Development of a chimaeric receptor approach to study signalling by tumour necrosis factor receptor family members

Duorong XU^{*†}, Zhenqi SHI^{*}, Jay McDONALD^{*‡}, George PAN^{*}, Xuemei CAO^{*}, Xueqing YU[†] and Xu FENG^{*1}

^{*}Department of Pathology, University of Alabama at Birmingham, 1670 University Blvd., VH G046B, Birmingham, AL 35294, U.S.A., [†]Department of Nephrology, the First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong Province 510080, People's Republic of China, and [‡]Veterans Administration Medical Center, Birmingham, AL 35233, U.S.A.

Members of the tumour necrosis factor receptor family play a pivotal role in cell differentiation, function and apoptosis. However, signalling by many members of the family remains to be elucidated. In the present study, we developed a chimaeric receptor approach for studying signalling by receptors belonging to this family. The chimaeric receptor comprises the human Fas external domain linked to the transmembrane and cytoplasmic domains of a tumour necrosis factor receptor family member of interest. When the chimaera is expressed in mouse cells, the clustering of the chimaera induced by a human Fas-activating antibody activates the intracellular domain of the chimaera with-

out affecting its endogenous counterpart. Since the antibody recognizes only human Fas, this approach can be used to dissect signalling by any tumour necrosis factor family member using any type of mouse cell including those endogenously expressing Fas. Moreover, we also showed that the chimaeric receptor approach can be used to study signalling at any stage of cell differentiation or function.

Key words: chimaeric receptor, human fas-activating antibody, intracellular signalling, receptor, tumour necrosis factor receptor.

INTRODUCTION

The TNF (tumour necrosis factor) family has been recognized as one of the most important groups of proteins that regulate a variety of biological processes such as host defence, inflammation, autoimmunity, apoptosis and organogenesis [1]. The TNF family comprises about 18 members, which exert diverse functions by binding and activating their receptors belonging to the TNFR (TNF receptor) family [1]. Most members of the TNFR family are type I transmembrane proteins that are characterized by lack of intrinsic enzymic activity and thus initiate intracellular signalling primarily by recruiting various adaptor proteins through functional motifs in their cytoplasmic domains [2,3]. The identification and characterization of the functional motifs in the cytoplasmic domains of the receptors represent an initial and crucial step in elucidating intracellular signalling pathways initiated by the receptors, especially for those newly identified.

A widely used strategy for dissecting the functional motifs of membrane-bound receptors involves expressing mutated forms of a receptor in cell types that do not endogenously express this receptor and then examining how the mutants regulate the differentiation and/or function of the cells. However, despite its popularity, this strategy has a serious drawback. If a cell type derived from a normal tissue does not endogenously express a protein, this protein must not play a role in the function of the cell. Consequently, studies using this strategy may not produce biologically relevant results. This has been proved previously to be a serious concern in the study of the signalling by the membrane-bound kinase receptor, c-fms [4]. Thus it is of paramount importance to study protein function in physiologically relevant cell types, namely the cells in which a protein of interest is not only endogenously expressed but in which the protein also plays a functional role. In principle, this may be achieved by expressing mutated forms of a protein in physiologically relevant cells in which the endogenous

receptor has been deleted. Often, such cells may be isolated from knockout mice lacking the gene for the receptor. However, in reality, it is not always achievable. First, knockout mice for many newly identified genes may not be available. Secondly, knockout for certain genes may be embryonically lethal.

In the present study, we developed a chimaeric receptor strategy for identifying functional domains of the TNFR family members using physiologically relevant mouse cells. This approach takes advantage of a commercially available Fas-activating antibody that specifically recognizes human Fas but not its mouse counterpart. The chimaeric receptor comprises the human Fas external domain linked to the transmembrane and intracellular domains of another TNFR family member. When the chimaeric receptor is expressed in mouse cells, the antibody activates the chimaeric receptor without affecting the endogenous receptor. To validate this experimental approach, we engineered a chimaeric receptor consisting of the human Fas external domain linked to the transmembrane and cytoplasmic domains of mouse RANK (receptor activator of nuclear factor κ B), a TNFR family member involved in osteoclast formation and function [5]. Our results demonstrated that this chimaeric receptor is capable of stimulating osteoclast formation and function on the ligation by the Fas-activating antibody using primary mouse osteoclast precursors. We also showed that the chimaeric receptor approach has a unique feature that permits the investigation of cell signalling at any stage of cell differentiation or function.

