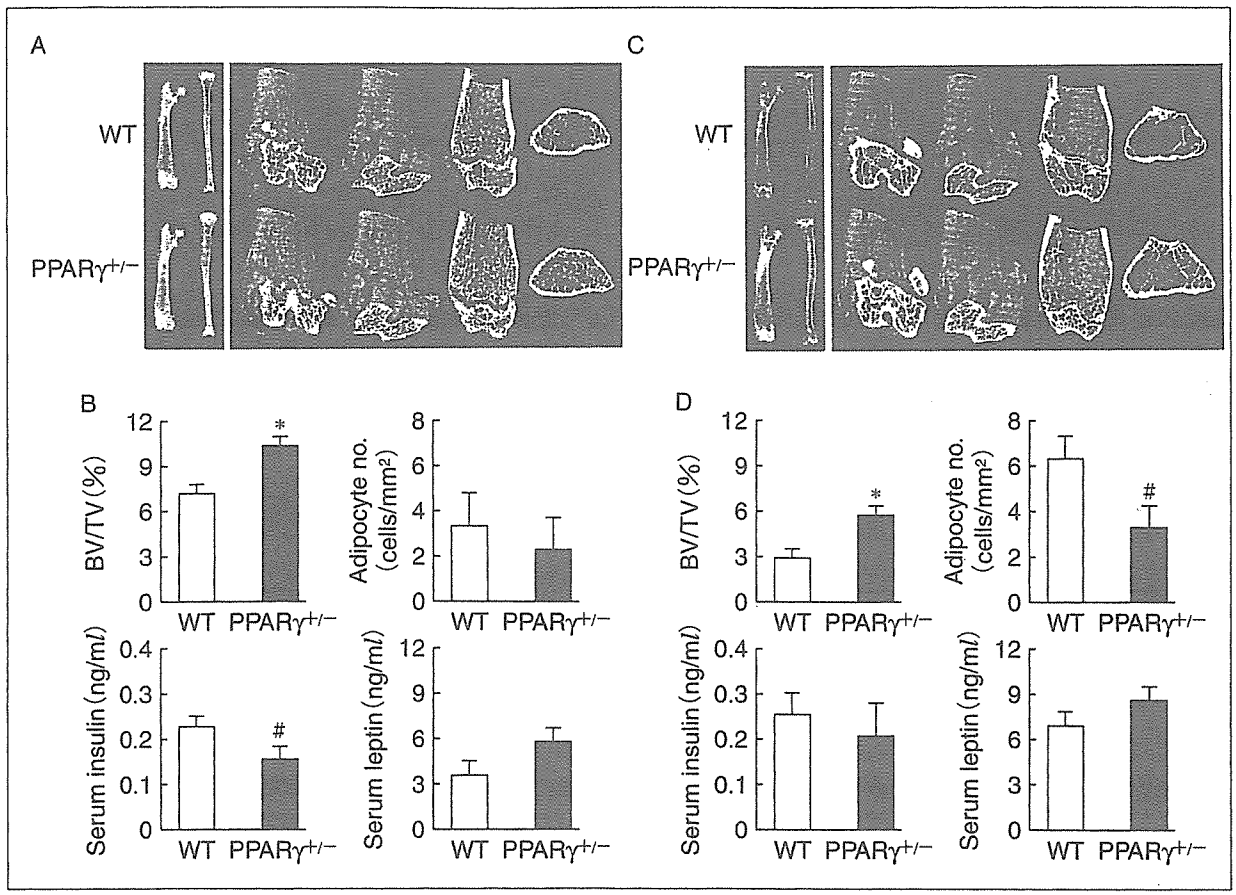

図1　8週齢(A, B)および52週齢(C, D)における野生型(WT)とPPARγヘテロノックアウトマウスの骨組織評価
A, C：下肢骨の単純レントゲン像および3次元μCT画像．B, D：骨量，骨髄脂肪細胞数，血清インスリン値，血清レプチン値．WTに対し*$p<0.01$, #$p<0.05$　　(文献[7]より引用改変)

IGF-I)は，ともに重要な骨代謝調節因子として知られる．まず，インスリンは，*in vitro*の検討で，骨芽細胞の増殖，分化やコラーゲン合成促進作用が報告され[9]，*in vivo*の検討では，ストレプトゾトシンによるインスリン分泌不全ラットで急速な骨量減少がみられると報告されている[10]．臨床的にも，I型糖尿病(IDDM)は骨粗鬆症を合併する場合が多いとされている[11]．II型糖尿病(NIDDM)でも，以前には骨量減少が指摘されていたが，近年では，体重増加に伴った骨量増加や病期あるいはインスリン反応性による骨量変化の違いが指摘されている．とくに，インスリン分泌が亢進している初～中期では骨量増加が，インスリン分泌が低下してくる末期では骨量減少が報告されている[11]．次に，IGF-Iは骨形成促進能をもつ成長因子の一つとしてよく知られるが，*in vitro*の検討で，成長ホルモンが肝臓に作用して分泌される全身性のもの以外に，局所で主に骨芽細胞から産生されるautocrine/paracrine factorとして重要であることが明らかになっている．*In vivo*の検討でも，マウスの交差実験で骨密度とIGF-I濃度が有意に相関していることが報告され[12]，臨床的には，閉経後女性で骨密度と血中IGF-I濃度の有意な正相関が認められ[13]，先天性のIGF-I欠乏疾患であるLaron症候群では著しい骨量減少が報告されている．このように，インスリンおよびIGF-Iを介するシグナルは，ヒトの骨代謝異常病態においても重要な役割を果たしている．しかし，これらの機能解析に有用であるインスリン受容体やIGF-I受容体などのノックアウトマウスは生後すぐに死亡してしまうため，骨代謝における作用を検討するのが難しかった．インスリンおよびIGF-Iは，それぞれ別のチロシンリン酸化型受容体に結合した後，共通の基質をリン酸化することによって，さらに下流のシグナルを活性化することが知られる．この

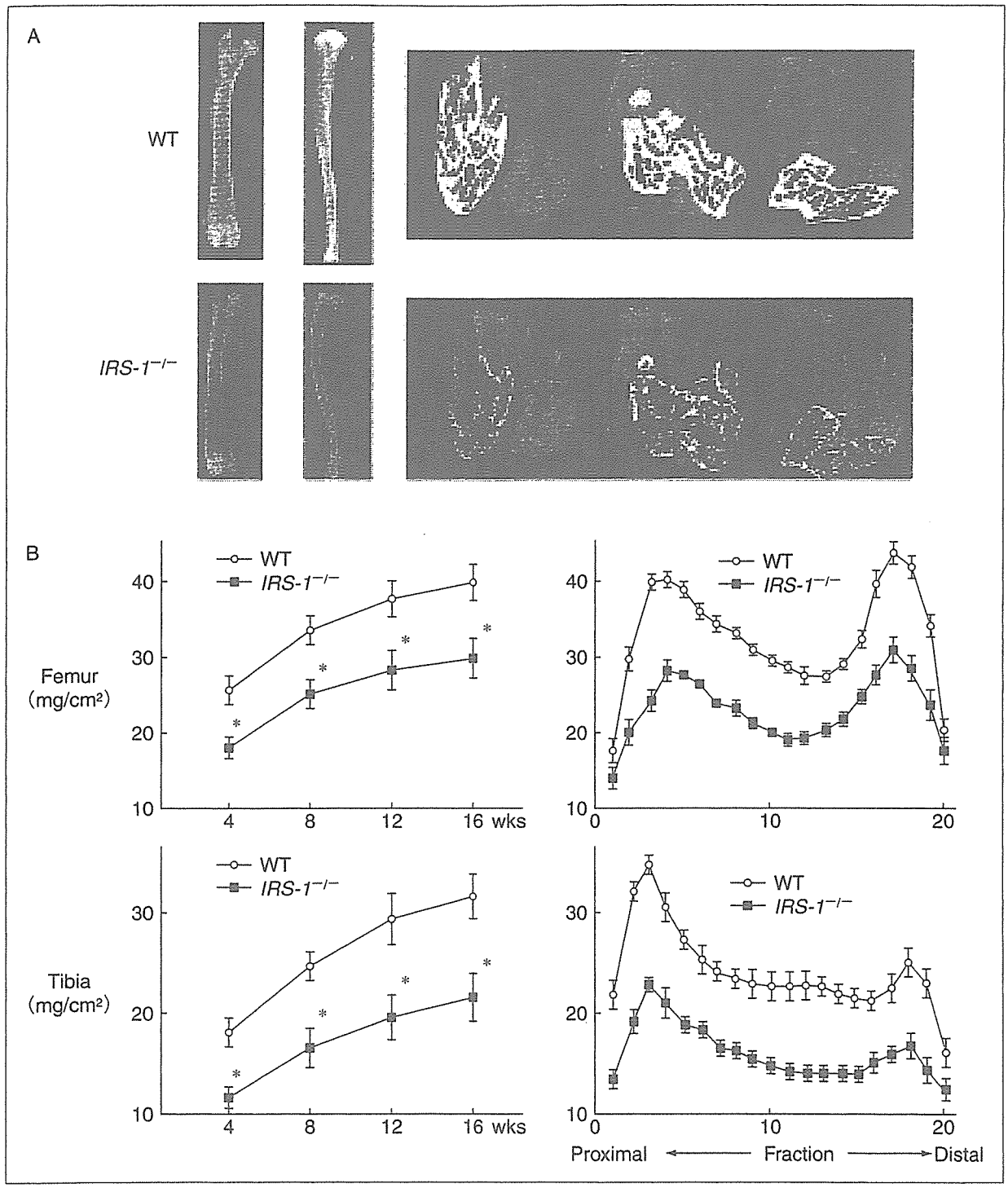

図2 8週齢における野生型(WT)とIRS-1ノックアウトマウスの骨組織評価
A：下肢骨の単純レントゲン像および3次元μCT画像．B：骨密度，WTに対し*$p<0.01$．(文献[15]より引用改変)

