrc1*).

Interestingly, KEGG pathway analysis described in Chapter 2 indicated the involvement of *Gpr137b* in the (IL-4) signaling pathway besides the osteoclast differentiation pathway. Thus, I

hypothesized that *Gpr137b* could be involved in macrophage polarization as IL-4 is well known to induce M2 macrophage polarization. Gene expression microarray analysis of *Gpr137b*-WT and -KO clones treated with IL-4 revealed that *Gpr137b*-KO attenuated the IL-4-induced expression of M2 macrophage-related genes such as mannose receptor (*Cd-206*), C-C motif chemokine ligand (*Ccl-22*), *Arginase1*, and *heparin-binding EGF-like growth factor* (*Hb-egf*), which was subsequently verified by qRT-PCR.

To determine whether *Gpr137b* has any impact on M1 macrophage polarization, *Gpr137*b-WT and -KO clones were then treated with LPS, a well-known inducer of M1 macrophage polarization. qRT-PCR analysis revealed that the LPS-induced expression of representative M1 macrophage markers such as *Cxcl10*, *Il1a*, *Nos2* and *Gbp5* was not affected by *Gpr137b*-KO. This result suggests that *Gpr137b* may not be associated with M1 macrophage polarization of RAW264 cells.

Conclusion

My findings in this study markedly expanded my views about bone remodeling and macrophage polarization. Elucidating the function of *Gpr137b* in osteoclast differentiation will make it a promising target for the novel anti-resorptive agent. Further, given the fact that macrophages affect many physiological or pathological processes, the current study will shed light on the utilization of *Gpr137b* as a potential drug target in the clinical application including helminth infection, inflammation allergy, rheumatoid arthritis and tissue repair.

審査結果の要旨

昨今の高度高齢化社会において，骨粗鬆症や関節リウマチなどの骨量減少・骨破壊をもたらす疾患の罹患者は国内だけで１千万人にも上るとされ,その対策は喫緊の課題である。現在，これらの疾患の治療において，骨吸収を担う破骨細胞を標的とする種々の骨吸収阻害剤が用いられているが，特に治療効果が高いとされるビスフォスフォネート剤服用中の歯科処置による顎関節壊死に代表されるように，重篤な副作用の問題により使用が制限される場合も多い。破骨細胞の分化や機能制御に関わる遺伝子やタンパク質を新たに同定し，これらの機能を分子レベルで解明できれば，作用機序が異なる新規骨吸収阻害剤の開発に繋がり，上記課題の解決への糸口となる。そこで本学位申請者は，破骨細胞への分化能を有するマウスマクロファージ様細胞株 RAW264 を用いて,破骨細胞分化に伴い発現が上昇する遺伝子を探索し，その結果見出された遺伝子の機能についてゲノム編集技術を用いて

解析した。

第 1 章では, RAW264 細胞を破骨細胞分化誘導因子である receptor activator of nuclear factor kappa-B ligand（RANKL）で処理することにより破骨細胞分化を誘導し，それに伴って発現が変動する遺伝子を網羅的遺伝子発現解析（RNA-seq）により探索した。発現量の指標である reads per kilobase of exon per million mapped reads (RPKM)値が 50 以上，かつ破骨細胞分化に伴う発現変動が 10 倍以上という条件を満たす遺伝子を選抜した結果, 発現上昇遺伝子を 56 種類見出し, これらの中で特に *Gpr137b* に着目した。*Gpr137b* がコードするタンパク質は，G タンパク質共役型受容体（GPCR）の 1 つである。GPCR はヒトでは約 800 種類存在し, 現在用いられている薬剤の約半数は GPCR を標的としている。また，GPCR のうち約 100 種類は，リガンドが未だに同定されていない，所謂「オーファン GPCR」であり，*Gpr137b* もこれに属する。オーファン GPCR は，新たな薬剤開発のための標的分子としてリガンド探索を含めた研究が盛んに行われているが，これまでに破骨細胞におけるオーファン GPCR に関する研究報告はほとんど存在しない。そこで，破骨細胞における *Gpr137b* の機能を解明するために，CRISPR/Cas9 系を用いたゲノム編集により，*Gpr137b* 遺伝子ノックアウト（*Gpr137b*-KO）RAW264 細胞クローンを作製した。なお, マウスでは *Gpr137b* と 97%の相同性を示す偽遺伝子 *Gpr137b-ps* が存在するが，得られた *Gpr137b*-KO 細胞クローンでは，*Gpr137b* 遺伝子の両アリルのみに 188 塩基対の欠損が認められ，*Gpr137b-ps* 遺伝子は無傷であることが確認された。

第 2 章では，前章で作製した *Gpr137b*-KO RAW264 細胞およびコントロール細胞を RANKL 存在下で培養し，これらの破骨細胞分化能について解析した。その結果，*Gpr137b*-KO RAW264 細胞では，*Trap*，*Ctsk*，および *Mmp9* 等の破骨細胞マーカー遺伝子の RANKL による発現誘導が顕著に抑制されていたことから, *Gpr137b* が破骨細胞分化に重要な役割を担う遺伝子であることが示唆された。さらに，マイクロアレイを用いた網羅的遺伝子発現解析およびパスウェイ解析の結果，*Gpr137b* が破骨細胞分化に決定的な役割を果たす nuclear factor kappa-B パスウェイの制御に関わることが示唆された。以上より, マクロファージ様細胞株である RAW264 の破骨細胞分化に伴い発現が上昇する遺伝子として *Gpr137b* を同定したが，一方で *Gpr137b* は，分化前の RAW264 細胞やマクロファージ前駆細胞（単球）を含む骨髄細胞においても，他の主要な臓器と比較して顕著に高いレベルで発現していた。

第 3 章では，*Gpr137b* がマクロファージの機能にも関与している可能性について追求した。マクロファージは炎症性である M1 と抗炎症性である M2 の 2 種類のサブクラスに大別され, 様々な薬剤やサイトカインにより各サブクラスへの極性化が制御される。そこで，*Gpr137b*-KO RAW264 細胞クローンを用いて解析を行った結果, *Gpr137b* は interleukin-4 が誘導する M2 極性化に関わるが，lipopolysaccharide が誘導する M1 極性化には関与しないことが明らかとなった。

本申請論文は，オーファン GPCR である *Gpr137b* が破骨細胞に選択的に高発現し，破骨細胞分化に重要な役割を担う遺伝子であることを明らかにした初めての論文である。本知見は，*Gpr137b* やその下流のシグナル伝達因子を標的とすることにより，副作用のリス

クが低く，且つ既存薬剤と作用機序が異なる新規骨吸収抑制剤の開発の可能性を示すことから，生化学，および創薬科学に大きく貢献するものである。よって，本論文の審査ならびに最終試験の結果と併せて，博士（応用生命科学）の学位を授与することを適当と認める。