MATERIALS AND METHODS

Construction of human Fas/mouse RANK chimaeric cDNA

Using the standard molecular cloning techniques, we engineered a chimaeric cDNA comprising human Fas external domain (from

Abbreviations used: BMM, bone-marrow macrophages; Epo-R, erythropoietin receptor; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; I κ B, inhibitory κ B; JNK, c-Jun N-terminal kinase; M-CSF, monocyte/macrophage colony-stimulating factor; α -MEM, α -minimum essential medium; RANK, receptor activator of nuclear factor κ B; RANKL, RANK ligand; TNF, tumour necrosis factor; TNFR, TNF receptor; TRAP, tartrate-resistant acid phosphatase.

¹ To whom correspondence should be addressed (email xfeng@path.uab.edu).

amino acids 1–173) linked in frame with the transmembrane and cytoplasmic domains of mouse RANK (amino acids 210–625). The cDNA fragment encoding a human Fas external domain was amplified by PCR using a human Fas cDNA clone and the following pair of primers: a forward primer (5'-TCCTCTAGAG-CCCTGGCTGCCAGGCGGAGCTGCCTCTTATT-3') containing an *Xba*I site (underlined) at its 5'-end and a reverse primer (5'-CTTACTAGTGTAGATCTGGATCCTTCCTCTTTGCACTTGGT-3') containing an *Spe*I site (underlined) at its 5'-end. The Fas cDNA fragment was subcloned into pBluescript II SK+ vector (Stratagene, La Jolla, CA, U.S.A.) between *Xba*I and *Spe*I sites to generate SK-hFas. cDNA encoding the RANK transmembrane and cytoplasmic domains was amplified by reverse transcriptase-PCR using total RNA from mouse BMMs (bone-marrow macrophages) with a forward primer (5'-TACACTAGTGCTTACCTGCCAGTCTCATCGTTCTGCTC-3') containing *Spe*I site (underlined) at its 5'-end and a reverse primer (5'-TACGGATCCAGTTGCGCACTGCGCCCCACCCTGCTCCTGCACCGGCCG-3') containing *Bam*HI site (underlined) at its 5'-end. The RANK cDNA fragment was subcloned into SK-hFas between *Spe*I and *Bam*HI to give rise to SK-hFas-mRANK. The orientation and sequence of the chimaeric cDNA was confirmed by sequencing.

Preparation of retrovirus encoding the chimaeric receptors

Retrovirus encoding the chimaera was prepared using pMX-puro vector [6] and the Plat-E packaging cells [7]. The chimaeric cDNA from SK-hFas-mRANK was subcloned into pMX-puro to generate pMX-puro-hFas-mRANK. Plat-E cells were cultured in Dulbecco's modified Eagle's medium with 10 % (v/v) heat-inactivated FBS (fetal bovine serum) supplemented with puromycin and blasticidin as described previously [7,8]. pMX-puro-hFas-mRANK was transiently transfected into Plat-E cells using LIPOFECTAMINE™ Plus reagent (Invitrogen, Carlsbad, CA, U.S.A.). Virus supernatant was collected at 48, 72 and 96 h after transient transfection.

Preparation and infection of BMMs

Bone-marrow cells were isolated from long bones of 4–8-week old C3H mice (Harlan Industries, Indianapolis, IN, U.S.A.) as described previously [9]. BMMs were prepared by culturing bone-marrow cells in α -MEM (α -minimum essential medium) containing 10 % heat-inactivated FBS in the presence of 0.1 vol. of culture supernatant of M-CSF (monocyte/macrophage colony-stimulating factor)-producing cells for 2 days as described in [10]. Cells were then infected with retrovirus for 24 h in the presence of 0.1 vol. of culture supernatant of M-CSF-producing cells and 8 μ g/ml polybrene. Infected cells were selected by treating the cell culture with 2 μ g/ml puromycin for 2–3 days. Selected cells were then used for various studies.