基質がインスリン受容体基質(insulin receptor substrate；IRS)であり，いくつかのサブタイプが特定され，その主たる機能はIRS-1(約180kD)およびIRS-2(約190kD)によってなされると考えられている．IRS-1,2は，肝細胞，筋肉細胞，脂肪細胞など，インスリン標的細胞を含む広範な組織に発現分布を示し，なかでもIRS-1は間葉系細胞で，IRS-2は血球系細胞で，比較的優位な発現を認め，IRS-1ノックアウトマウスでは明らかな骨代謝回転の低下による骨量低下がみられ，背景には骨芽細胞の増殖，分化能の低下，インスリンやIGF-I刺激に対する反応性の低下，骨芽

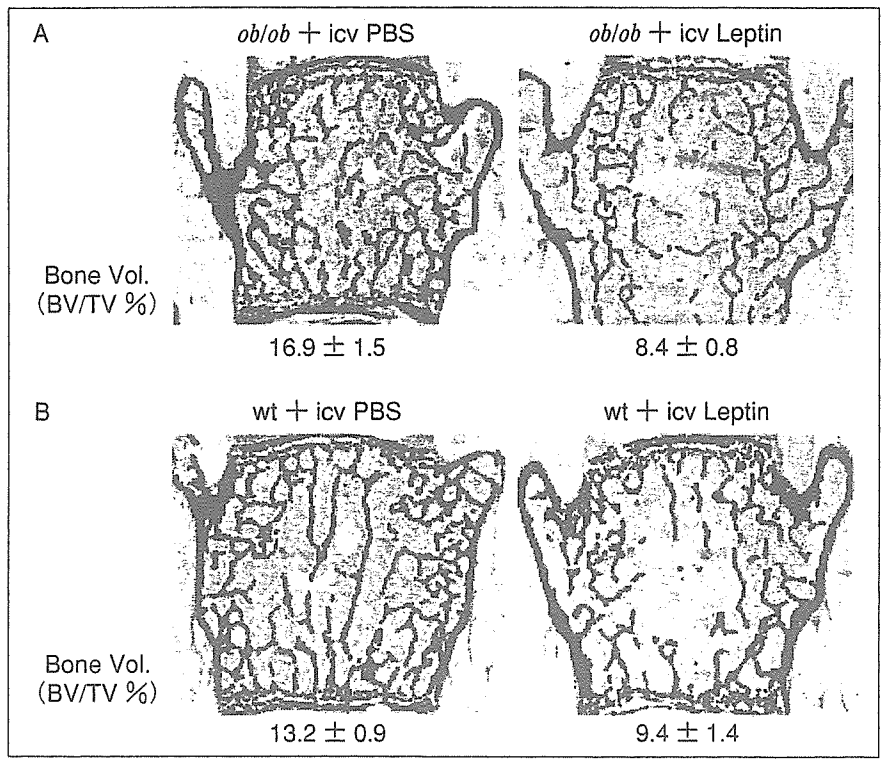

図3　16週齢における野生型(WT)と*ob/ob*マウスの骨組織評価(腰椎)
A, B：PBS(左)およびレプチン(右)の脳室内投与．WTに対し$p<0.05$
(文献[16]より引用改変)

細胞での破骨細胞形成支持能の低下が存在することが明らかにされた[14]．一方，IRS-2ノックアウトマウスにおいても骨量低下は認められたが，その程度はIRS-1ノックアウトマウスほど著明ではなく，骨芽細胞機能の低下も少なかったが，破骨細胞機能の亢進を認めた[15]．

(3) レプチン(図3)

レプチンは脂肪細胞から分泌される16kDのホルモンである．これまでに，視床下部弓状核にあるレプチン受容体を介し，摂食抑制ペプチド(α-MSH, CART)の発現亢進，摂食亢進ペプチド(NPY)の発現抑制により，摂食抑制作用とエネルギー消費亢進作用を有し，肥満や体重増加を抑制することが知られている．また，血圧調節，免疫能調節などにも関与するとされている．しかし，肥満では血中レプチン濃度が上昇しているにもかかわらず，過食行動や熱産生低下は是正されないため，レプチン抵抗性の状態にあると考えられている．

これまでに，レプチン欠損マウス(*ob/ob*)およびレプチン受容体欠損マウス(*db/db*)では生後から著しい肥満を呈し，性腺機能低下，高コルチゾール血症があるにもかかわらず，高い骨量を維持していることが報告され[16]，臨床的にも，レプチン欠乏症lipodystrophy(脂肪異栄養症)では骨量が高いことが知られているが，レプチンの骨代謝への作用には，以下に記すような骨形成促進と抑制，中枢性と局所性の報告があり，一定の見解が得られていない．

1) レプチンの間接作用(視床下部を介する)

Ducyらは，レプチンを第三脳室に投与すると，*ob/ob*，Wild Type(WT)のいずれにおいても骨量が減少することを報告した[16]．すなわち，レプチンにはエネルギー代謝調節作用と同じく，視床下部を介した骨形成抑制作用があることを示唆した．さらに，Takedaらは，レプチンのエネルギー代謝調節作用に関する既知の視床下部の神経因子やニューロンは，レプチンの骨形成促進作用に関与しておらず，エネルギー代謝調節作用とは異なった経路を介した骨形成抑制作用を発現しているとした[17]．

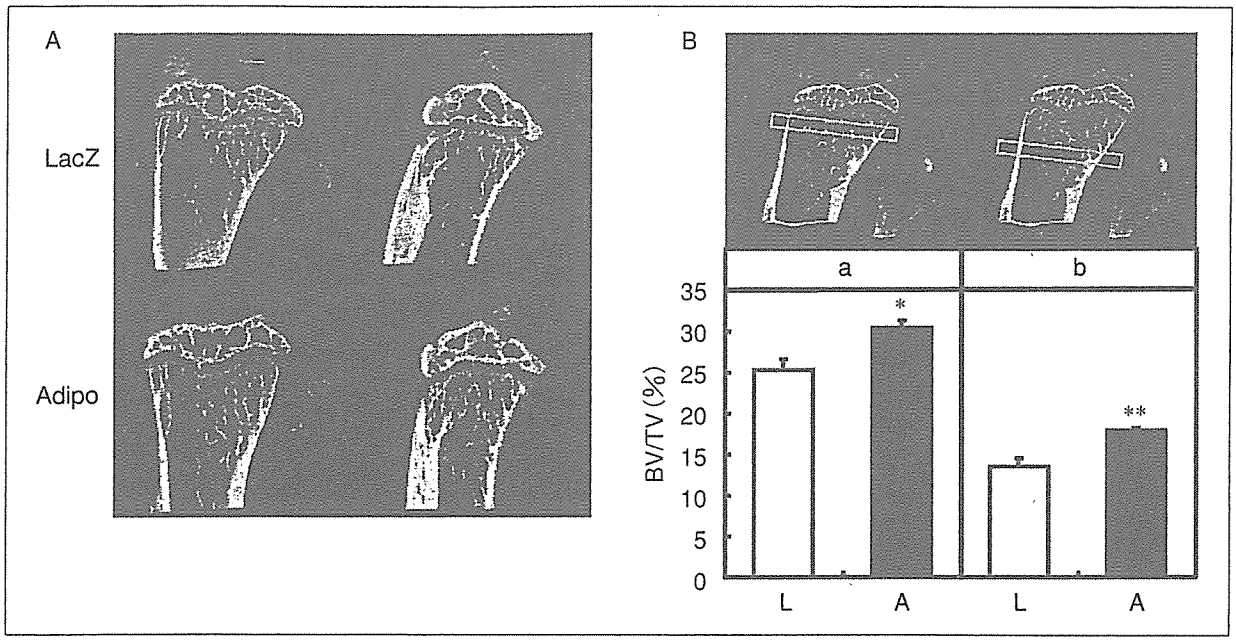

図4　8週齢の野生型(LacZ)とアディポネクチン強発現(Adipo)マウスの骨組織評価
A：脛骨近位部の3次元μCT画像，B：脛骨近位成長帯から50～250μm(a)，500～700μm(b)遠位部における海綿骨量．LacZに対し*$p<0.05$, **$p<0.01$　(文献[22]より引用改変)