In vitro osteoclast formation assays and bone resorption assays

Infected BMMs were cultured in 24-well tissue culture plates (1×10^5 cells/well) in α -MEM containing 10 % heat-inactivated FBS in the presence of 0.01 vol. of culture supernatant of M-CSF-producing cells and 100 ng of GST-RANKL (glutathione S-transferase-RANK ligand) [11]. In osteoclastogenesis assays involving the use of the chimaeric receptor, human Fas-activating antibody (Upstate Biotechnology, Lake Placid, NY, U.S.A.) was added at concentrations as indicated in individual assays. Osteoclasts were stained for TRAP (tartrate-resistant acid phosphatase) activity using a commercial kit (387-A; Sigma). Bone resorption assays were performed by generating osteoclasts on whale dentin slices from infected or uninfected BMMs as

described above. Dentin slices were harvested 4–5 days after the osteoclasts were formed. Cells were removed from the dentin slices with 0.25 M ammonium hydroxide through mechanical agitation. Dentin slices were then subjected to scanning electron microscopy as described in [12].

Flow cytometric analysis

Infected BMMs (up to 1×10^6 cells) were suspended in 200 μ l of PBS/azide. Cells were then blocked with 1 μ g of 2.4G2 antibody [13] for 30 min on ice. Under dim light, 20 μ l of anti-human Fas antibody conjugated with phycoerythrin (sc-21730PE; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or control IgG was added to the cell suspension and cells were incubated on ice for 30 min. Cells were washed twice with 1 ml cold PBS/azide and resuspended in 300 μ l cold PBS/azide. Cold 0.5 % of paraformaldehyde solution (200 μ l) was added to fix the cells. Flow cytometric analysis was performed using a Becton-Dickinson FACScan (Becton-Dickinson Immunocytometry Systems, Mountain View, CA, U.S.A.).

Western-blot analysis

Infected or uninfected BMMs were cultured in serum-free α -MEM in the absence of M-CSF for 16 h before treatment with RANKL or Fas-activating antibody. Cells were washed twice with ice-cold PBS and then lysed in a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na_3VO_4 , 1 mM NaF, and $1 \times$ protease inhibitor cocktail 1 (P-2850; Sigma) and $1 \times$ protease inhibitor cocktail 2 (P-5726; Sigma). Western-blot analysis was performed largely as described in [14]. Cell lysates (40 μ g) were boiled in the presence of SDS sample buffer [0.5 M Tris/HCl, pH 6.8, 10 % (w/v) SDS, 10 % (v/v) glycerol and 0.05 % (w/v) Bromophenol Blue] for 5 min and loaded on to SDS/10 % polyacrylamide gel for electrophoresis. Proteins were transferred on to nitrocellulose membranes (catalogue no. 162-0147) from Bio-Rad (Hercules, CA, U.S.A.) using a semi-dry blotter (Bio-Rad). Membranes were blocked in blocking solution [5 % (v/v) non-fat dried milk in TBS (Tris-buffered saline) containing 0.1 % Tween 20] for 1 h to prevent non-specific binding and then washed three times with TBS-T (TBS containing 0.1 % Tween 20). Membranes were incubated with primary antibodies in TBS-T containing 5 % bovine albumin (Sigma; catalogue no. A-7030) overnight at 4 °C. Next day, membranes were washed three times with TBS-T and incubated with secondary antibody in TBS-T containing 5 % non-fat dried milk for 1 h. Membranes were washed extensively and chemiluminescence detection assay was performed using SuperSignal West Dura kit from Pierce (Rockford, IL, U.S.A.).

RESULTS

Construction of a chimaeric receptor containing the human Fas external domain for studying signalling by TNFR family members

Although many members of the TNFR family are believed to be activated by ligand-induced trimerization [1], recent results indicated that some TNFR family members, including Fas, are self-assembled as trimers before ligand binding and the activation of the preassembled receptors is triggered by the ligand-induced rearrangement of the assembled trimers [15,16] (Figure 1A). Interestingly, the binding of human Fas by an antibody recognizing the human Fas extracellular domain is also capable of activating Fas [17,18]. Thus we hypothesized that a chimaeric receptor consisting of the human Fas external domain linked to

Figure 1 Signalling by a TNFR family member and a chimaeric receptor

(A) Model of signalling by a TNFR family member. Ligand, a TNF family member; Receptor, a TNFR family member. (B) A chimaeric receptor comprising the human Fas external domain linked to the transmembrane and cytoplasmic domains of a TNFR family member. (C) Model of signalling by the chimaeric receptor on mouse cells. Fas-AB, Fas-activating antibody.

the transmembrane and intracellular domains of a TNFR family member may be used to dissect signalling by this TNFR family member in mouse cells (Figure 1B). When this chimaeric receptor is expressed in physiologically relevant mouse cells that endogenously express the TNFR family member, Fas-activating antibody will activate the chimaeric receptor without affecting the endogenous receptor (Figure 1C). If this hypothesis is true, the functional motifs in the cytoplasmic domain of the TNFR family member may be identified by introducing deletion/point mutations in the cytoplasmic region of the chimaeric receptor.

To test our hypothesis, we engineered a chimaeric receptor comprising of human Fas external domain (amino acids 1–173) linked in frame to the transmembrane and cytoplasmic domains of mouse RANK (amino acids 210–625) (Figure 2A). RANK is a TNFR family member that plays an important role in the differentiation, survival and function of osteoclasts, the principal bone-resorbing cells [5]. Osteoclasts differentiate from mononuclear cells of monocyte/macrophage lineage [5]. In addition to RANKL, osteoclast differentiation also requires M-CSF [5]. BMMs are widely used primary osteoclast precursors [9].

To determine whether the chimaeric receptor is capable of mediating osteoclast differentiation using Fas-activating antibody

Figure 2 A chimaeric receptor consisting of the human Fas external domain linked to the transmembrane and cytoplasmic domains of mouse RANK is capable of mediating osteoclast formation using Fas-activating antibody as a surrogate

(A) Schematic diagram of the chimaeric receptor. hFas Ext domain, human Fas external domain; TM, mouse RANK transmembrane domain; RANK Cyto Domain, mouse RANK cytoplasmic domain; (B) Flow cytometric analysis. The surface expression of the chimaera on infected BMMs (Infected) was determined by flow cytometric analysis as described in the Materials and methods section. Uninfected BMMs (Uninfected) were used as control. (C) Osteoclastogenesis assay. Uninfected BMMs or BMMs infected with virus encoding the chimaeric receptor were treated with M-CSF (20 ng/ml) plus RANKL (100 ng/ml) or M-CSF (20 ng/ml) plus Fas-activating antibody (Fas-AB) (100 ng/ml). Cultures were stained for TRAP, a marker for osteoclasts.

as a surrogate of RANKL, we expressed the chimaeric receptor in BMMs by retroviral technology. Flow cytometric analysis with antibody against the external domain of human Fas demonstrated that the chimaera was expressed on the cell surface (Figure 2B). Then, we treated infected BMMs expressing the chimaera with M-CSF (20 ng/ml) plus RANKL (100 ng/ml) or M-CSF (20 ng/ml) plus human Fas-activating antibody (100 ng/ml) (Figure 2C, lower panel). The infected cells differentiated into large multinucleated TRAP positive osteoclasts in response to M-CSF and RANKL treatment, indicating that the endogenous RANK in the infected cells is still functional. Importantly, when infected BMMs were treated with M-CSF and the Fas-activating antibody, they also formed osteoclasts, confirming that the chimaeric receptor is working. As negative control, uninfected BMMs failed to form osteoclasts in response to M-CSF and Fas-activating antibody treatment (Figure 2C, upper panel). Moreover, our subsequent studies showed that the Fas-activating antibody promoted osteoclast differentiation in a dose-dependent manner

Figure 3 Human Fas antibody stimulates osteoclast formation in a dose-dependent manner

(A) Osteoclastogenesis assays. The assays were performed in 24-well plates as described in Figure 2(C). Each condition had four replicates (wells). A representative area of the cultures from each condition is shown. (B) Quantification of the data. The multinucleated TRAP-positive cells (> 3 nuclei) in four random view areas (at 100 $\times$ magnification) in each of the four replicates were counted. Results are representative of the averages of the 16 counts $\pm$ S.D.