2) レプチンの直接作用

レプチンは骨髄の脂肪細胞でも産生され，その受容体は骨形成を担う骨芽細胞にも存在している．*In vitro*の検討では，骨芽細胞の増殖，分化を促進し[18]，破骨細胞分化因子(RANKL)を介して破骨細胞への分化を抑制する[19]．また，軟骨細胞の増殖を促進することも報告されている[20]．*In vivo*の検討では，レプチンを全身投与すると，*ob/ob*, WT, OVX, 先天性レプチン欠乏症で骨成長が進み，骨量が増加することが明らかにされており，成人女性の血中レプチン濃度と骨密度は正相関するとの報告もある[21]．つまり，レプチンの骨組織に対する直接作用は，視床下部を介した中枢作用とは逆に，骨形成を促進して骨吸収を抑制することになる．

(4)アディポネクチン(図4)

アディポネクチンは，血中に5～30μg/mlと高濃度に存在し，脂肪細胞でのみ産生，分泌されるにもかかわらず，肥満者や男性において低下し，減量によって増加する特異なアディポサイトカインである．さらに，肥満度が同じでも，心筋梗塞や狭心症といった動脈硬化性疾患，および糖尿病で血中アディポネクチン濃度は低下する．これまでに，アディポネクチンはIRS-Iシグナルを介したPI3-kinaseの活性および糖輸送を上昇させ，インスリン感受性を増強させること，また，脂肪酸の酸化およびクリアランスに重要な脂肪酸輸送蛋白1型(fatty acid transport protein 1；FATP-1)の遺伝子発現を増強させることがわかっている．一方で，脂肪蓄積で脂肪組織より過分泌されるアディポサイトカインであるTNF-αは同じ経路に作用し，アディポネクチンと逆の作用を示すが，アディポネクチンとTNF-αは互いの作用を抑制し合うのみならず，その産生場所である脂肪組織において転写レベルでの調節により互いの産生を抑制し合う．つまり，アディポネクチンは，インスリン抵抗性惹起因子であるTNF-αの産生と機能を抑制することによってもインスリン感受性を増強させる．また，アディポネクチンは血管壁に作用し，単球の接着，平滑筋細胞の増殖，遊走，マクロファージの泡沫化を抑制して抗動脈硬化作用を発揮し，TIMP-1産生上昇を介して急性冠症候群に対しても抑制作用を示す．

近年，アディポネクチンレセプターが同定され，その発現がユビキタスであったことから，レプチンのみならずアディポネクチンも骨代謝になんらかの影響を及ぼすことが容易に想像さ

れた．そして，最近，*In vivo*の検討で，アデノウイルスを用いたアディポネクチン強発現マウスの海綿骨量が著明に増加し，破骨細胞数の減少とNTxの減少を伴うことが明らかとなり[22]，*In vitro*の検討では，アディポネクチンが破骨細胞の分化，吸収活性を抑制すること，骨芽細胞の分化，石灰化を促進することが明らかにされた[22]．さらに，骨芽細胞の増殖，分化促進作用は，MAPKシグナルを介したものであることもわかった[23]．また，アディポネクチンのパラログであるCartducinが軟骨細胞の増殖，分化を促進することも報告されている[24]．

肥満と骨折率

肥満は骨量の増加因子であるのみならず，骨折率の増加とも関連していることが多数報告されている．これは，肥満だとバランス制御が悪く，転倒率が上昇することが考えられる．さらに，骨量の増加は骨への加重が増加していることを意味することから，荷重の増加が骨折に寄与することも想定される．そのため，肥満では骨量増加がみられるものの，骨折率の低下には結びつかない．

肥満での骨折の危険性を論じるには，糖尿病と非糖尿病を分けて考える必要がある．1948年にAlbrightらが糖尿病の長期罹患患者に易骨折性が生じると報告して以来，糖尿病と骨代謝異常との関連性が検討されてきた．インスリン欠乏が特徴的であるⅠ型糖尿病では骨量減少が生じ，その結果，骨折頻度が増加するという一定した見解が得られている[25]．また，Ⅱ型糖尿病においても皮質骨での有意な骨密度の減少を認め，Ⅱ型糖尿病における骨折の危険性が閉経後女性においてとくに増加すること[26]，そして，いったん骨折を発症した際には骨折治癒過程が遅延することが報告された[27]．糖尿病では，高血糖の持続やインスリンおよびIGF-Ⅰの欠乏による骨芽細胞機能の低下が特徴であり[28]，細胞内ソルビトールの蓄積が骨芽細胞機能の傷害に関与していることが報告されている[29]．それゆえ，糖尿病においては持続的高血糖が骨基質蛋白の糖化(グリケーション)を促し，たとえ骨の量的変化を認めなくても質的変化が起こり，その結果として骨脆弱性がもたらされる可能性がある．糖尿病では骨密度に依存せずに骨折率が増加する報告もあることから[30]，糖尿病では骨密度の低下はなくとも骨質の劣化により骨折率が上昇するものと考えられる．

また，過去の報告から，5％以上の減量で骨量減少が生じ，体重減少に伴い骨折率は上昇するとされる．摂取カロリーが減少すると，PTHが上昇し，エストロゲンの低下とそれに伴う骨吸収が亢進する．逆に，カルシウム摂取量が増加すると，減量に伴う骨吸収の亢進が抑制される．このことから，減量により腸管でのカルシウム吸収量が低下し，二次性副甲状腺機能亢進症が起こると考えられる．さらに，女性では減量の速度と血清エストロゲン濃度の低下に相関がみられ，とくに，閉経後は脂肪組織局所で生合成されるため，脂肪量の減少により血清エストロゲン濃度が低下し，腸管でのカルシウム吸収がさらに低下すると考えられる．ほかに，食事制限によりIGF-Ⅰが低下し，コルチゾールが増加することで骨形成が低下することも報告されている．しかし，いまだ確固たる機序の解明はなされていない．

おわりに

肥満と骨量および骨質の関係を説明する明確な機構はいまだ存在しなかった．力学的負荷が骨代謝に影響を与えていることは臨床研究から疑いようのない事実であり，生理活性物質が骨代謝に少なからず影響を与えていることはこれまでの知見から明らかである．しかし，明確な説明ができうるまでの解明には至っていない．生活習慣病，メタボリックシンドロームが脚光を浴びている現代において，肥満，骨粗鬆症の有効な予防対策や治療法の確立が早急に必要とされている．それを反映するように，肥満と骨代謝に関する論文報告数が急増していることから，これからの医学研究におけるホットトピックスになることは間違いないと思われる．新たな展開に期待したい．

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Ⅳ-Ⅱ　分担研究刊行物

C）RA骨髄細胞由来病因遺伝子解明と治療法開発研究

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Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 regulates the induction of Langerhans cell maturation

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Recently, we reported that Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 (SHPS-1) plays an important role in the migration of Langerhans cells (LC). Here, we show that SHPS-1 is involved in the maturation of LC. Immunofluorescence analysis on epidermal sheets for I-A or CD86 revealed that LC maturation induced by 2,4-dinitro-1-fluorobenzene (DNFB) or by TNF-α was inhibited by pretreatment with an anti-SHPS-1 monoclonal antibody (mAb) or with CD47-Fc fusion protein, a ligand for SHPS-1. Further, FACS analysis demonstrated that I-A^+ LC that had emigrated from skin explants expressed CD80 or CD86, whereas CD47-Fc protein reduced $CD80^{high+}$ or $CD86^{high+}$ cells. CD47-Fc protein also reduced the up-regulation of surface CD80 or CD86 by LC remaining in the skin explants. In SHPS-1 mutant mice, we observed that the up-regulation of surface CD86 and CCR7 by LC induced by DNFB as well as that of surface CD80 and CD86 by LC in skin explants was attenuated. Finally, contact hypersensitivity (CHS) response was suppressed in SHPS-1 mutant mice and in wild-type mice treated with an anti-SHPS-1 mAb. These observations indicate that SHPS-1 plays an important role in the maturation of LC *ex vivo* and *in vivo*, and that SHPS-1-CD47 interaction may negatively regulate CHS.

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Introduction

DC are potent antigen-presenting cells that play a crucial role in the initiation of immune responses to pathogens. Langerhans cells (LC) are specialized immature DC in the skin that reside in the suprabasal layers of the epidermis. When LC encounter exogenous antigens, including haptens and microorganisms, they capture and process them to generate MHC/peptide complexes on their surface. LC migrate from the epidermis to draining lymphoid tissues in order to initiate naive T cells and to present the MHC/peptide complexes to

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Abbreviations: **CCR7:** CC chemokine receptor 7 · **CHS:** contact hypersensitivity · **DNFB:** 2,4-dinitro-1-fluorobenzene · **LC:** Langerhans cell · **SHPS-1:** Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 · **SIRP α1:** signal-regulatory protein α1

T cells with the necessary adhesion molecules and costimulatory signals [1–7]. During this migration, LC mature from a dormant into an activated functional state and down-regulate their endocytotic activity. The maturation of LC is characterized by increased expression of cell-surface molecules such as MHC class II, CD11c, CD40, CD80, CD86, CC chemokine receptor 7 (CCR7) and Langerin and by decreased expression of E-cadherin [7–13]. Recently, it has been shown that this functional maturation of LC is affected by various factors such as corticosteroids, hyaluronan, ultraviolet irradiation, prostaglandin E2-EP4 signaling, and ligation of E-cadherin or mycophenolate mofetil [11, 14–18].