(Figure 3A). Especially, Fas-activating antibody at a concentration as low as 1 ng/ml was still capable of mediating osteoclast formation and reached the maximal capacity at a concentration of 100 ng/ml (Figure 3B). To examine whether osteoclasts generated using the chimaera strategy are capable of resorbing bone, we performed bone resorption assays (Figure 4). Osteoclasts generated using the chimaera approach resorbed bone as efficiently as those formed using the endogenous RANK (Figure 4, resorption indicated by irregular pits), confirming that they are functional osteoclasts.

These results indicate that a chimaera comprising the human Fas external domain linked to the transmembrane and cytoplasmic domains of a TNFR family member is capable of activating signals that are normally activated by the endogenous receptor, using a human Fas-activating antibody as a surrogate of the biological ligand of the TNFR family member. Thus this chimaeric receptor can serve as a useful tool to dissect signalling initiated by any TNFR family member.

Chimaeric receptor approach possesses a unique feature that permits the study of cell signalling at any stage of cell differentiation or function

Chimaeric receptor approach may represent the best strategy for a structure–function study of a receptor belonging to the TNFR family when cells deficient in this receptor are not obtained. For instance, knockout mice lacking the receptor may be either embryonically lethal or have not yet been generated. Nonetheless, even in circumstances where such cells are available, the chimaeric receptor approach may still serve as a better choice. As shown in Figure 5(A), if cells lacking a receptor of interest are available, the dissection of functional domains of this receptor can be easily achieved by expressing the mutated forms of the receptor in the cells. However, some of the TNFR family members mediate not only cell differentiation, but also the survival, apoptosis and/or function of differentiated cells [1]. In such cases, if a motif plays an essential role in both cell differentiation and the

Figure 4 Osteoclasts generated through the chimaeric receptor are functional

Uninfected or infected BMMs plated on dentin slices were treated with M-CSF (20 ng/ml), M-CSF (20 ng/ml) plus RANKL (100 ng/ml) or M-CSF (20 ng/ml) plus FAS-activating antibody (Fas-AB) (100 ng/ml). Bone resorption assays were then performed as described in the Materials and methods section.

Figure 5 The chimaeric receptor possesses a unique feature

(A) Model of the use of knockout cells for a structure–function study of a receptor. (B) Model of how the chimaeric receptor may be used to study signalling at any stage of cell differentiation or function. hFas-receptor: a chimaeric receptor comprising of human Fas external domain linked to the transmembrane and cytoplasmic domains of a TNF family member. Fas-AB, Fas-activating antibody. Diff cells, differentiated cells.

survival/apoptosis/function of differentiated cells, the method using the cells lacking the receptor can only be used to elucidate its role in cell differentiation (Figure 5A). It will not be possible to know whether the same motif plays a role in the survival/apoptosis/function of differentiated cells, since deletion/muta-

tion of this motif will not give rise to differentiated cells for further study (Figure 5A).

In contrast, the chimaeric receptor has a unique feature that permits the study of signalling at any stage of cell differentiation or function. As shown in Figure 5(B), we hypothesized that the expression of a mutated chimaera in normal cells should not affect the expression of the endogenous receptor. To investigate whether a mutant mediates cell differentiation, the chimaera-expressing cells are treated with Fas-activating antibody. If the cells fail to differentiate in response to the Fas antibody treatment, the mutated motif/domain in the chimaera is essential for cell differentiation. To study further whether this motif/domain also plays a role in the survival/apoptosis/function of differentiated cells, the chimaera-expressing cells are first treated with its biological ligand (Ligand) to promote cell differentiation. On the completion of cell differentiation, the differentiated cells are treated with human Fas-activating antibody, which then activates the chimaeric receptor on the differentiated cells to reveal the role of the motif/domain in the survival/apoptosis/function of the differentiated cells (Figure 5B).