One signal-regulatory protein, Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 (SHPS-1) [19], is also known as signal-regulatory protein α1 (SIRP α1) [20], BIT (brain Ig-like molecule with tyrosine-based activation motifs) [21], P84 [22], MFR (macrophage fusion receptor) [23] and MyD-1 [24]. In mice, SHPS-1 is expressed in myeloid cells, including monocytes, macrophages and DC [25]. SHPS-1 is a transmembrane glycoprotein whose extracellular domain comprises three immunoglobulin-like domains with multiple N-linked glycosylation sites, while the cytoplasmic domain of SHPS-1 contains four tyrosine residues that form two ITIM [26]. SHPS-1 was initially discovered as a tyrosine-phosphorylated transmembrane protein that binds SHP-1 or SHP-2. IAP/CD47, an integrin-associated protein, has been identified as an extracellular ligand for SHPS-1 [27]. The CD47-SHPS-1 system has been shown to regulate a novel cell-cell communication system that is important in immunology and hematology. CD47 on red blood cells binds to SHPS-1 on macrophages, thereby inhibiting macrophage activation and phagocytosis [28, 29]. The interaction of SHPS-1 with CD47 negatively regulates cellular responsiveness during T cell activation and during the induction of antigen-specific cytotoxic T lymphocytes by DC and monocytes [24, 30]. Ligation of SHPS-1 prevents the functional maturation of immature DC and suppresses IL-12 production by mature DC [31], although it has not been elucidated whether the ligation of SHPS-1 on LC affects the maturational state of LC.

We recently demonstrated that LC/DC migration is significantly inhibited by the ligation of SHPS-1 and that LC migration is impaired in SHPS-1 mutant mice that lack the intracellular domain of SHPS-1 [32]. In this study, we focused on LC maturation and investigated whether that process is affected by the ligation of SHPS-1. We now demonstrate that LC maturation induced by haptens, TNF-α or a skin explant culture is significantly inhibited by the ligation of SHPS-1 with an anti-SHPS-1 mAb or with a CD47-Fc fusion protein. We further show that LC maturation induced by haptens or a skin explant culture is impaired in SHPS-1 mutant mice. Finally, we show that the contact hypersensitivity (CHS) response is markedly attenuated in mice treated with the anti-SHPS-1 mAb before sensitization or in SHPS-1 mutant mice.

Results

The ligation of SHPS-1 suppresses 2,4-dinitro-1-fluorobenzene-induced maturation of LC

The expression levels of I-A, CD40, the costimulatory molecules CD80 and CD86 and the DC marker CD11c are considered to reflect the maturational state of LC [7, 10, 12, 33]. It has been reported that application of a hapten, such as DNFB, up-regulates I-A [7, 8, 12] and elevates the expression of CD86 but not CD80 in the I-A$^+$ population of murine epidermal cells [14] or migrated dermal LC [34]. Similar to a previous report, we did not observe the expression of CD80 on LC *in situ* or following 2,4-dinitro-1-fluorobenzene (DNFB) painting on the skin. Therefore, we evaluated whether the ligation of SHPS-1 affects the surface expression of I-A or CD86 by immunofluorescence analysis of epidermal sheets. To evaluate the level of expression of surface molecules, we assessed the I-A$^+$ area in each cell by computer image analysis (NIH image) [35]. Application of DNFB markedly increased I-A expression, whereas *in vivo* pretreatment with an anti-SHPS-1 mAb (Fig. 1A) or with CD47-Fc protein (Fig. 1B) significantly reduced the up-regulation of I-A expression. Similarly, application of DNFB significantly elevated CD86 expression per I-A$^+$ cell, whereas *in vivo* pretreatment with CD47-Fc protein markedly inhibited up-regulation of CD86 expression (Fig. 1C). Representative images are shown in Fig. 1D. We could not observe CD80 expression on I-A$^+$ cells in the epidermis (data not shown).

The ligation of SHPS-1 suppresses LC maturation induced by TNF-α treatment

TNF-α has been reported to play an important role in the maturation of LC or DC *in vitro* [10, 17, 36]. Therefore we examined whether *in vivo* treatment with TNF-α affected the surface expression of I-A by immunofluorescence analysis of epidermal sheets. Intradermal injection of TNF-α (50 ng/mL) significantly increased I-A expression compared with injection of rat IgG (as a control). We then investigated whether ligation of SHPS-1 affected the TNF-α-induced maturation of LC. *In vivo* pretreatment with an anti-SHPS-1 mAb significantly inhibited the up-regulation of I-A expression of LC (Fig. 2A). Representative images are shown in Fig. 2B.

The ligation of SHPS-1 inhibits LC maturation induced by epidermal explant culture

Larsen *et al.* established a skin culture system, which uses murine ear skin to investigate the emigration and maturation of DC [37]. Since we wanted to investigate the maturation of LC rather than DC, we modified the method to use epidermal sheets rather than whole skin sheets. Interestingly, most cells emigrating into the medium during 48 h expressed both I-A and CD80 (Fig. 3A) or CD86 (Fig. 3B). The addition of CD47-Fc protein into the medium slightly but significantly reduced the percentage of I-A^+ $CD80^{high+}$ emigrated cells (Fig. 3A and B) and I-A^+ $CD86^{high+}$ emigrated cells (Fig. 3C and D). Immunofluorescence analysis revealed that I-A^+ cells remaining in the epidermal sheets 24 h after incubation expressed CD80 (Fig. 3E) and CD86 (Fig. 3F), while I-A^+ cells in the epidermal sheets before incubation hardly expressed CD80 or CD86 (data not shown). These observations ensure that the epidermal explant culture system is useful for investigating LC maturation. Immunofluorescence analysis of epidermal sheets clearly showed that treatment with CD47-Fc protein inhibited the up-regulation of CD80 (Fig. 3E) and CD86 expression (Fig. 3F). Up-regulation of CD205 expression in LC was also observed following explant culture (data not shown). The up-regulation of CD205 was inhibited by CD47-Fc (Fig. 3G).

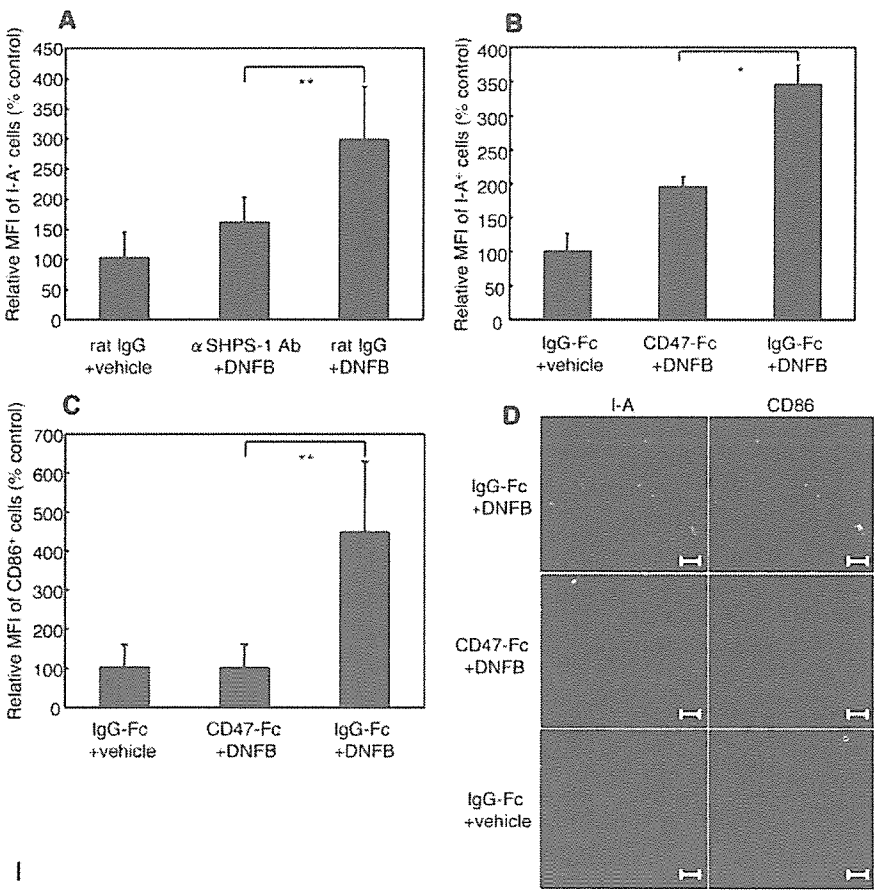


Figure 1. The ligation of SHPS-1 suppresses the DNFB-induced maturational state of LC. Anti-SHPS-1 mAb, CD47-Fc protein, or isotype-matched controls were intradermally injected before DNFB application. Twenty-four hours after the DNFB application, epidermal samples were collected and stained with FITC-conjugated anti-mouse I-A^b mAb and PE-conjugated anti-mouse CD86 mAb. (A) Application of DNFB significantly increased the MFI of I-A expression, whereas the *in vivo* pretreatment of anti-SHPS-1 mAb significantly reduced the up-regulation of I-A expression. The ** indicates statistical significance compared with treatment of control antibody and DNFB ($p < 0.01$, $n = 4$). (B) CD47-Fc significantly reduced the up-regulation of I-A expression. The * indicates statistical significance compared with treatment of IgG-Fc and DNFB ($p < 0.05$, $n = 4$). (C) Application of DNFB significantly increased the MFI of CD86 expression, whereas *in vivo* pretreatment with CD47-Fc significantly reduced the up-regulation of CD86 expression. The ** indicates statistical significance compared with treatment of IgG-Fc and DNFB ($p < 0.01$, $n = 4$). (D) Representative images after treatment with CD47-Fc are shown. Bars indicate 20 μm.