The feasibility of this feature critically depends on whether the chimaeric receptor can mediate the survival/apoptosis/function of the differentiated cells after the completion of cell differentiation. In the present study, we used the chimaeric receptor shown in Figure 2(A) to test this possibility, since RANK plays a key role in not only osteoclast differentiation but also in the function and survival of differentiated cells (mature osteoclasts) [5]. We treated BMMs expressing chimaera with M-CSF and RANKL. After osteoclasts were formed, we removed M-CSF and RANKL and added PBS as a negative control, RANKL as positive control or Fas-activating antibody (Figure 6). Most of the osteoclasts died 24 h later in the culture with PBS, whereas all cells in the culture with RANKL survived, confirming that RANKL is a potent factor supporting the survival of osteoclasts. Importantly, all cells in the culture treated with Fas-activating antibody also survived. A set of the cultures was maintained for 5 days (Figure 6, bottom panel). At the end of the 5-day period, virtually no cells survived in the culture with PBS, whereas approx. 50% of cells in the cultures treated with either RANKL or Fas-activating antibody survived. These results indicate that the chimaera was capable of regulating the survival of osteoclasts after the osteoclasts were formed. Thus the chimaeric receptor can be used to study signalling by a receptor at any stage of cell differentiation or function.

Chimaeric receptor activates signalling pathways that are normally activated by the endogenous receptor

Finally, we examined whether the chimaeric receptor is capable of initiating cell signalling activated by the endogenous RANK. It has been shown that RANK activates various intracellular signalling pathways, including nuclear factor κ B [19,20], JNK (c-Jun N-terminal kinase) [19,21], ERK (extracellular-signal-regulated kinase) [14,19] and p38 [14,22,23]. Activation of these signalling pathways was assessed by performing Western-blot analysis with antibodies against phosphorylated I κ B (inhibitory κ B), JNK, ERK or p38 as described previously [14]. As positive controls, we treated BMMs with RANKL for various times and then determined the activation of the four pathways in the RANKL-treated BMMs (Figures 7A–7D, lanes 1–3). Consistent with a previous study [14], RANKL activates all four pathways. Moreover, as negative controls, we also treated BMMs with human Fas-activating antibody for various times to examine whether the antibody can activate these signalling pathways in uninfected BMMs and our results indicated that the antibody failed to do so (Figures 7A–7D, lanes 4–6). When BMMs expressing the

Figure 6 The chimaeric receptor is capable of supporting the survival of mature osteoclasts

Infected BMMs were treated with M-CSF and RANKL for 4 days to form osteoclasts. After the formation of osteoclasts, the cultures were treated with PBS as negative control, RANKL as positive control or Fas-activating antibody (Fas-AB) for either 24 h or 5 days. The cultures were stained for TRAP.

chimaera were treated with Fas-activating antibody, all four signalling pathways were activated (Figures 7A–7D, lanes 7–9). These results further support that the chimaeric receptor approach is a useful tool to perform a structure–function study of members of the TNFR family in cell differentiation, survival, apoptosis and/or function.

DISCUSSION

Since its discovery in the early 1970s [24], the TNF family has emerged as one of the most important families of proteins that mediate diverse biological functions [1]. The TNF family members regulate biological functions through binding to membrane-bound receptors belonging to the TNFR family [1]. Members of the TNFR family lack intrinsic kinase activity and thus they initiate signalling by interacting intracellular-signalling molecules such as TRAF (TNF receptor associated factor), TRADD (TNFR-associated death domain) and FADD (Fas-associated death domain) [2,3]. As such, the structure–function study (e.g. identification of the functional motifs/domains in these receptors that mediate cellular functions) of the TNFR family members is an integral part of our effort to elucidate signalling mechanisms of these receptors. In the present study, we have developed a chimaeric receptor approach that can be used to perform a structure–function study of the TNFR family members.