LC maturation is attenuated in SHPS-1 mutant mice

Wild-type mice and mice with a mutation in SHPS-1 that lacks most of the cytoplasmic domain of the protein were painted with 0.5% DNFB and after 24 h epidermal samples were analyzed for expression of I-A$^+$ or CD86$^+$. CD86$^+$ cells were scarcely observed in the epidermis of SHPS-1 mutant mice or wild-type mice before DNFB application (data not shown). Application of DNFB remarkably increased the fluorescent intensity of CD86 on LC in wild-type mice, whereas CD86 expression after DNFB application was impaired in SHPS-1 mutant mice (Fig. 4A). Representative images are shown in Fig. 4B. Furthermore, application of DNFB remarkably increased the fluorescence intensity of I-A on LC in wild-type mice compared with SHPS-1 mutant mice (Fig. 4B). Epidermal explants from wild-type mice and from SHPS-1 mutant mice were incubated for 24 h and were then stained for I-A, CD80, or CD86 as described for Fig. 3E and F. Immunofluorescence analysis of epidermal sheets revealed that up-regulation of CD80 (Fig. 4C) and CD86 (Fig. 4D) was markedly inhibited in SHPS-1 mutant mice compared with wild-type mice.

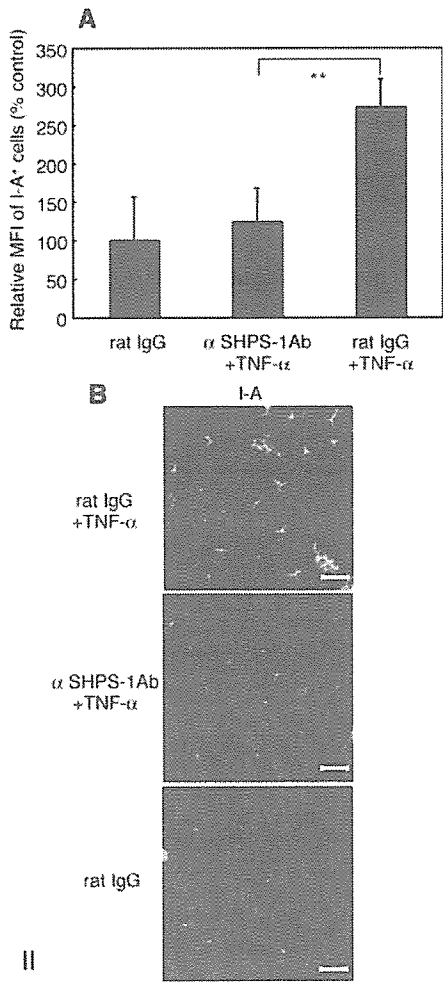


Figure 2. The ligation of SHPS-1 suppresses LC maturation induced by TNF-α- treatment. Anti-SHPS-1 mAb or rat IgG in combination with TNF-α was injected intradermally as described in the *Materials and methods*. One hour after the injection, epidermal samples were collected and stained for I-A. (A) Combined injection of rat IgG and TNF-α significantly increased the MFI of I-A expression, whereas combined injection of anti-SHPS-1 mAb and TNF-α significantly reduced the up-regulation of I-A expression. The ** indicates statistical significance compared with treatment of control antibody and TNF-α ($p < 0.01$, $n = 4$). (B) Representative images are shown. Bars indicate 20 μm.

Moreover, recent studies have shown that mature DC (but not immature DC) express CCR7, a chemokine receptor also known to regulate the entry of lymphocytes and LC into lymph nodes [38, 39]. Therefore, we further studied whether DNFB application affects the expression of CCR7 on LC residing in the epidermis. CCR7$^+$ cells were scarcely observed in the epidermis of SHPS-1 mutant mice or in wild-type mice before DNFB application (data not shown). The expression of CCR7 on I-A$^+$ cells in the epidermis of SHPS-1 mutant mice and of wild-type mice was hardly observed after application of vehicle (Fig. 5B). In contrast, application of DNFB increased the fluorescence intensity of CCR7 on LC in wild-type mice, whereas no increase of CCR7 expression was observed following DNFB application in SHPS-1 mutant mice (Fig. 5A and B).

The CHS response is attenuated in SHPS-1 mutant mice

To evaluate whether *in vivo* treatment with anti-SHPS-1 mAb influences the CHS response, we injected anti-SHPS-1 mAb intradermally before sensitization or elicitation. The CHS response to DNFB was significantly diminished by the treatment with anti-SHPS-1 mAb before sensitization (Fig. 6A). In contrast, the CHS response was not affected by the anti-SHPS-1 mAb treatment just before elicitation (data not shown). Furthermore, we assessed the ability to develop a CHS reaction in SHPS-1 mutant mice. The CHS response to DNFB was drastically decreased in SHPS-1 mutant mice compared with wild-type mice (Fig. 6B). We further examined the proliferation activity of regional lymph node cells in DNFB-sensitized mice. Addition of DNBS into the culture media induced marked proliferation of lymph node cells from wild type mice, while this proliferation was significantly impaired in SHPS-1 mutant mice (Fig. 6C). Similarly, DNBS stimulated the production of IFN-γ by lymph node cells in wild-type mice, whereas it was significantly reduced in SHPS-1 mutant mice (765 ± 262 *vs.* 133 ± 86 pg/mL; $n = 7$, $p < 0.05$).

Discussion

We showed in this study that ligation of SHPS-1 with an anti-SHPS-1 mAb or with CD47-Fc protein reduced the hapten-induced expression of I-A and CD86 by LC *in vivo*. Because expression of CD80 was hardly observed under the experimental conditions of DNFB painting on the skin *in vivo*, we studied the phenotypic maturational change of LC using a modified technique for the skin explant culture system [37]. As Hoetzenecker *et al.* [11] reported that expression by CD80 of LC in the epidermis was hardly observed in explant cultures of whole skin, we used the epidermis rather than the whole skin for skin explant culture [35]. That epidermal explant method enabled us to observe a significant induction of the expression of CD80 as well as of CD86 on LC remaining in the epidermis. Induction of the expression of those molecules was significantly inhibited by the addition of CD47-Fc protein. Thus, the epidermal skin explant culture system is useful for investigating the phenotypic maturation of LC by exogenous stimulants. We previously showed that SHPS-1 is selectively expressed on LC in the epidermis [32], which indicated that ligation of SHPS-1 with its ligands exclusively affects LC but not keratinocytes. These results indicate that engagement of SHPS-1 negatively regulates the phenotypic maturation of LC *in vivo* and *ex vivo*. We further observed that most cells migrating into the

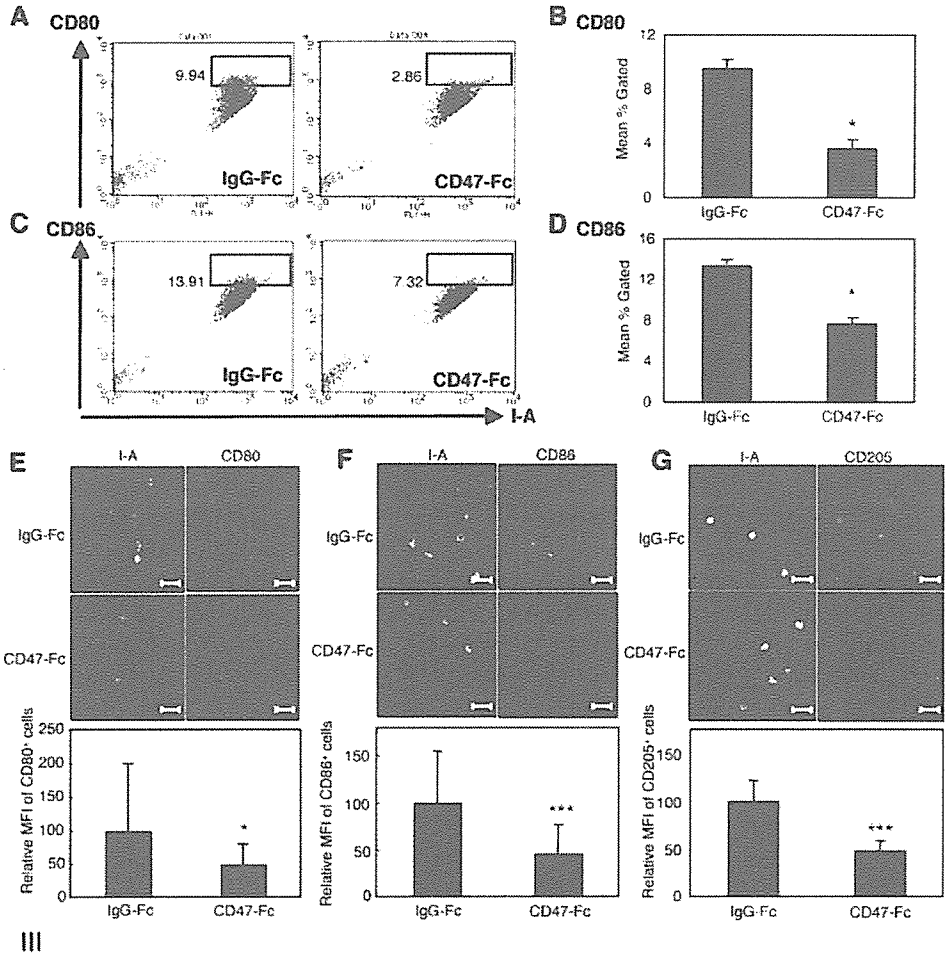
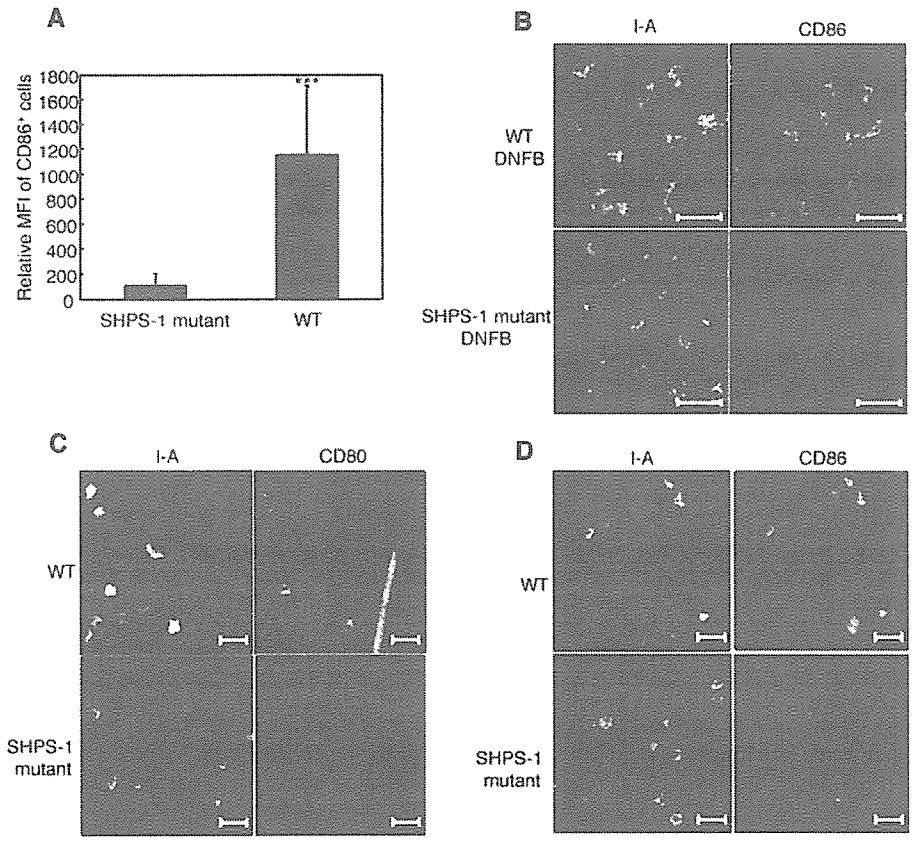


Figure 3. The ligation of SHPS-1 inhibits LC maturation induced by epidermal explant culture. The epidermal sheets were separated from the dermis by incubation of the dorsal halves of the ears in dispase. The epidermal sheets were cultured in 24-well tissue culture plates in 1.5 mL culture medium for 24 or 48 h at 37°C. (A, C) Cells that had emigrated into the culture medium during 48 h were evaluated by FACS. The emigrated cells typically expressed both I-A and CD80 or CD86. The addition of CD47-Fc protein into the medium obviously reduced the percentage of I-A$^+$ CD80^{high+} or I-A$^+$ CD86^{high+} emigrated cells, respectively. (B, D) Mean values of the percentage of I-A$^+$ CD80^{high+} or I-A$^+$ CD86^{high+} emigrated cells from three experiments are shown. Bars indicate SD. $^*p < 0.05$ (E, F, G) The epidermal sheets were collected after 24 h incubation and were stained for both I-A and either CD80, CD86 or CD205. The immunofluorescence analysis of epidermal sheets revealed that remaining I-A$^+$ cells in the epidermal sheets expressed CD80, CD86 and CD205, whereas additional treatment with CD47-Fc protein significantly inhibited the up-regulation of the expression. Bars in the photographs indicate 50 μm. $^*p < 0.05$, $^{***}p < 0.001$.

culture medium following epidermal explant culture expressed I-A, CD80 and CD86, indicating that the emigrated LC are in the maturation process. Ligation of SHPS-1 with CD47-Fc protein partially but significantly reduced the percentages of I-A$^+$ CD80^{high+} and I-A$^+$ CD86^{high+} cells in the emigrated LC population. The inhibitory effects of SHPS-1 ligation on maturation seem to be stronger in epidermal residing LC than emigrated cells. As it has been shown that binding of E-cadherin suppresses LC-like DC maturation [17], the reason why epidermal LC did not well maturate as compared to emigrated LC may be attributed to the homophilic binding of E-cadherin between LC and keratinocytes in the epidermis. We assume that ligation of SHPS-1 may thus cooperate with E-cadherin signals and greatly suppress LC maturation in the epidermis.

We further showed that the phenotypic maturation of LC by a hapten and *ex vivo* explant culture is suppressed in SHPS-1 mutant mice, which lack most of the intracellular domain of SHPS-1. Four tyrosine residues in the intracellular domain of SHPS-1 are believed to play a critical role in the transmission of the signals [20]. Phosphorylated tyrosine residues of SHPS-1 recruit the tyrosine phosphatases SHP-1 and SHP-2, which transmit the signals thereafter. The mutant protein in SHPS-1 mutant mice is not tyrosine phosphorylated, nor does it form a complex with SHP-1 or SHP-2 [40]. The SHPS-1 mutant mice have several characteristic features in the dysfunction of LC/DC and macrophages, including the motility of LC and the phagocytosis of macrophages. The finding that maturation of LC is attenuated in SHPS-1 mutant mice suggests that SHPS-1 is a prerequisite molecule for the LC functions including maturation.



IV

Figure 4. LC maturation induced by DNFB or by epidermal explant culture is attenuated in SHPS-1 mutant mice. SHPS-1 mutant mice and wild-type mice were painted with 0.5% DNFB on the ear and after 24 h the epidermal samples were analyzed for expression of I-A$^+$ or CD86$^+$ as described in legend of Fig. 1. (A) In wild-type mice, the MFI of CD86 expression increased after application of DNFB, whereas in SHPS-1 mutant mice, the up-regulation of CD86 expression was significantly suppressed. The *** indicates statistical significance compared with SHPS-1 mutant mice ($p < 0.001$, $n = 4$). (B) Representative images are shown. (C, D) Epidermal explants from wild-type mice and from SHPS-1 mutant mice were incubated for 24 h and were stained for I-A, CD80 or CD86. The up-regulation of CD80 (C) and CD86 (D) were obviously inhibited in SHPS-1 mutant mice compared with wild-type mice. Bars indicate 50 μm.

Especially, the phosphorylation of those four tyrosine residues in the intracellular domain of SHPS-1 and the consequent signal transduction is critical for LC maturation. Similarly, engagement of SHPS-1 has been shown to dephosphorylate the tyrosine residues in the intracellular domain of SHPS-1, resulting in the dissociation of SHP-2 [35]. In conjunction with the finding that ligation of SHPS-1 attenuates the maturation of LC, we assume that the maturation of LC requires the signal transduction *via* the phosphorylation of these tyrosine residues. This is further supported by the observation that the TNF-α-induced up-regulation of I-A expression by LC is attenuated by the ligation of SHPS-1, because it has been reported that TNF- α induces both the maturation of DC and the phosphorylation of tyrosine residues of SHPS-1 and the consequent recruitment of SHP-2 [10, 17].