Figure 7 The chimaeric receptor is capable of activating signalling pathways normally activated by RANK

Infected or uninfected BMMs were treated with RANKL or Fas-activating antibody (Fas-AB) for various time periods. Activation of $\text{I}\kappa\text{B}$ /nuclear factor κB , JNK, ERK and p38 pathways were assessed as phosphorylation of $\text{I}\kappa\text{B}$, JNK, ERK and p38 using Western-blot analysis with antibodies against phospho- $\text{I}\kappa\text{B}$ (p- $\text{I}\kappa\text{B}$), phospho-JNK (p-JNK), phospho-ERK (p-ERK) and phospho-p38 (p-p38). Immunoblots were stripped and then reprobed with antibodies against $\text{I}\kappa\text{B}$, JNK, ERK and p38 as loading controls. Min, minute. Phosphorylation of (A) $\text{I}\kappa\text{B}$, (B) JNK, (C) ERK and (D) p38.

Chimaeric receptors have been used previously to study signalling by membrane-bound receptors [8,25]. However, it has proved to be quite difficult to find an appropriate external domain for a chimaeric receptor. First, after the binding by its biological ligand, the external domain of a chimaeric receptor should be capable of activating the cytoplasmic domain of the chimaeric receptor to initiate intracellular signalling. Secondly, the receptor from which the external domain is derived must not be expressed in cells used for the study to prevent unwanted signalling from the receptor, which will often complicate the interpretation of the results. For instance, we previously constructed a chimaeric receptor consisting of the external domain of Epo-R (erythropoietin receptor) linked to the transmembrane and cytoplasmic domains of c-fms (the receptor for M-CSF) to study M-CSF-induced signalling in proliferation and differentiation of osteoclast precursors (BMMs) [8]. Given that c-fms is activated by M-CSF-induced homodimerization, we had to find an external domain that dimerizes on binding by its biological ligand. After testing several domains, we eventually chose to use the external domain of Epo-R because (i) after binding by its ligand erythropoietin,

Epo-R will dimerize and (ii) the cells under study (BMMs) do not express Epo-R.

Recently, we attempted to create a chimaeric receptor to study signalling by RANK, another important receptor involved in osteoclast differentiation and function. We initially used the mouse TNFR1 (tumour necrosis factor receptor 1) external domain (W. Liu and X. Feng, unpublished work). However, the use of the chimaeric receptor containing the TNFR1 external domain has a drawback since many cell types, including BMMs, express TNFR1 and/or TNFR2 (TNF α binds to both TNFR1 and TNFR2). Although this chimaeric receptor may be used to study RANK signalling using BMMs from TNFR1^{-/-} and TNFR2^{-/-} double knockout mice, it will not work in established macrophage cell lines such as RAW 264.7, which is a RANKL-responsive osteoclast precursor cell line [14]. Primarily on the basis of the availability (commercially) of a Fas-activating antibody that recognizes human Fas but not its mouse counterpart, we improved our approach by using the human Fas external domain. Since the Fas-activating antibody recognizes only human Fas, the chimaeric receptor comprising human Fas external domain is superior in that it can be used to perform a structure–function study of any TNFR family member using any physiologically relevant cell type of mouse origin, including primary cells isolated from mice and established mouse cell lines. Moreover, this approach has a unique feature that makes it possible to perform a structure–function study of a TNFR family member at any stage of cell differentiation or function. Our studies have demonstrated the feasibility of this feature. Thus this chimaeric receptor approach can be used to study signalling by a member of the TNFR family at a cellular stage probably not studied before.

We report our findings to advocate the further application of this innovative approach to the study of signalling by other TNFR family members. We are currently creating multiple cloning sites between *SpeI* and *BamHI* in the construct shown in Figure 2(A) to facilitate the cloning of cDNA fragments encoding the trans-membrane and cytoplasmic domains of other TNFR family members. The constructs will be available to investigators who want to use this approach to study signalling by a receptor of interest.

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