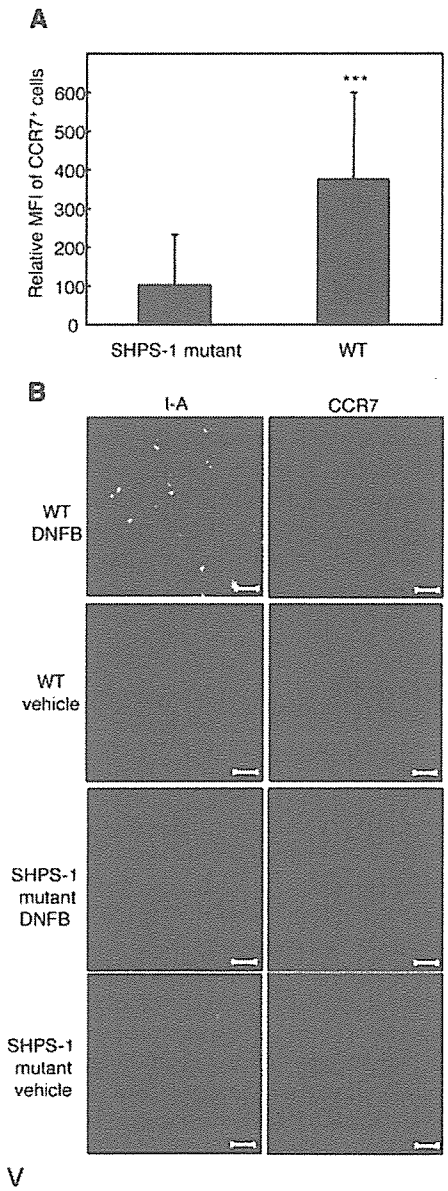


Figure 5. Up-regulation of CCR7 expression on LC induced by DNFB is attenuated in SHPS-1 mutant mice. (A) SHPS-1 mutant mice and wild-type mice were painted with 0.5% DNFB and after 24 h the ears were removed for analysis of I-A$^+$ or CCR7$^+$ LC. In wild-type mice, the MFI of CCR7 expression increased after application of DNFB, whereas in SHPS-1 mutant mice the up-regulation of CCR7 expression was significantly suppressed. The *** indicates statistical significance compared with SHPS-1 mutant mice (p <0.001, n = 6). (B) Representative images are shown. Bars indicate 20 μm.

We previously reported that the motility of LC is attenuated in wild-type mice by the engagement of SHPS-1 and in SHPS-1 mutant mice [32]. As immature DC, following induction of maturation after stimulation by inflammatory cytokines or haptens, up-regulate CCR7 [39, 41, 42], our next question was whether or not the expression of CCR7 on LC is impaired during the maturational changes following hapten application to SHPS-1 mutant mice. We found that expression of CCR7 on LC is suppressed following DNFB application in SHPS-1 mutant mice. Because expression of CCR7 on LC and its ligands, macrophage inflammatory protein-3β/CCL19 or a secondary lymphoid chemokine/CCL21 in lymph vessels and draining lymph nodes, is critical for the migration of LC from the epidermis into draining lymph nodes [38, 39], it is conceivable that the suppression of LC motility in SHPS-1 mutant mice might be attributed, at least in part, to the attenuated induction of CCR7. Because SHPS-1 is known to be involved in cell motility and adhesion, the inhibition of LC motility could be influenced both by the down-regulation of CCR7 expression and by the attenuated cell motility in SHPS-1 mutant mice.

We confirmed that the CHS response is suppressed in mice treated with SHPS-1 ligation before sensitization and in SHPS-1 mutant mice. Recently, three different groups reported three distinct results of CHS response by transient depletion of LC using mice that express diphtheria toxin receptor on LC; that is, (i) diminished (but not abrogated) [43], (ii) enhanced [44], and (iii) unchanged CHS response [45]. Thus, LC may not be the only cells that are required for the induction of CHS. Although we did not examine, it is plausible to consider that SHPS-1 ligation and its deficiency may affect maturation and motility of dermal DC, which may result in the diminished CHS response. The observation that the engagement of SHPS-1 inhibited the CHS response during the sensitization phase but not the elicitation phase suggests that the maturation and migration of LC/DC are possibly important for priming T cells but not for local inflammatory responses induced by antigen specific T cells. It has been shown that CD80 and CD86 play critical roles in the initiation of primary immune responses in the skin, in alloreactive immune responses and in antigen-specific T cell activation [34,

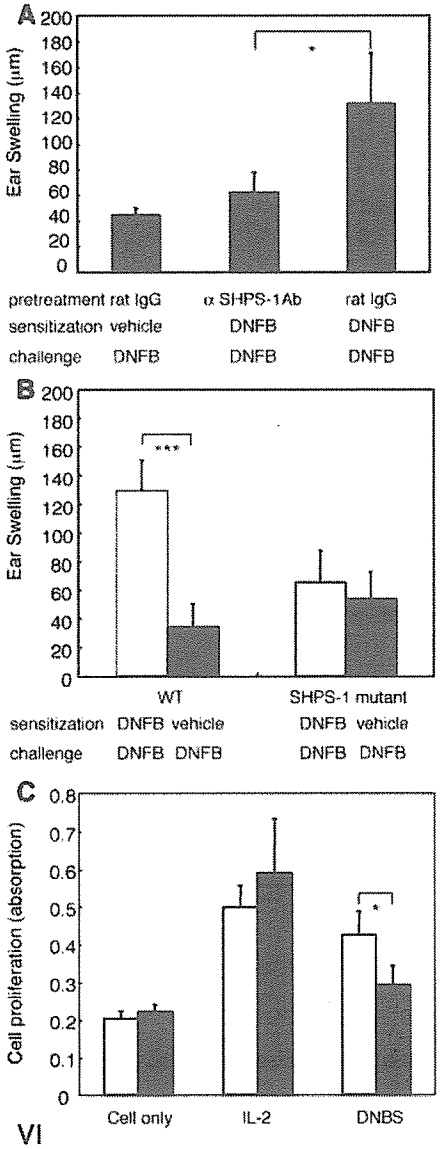


Figure 6. CHS responses are suppressed in mice treated with anti-SHPS-1 mAb before sensitization and in SHPS-1 mutant mice. The induction of the CHS reaction was conducted as described in the *Materials and methods*. CHS responses are expressed as the average increase of ear swelling with error bars representing SD for each group of six mice. (A) The CHS response to DNFB was significantly diminished by the treatment of anti-SHPS-1 mAb before sensitization compared with treatment of rat IgG. The * indicates statistical significance compared with the treatment of rat IgG and DNFB sensitization ($p < 0.05$, $n = 6$). (B) The CHS response to DNFB drastically decreased in SHPS-1 mutant mice compared with wild-type mice. The *** indicates statistical significance compared with the treatment of vehicle sensitization and DNFB challenge ($p < 0.001$, $n = 6$). (C) Five days after sensitization with DNFB, regional lymph nodes of the mice were collected and cultured with DNBS or IL-2, and cell proliferation was assessed as described in *Materials and methods*. Mean values of cell proliferation by DNBS were significantly impaired in SHPS-1 mutant mice, while those by IL-2 (50 U/mL) were unchanged. Representative data are shown from two separate experiments. The * indicates statistical significance compared with the SHPS-1 mutant mice and wild-type mice ($p < 0.05$, $n = 3$).

46]. We showed in this study that the expression of CD80 and CD86 was suppressed by SHPS-1 ligation. Thus, it is suggested that SHPS-1 plays important roles in the induction of both maturation and motility of LC and possibly of DC, which could be essential in the sensitization phase of CHS.

CD47, an SHPS-1 ligand, is widely expressed by a variety of cell types, including lymphocytes and endothelial cells [47]. Because keratinocytes express CD47 [35], maturation and migration might be negatively regulated by engagement with this molecule on cells surrounding LC *in situ*. We recently reported that LC also express CD47 of which ligation negatively regulates the maturation and motility of LC [35]. Thus, ligation of both SHPS-1 and CD47 on LC suppresses LC function, inhibiting priming T cells and consequent establishment of the immune response. It is possible that SHPS-1 and CD47 on LC bind each other or in a *cis* fashion.■ check sentence &block, So far, it is unclear whether the expression of SHPS-1 and CD47 is down-regulated during the maturational change and this is to be further studied.

Materials and methods

Mice

The generation of mutant mice that lack most of the cytoplasmic region of SHPS-1 has been previously described [48]. Mice were bred and maintained in the Institute of Experimental Animal Research of Gunma University or Kobe University Graduate School of Medicine under specific pathogen-free conditions. The mice were backcrossed onto the C57BL/6 background over five generations. Genotyping of the mice was performed by PCR analysis as previously reported [48]. Female C57BL/6 mice (6- to 8-week-old) were purchased from Charles River Japan (Tokyo, Japan). All animals were maintained in microisolator cages and exposed to a 12 h light/12 h dark cycle, with standard feed and water *ad libitum*. All animal experiments were conducted according to the Guidelines for Animal Experimentation at Kobe University Graduate School of Medicine or Gunma University.

Antibodies

Ascites were collected from BALB/c nu/nu mice that had been injected i.p. with the hybridoma producing rat anti-mouse-SHPS-1 mAb (p84) [49]. The p84 antibody was purified from the ascites using a protein A column. FITC- or PE-conjugated mouse anti-mouse I-A^b mAb (AF6–120.1) were purchased from BD Bioscience (Tokyo, Japan). PE-conjugated rat anti-mouse

CD80 mAb (RMMP2) and CD86 mAb (RMMP1) were purchased from Immunotech (Marseille, France). Rat anti-CD205 mAb was from UK-Serotec (Oxford, UK). Rat IgG was obtained from Sigma (St. Louis, MO). Goat polyclonal anti-CCR7 Ab and PE-conjugated donkey anti-goat IgG $F(ab')_2$ were purchased from Abcam (Cambridge, UK) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. CD47-Fc fusion protein was generated as previously described [50]. Briefly, CHO-Ras cells were transfected with pTracerCMV-hCD47-Fc. The CD47-Fc protein was then purified from the culture supernatant by column chromatography on Protein G HP (Amersham Pharmacia Biotech, Uppsala, Sweden). Human IgG-Fc fragment was obtained from Jackson Immunoresearch (West Grove, PA).

Preparation of murine epidermal sheets and immunofluorescence analysis

Murine epidermal sheets were prepared as previously described [32]. After fixation, the sheets were simultaneously incubated at room temperature for 30 min with FITC-conjugated mouse anti-mouse I-A^b mAb and PE-conjugated rat anti-mouse CD86 mAb, each diluted 1:100 in 5% BSA/PBS. In other experiments, the sheets were incubated at room temperature for 60 min with anti-mouse CCR7 Ab, washed with PBS, and thereafter was simultaneously incubated for 30 min with FITC-conjugated anti-mouse I-A^b mAb and PE-conjugated donkey anti-goat IgG $F(ab')_2$, each diluted 1:100 in 5% BSA/PBS. Finally, the sheets were washed with PBS and mounted on microscope slides in PermaFluor (Shandon, Pittsburgh, PA). The samples were analyzed using an Olympus Fluoview confocal laser scanning microscope (Olympus, Nagano, Japan). All specimens were scanned using the same emitting conditions. The relative MFI of I-A^b, CD86 or CCR7 were counted per one I-A^{b+} cell using NIH image. Similar experiments were made with SHPS-1 mutant mice.

LC maturation induced by DNFB application

Mice were painted with 10 μL 0.5% DNFB solubilized in acetone/olive oil (4/1) on both the dorsal and the ventral ear halves. Twenty-four hours later, the ears were collected for staining of LC as described above. To investigate the *in vivo* effects of anti-SHPS-1 mAb (p84), anti-SHPS-1 mAb or control rat IgG was injected intradermally at a dose of 60 μg/ear just before the DNFB application. Similarly, CD47-Fc protein or control human IgG-Fc was injected intradermally at 60 μg/ear just before the DNFB application.

LC maturation induced by TNF-α

Groups of mice (n = 5) received 30 μL intradermal injections of mouse rTNF-α (Sigma) (50 ng/ear) or an equivalent volume of carrier protein (0.1% bovine serum albumin) into the ear pinnae using 30-gauge stainless steel needles. Anti-SHPS-1 mAb or control rat IgG was injected intradermally at a dose of 60 μg/ear simultaneously. One h after the injection, the ears were collected for staining of LC as described above.

Skin organ culture

The epidermal sheets were prepared as previously described [32]. They were cultured in 24-well tissue culture plates in 1.5 mL culture medium (10% FCS, RPMI1640 with 20 μg/mL CD47-Fc protein or control human IgG-Fc) for 24 or 48 h at 37°C. At least six explants were cultured for each experimental condition. Cells that had emigrated from the epidermal skin explants into the culture medium during 48 h were evaluated phenotypically using a FACSCalibur flow cytometer and CELL QUEST (Becton Dickinson, San Jose, CA). In detail, cells that had emigrated were collected, washed in 1% BSA/PBS, and then were simultaneously incubated on ice for 30 min with FITC-conjugated mouse anti-mouse I-A^b mAb and PE-conjugated rat anti-mouse CD80 or CD86 mAb, each diluted 1:100 in 1% BSA/PBS. After a final washing, each sample was analyzed using a FACSCalibur flow cytometer and CELL QUEST. In another experiment, after 24 h the epidermal sheets were collected and fixed in acetone for 3 to 5 min at –20°C. After fixation, the sheets were washed in PBS and then were simultaneously incubated at room temperature for 30 min with FITC-conjugated mouse anti-mouse I-A^b mAb and PE-conjugated rat anti-mouse CD80, CD86 or CD205 mAb, each diluted 1:100 in 5% BSA/PBS. After washing with PBS, the sheets were mounted on microscope slides in PermaFluor. The samples were analyzed as described above.

CHS response and cell proliferation assay

Mice were sensitized by applying 25 μL DNFB solution (0.5% in acetone/olive oil, 4/1) on their shaved abdomens on day 0. On day 5, 10 μL 0.2% DNFB was applied to the dorsal and ventral aspects of the right ear, and the vehicle (acetone/olive oil) was applied to the left ear.

To investigate the *in vivo* effects of anti-SHPS-1 mAb, rat anti-SHPS-1 mAb or control rat IgG were injected intradermally in the right ear at a dose of 60 μg just before the DNFB application and 10 μL 1% DNFB or the vehicle (acetone/olive oil) was applied to the dorsal and ventral aspects of the right ear on day 0. On day 5, 10 μL 0.2% DNFB was applied to the dorsal and ventral aspects of the left ear. Ear swelling was measured in a blinded fashion with a digimatic micrometer (Mitutoyo, Kawasaki, Japan) 24 h after the challenge.

For cell proliferation assay, the regional lymph node cells were collected 5 days after DNFB sensitization. The cells (3×10^5) in 96-well plates were cultured with DNBS (Sigma, 30 μg/mL) in complete RPMI (RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10 mM HEPES, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 5×10^{-5} M 2-ME (Nacalai Tesque, Tokyo, Japan), and 1% penicillin/streptomycin/ amphotericin B (Bio-Whittaker., Walkersville, MD)) for 3 days. For the last 4 h, each well was pulsed with MTS reagent [CellTiter^{96} Aqueous One Solution Proliferation assay kit (Promega, Madison, WI)] and the proliferation activity of each well was measured using an ELISA reader (Nippon Bio-Rad Laboratories). Simultaneously, the supernatants after three days culture were collected and assessed the contents of IFN-γ using mouse IFN-γ ELISA kit (R&D System, Minneapolis, USA).

Statistical analysis

The statistical significance of differences between the means was determined using Student's *t*-test. Differences are considered statistically significant at $p < 0.05$. Each experiment was performed at least two times.

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Isolation and Expression Profiling of Genes Upregulated in Bone Marrow-Derived Mononuclear Cells of Rheumatoid Arthritis Patients

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Abstract

We have comprehensively identified the genes whose expressions are augmented in bone marrow-derived mononuclear cells (BMMC) from patients with Rheumatoid Arthritis (RA) as compared with BMMCs from Osteoarthritis (OA) patients, and named them *AURA* after augmented in RA. Both stepwise subtractive hybridization and microarray analyses were used to identify *AURA* genes, which were confirmed by northern blot analysis and/or reverse transcription polymerase chain reaction (RT–PCR). We also assessed their expression levels in individual patients by quantitative real-time RT–PCR. Of 103 *AURA* genes we have identified, the mRNA levels of the following 10 genes, which are somehow related to immune responses, were increased in many of the RA patients: *AREG* (=*AURA9*), FK506-binding protein 5 (FKBP5 = *AURA45*), C-type lectin superfamily member 9 (*CLECSF9* = *AURA24*), tyrosylprotein sulfotransferase 1 (*TPST1* = *AURA52*), lymphocyte G0/G1 switch gene (*G0S2* = *AURA8*), chemokine receptor 4 (CXCR4 = *AURA86*), nuclear factor-kappa B (NF-κB = *AURA25*) and two genes of unknown function (FLJ11106 = *AURA1*, BC022398 = *AURA2* and XM_058513 = *AURA17*). Since *AREG* was most significantly increased in many of the RA patients, we subjected it to further analysis and found that AREG-epidermal growth factor receptor signaling is highly activated in synovial cells isolated from RA patients, but not in OA synoviocytes. We propose that the expression profiling of these *AURA* genes may improve our understanding of the pathogenesis of RA.

Key words: stepwise subtraction; microarray; RA; OA; amphiregulin; synoviolin

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by arthritis that predominantly results in chronic inflammation of systemic joints associated with the overgrowth of synovial cells. This induces progressive cartilage and bone destruction in the joint and subsequent disability. Since RA pathogenesis is likely to involve genetic elements, a number of groups have subjected samples from healthy and affected individuals to DNA microarray analyses for a broad-scale comparison. These studies have provided

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