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Molecular cloning, expression analysis and assignment of the porcine tumor necrosis factor superfamily member 10 gene *(TNFSF10)* to SSC13q34→q36 by fluorescence in situ hybridization and radiation hybrid mapping

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Abstract. We have cloned the complete coding region of the porcine *TNFSF10* gene. The porcine *TNFSF10* cDNA has an ORF of 870 nucleotides and shares 85% identity with human *TNFSF10*, and 75% and 72% identity with rat and mouse *Tnfsf10* coding sequences, respectively. The deduced porcine TNFSF10 protein consists of 289 amino acids with the calculated molecular mass of 33.5 kDa and a predicted pI of 8.15. The amino acid sequence similarities correspond to 86, 72 and 70% when compared with human, rat and mouse sequences, respectively. Northern blot analysis detected *TNFSF10*-specific transcripts (~ 1.7 kb) in various organs of a 10-week-old pig, suggesting ubiquitous expression. Real-time RT-PCR studies of various organs from fetal (days 73 and 98) and postnatal stages (two weeks, eight months) demonstrated developmental and tissue-specific regulation of *TNFSF10* mRNA abundance. The chromosomal location of the porcine *TNFSF10* gene was determined by FISH of a specific BAC clone to metaphase chromosomes. This *TNFSF10* BAC clone has been assigned to SSC13q34→q36. Additionally, the localization of the *TNFSF10* gene was verified by RH mapping on the porcine IMpRH panel.

TNFα-related apoptosis-inducing ligand (TRAIL/Apo-2L) is encoded by the tumor necrosis factor superfamily member 10 gene *(TNFSF10)*. Currently over 40 distinct ligand-receptor systems of this superfamily are recognized in human. In pig, the positions of *TNFSF1A* and *TNFSF1B* were mapped to 7p11→q11 (Solinas et al., 1992). Sequence information of three other genes, *TNFSF4–6*, is available in public databases along with a growing number of porcine transcripts showing high homologies to human TNF ligand superfamily members. TRAIL has been shown to be important for the control of tumor growth and for modulation of the cellular immune system. An apoptosis-inducing effect of TRAIL was observed primarily in tumor cells and in Jurkat human T cells. This effect is initiated by binding of TRAIL to receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5), which signal apoptosis via their cytoplasmic death domains (Pan et al., 1997a, b; Sheridan et al., 1997). Other TRAIL binding proteins, TRAIL-R3 (DcR1), TRAIL-R4 (DcR2), and osteoprotegerin, lack functional death domains and have been discussed as decoy receptors (Degli-Esposti et al., 1997; Marsters et al., 1997; Sheridan et al., 1997).

Recent studies have shown that TRAIL is involved in the mediation of cytotoxic effects of immune cells, including T-cells, NK cells, macrophages and dendritic cells. Thus it has

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been suggested that TRAIL could play a role in suppressing tumor metastasis. This assumption was supported by the observation of increased susceptibility to tumor initiation and metastasis in TRAIL-deficient mice (Liu et al., 2001; Takeda et al., 2001; Cretney et al., 2002).

In addition to its anti-tumor effects, various immune modulatory mechanisms of TRAIL have been described. TRAIL is able to induce primary plasma cell apoptosis and to shorten the lifespan of neutrophils (Ursini-Siegel et al., 2002; Renshaw et al., 2003; Kamohara et al., 2004). Moreover TRAIL acts as a cell cycle inhibitor for T-cells independent of their differentiation status or antigen specificity (Song et al., 2000; Lunemann et al., 2002). Recently TRAIL was reported to inhibit the primarily T-cell mediated rejection of corneal allografts (Xie et al., 2003). Thus, expression of human TRAIL in transgenic pigs might be a strategy to protect pig tissues against cell-mediated rejection after xenotransplantation to primates (Klose et al., 2005). To further explore the feasibility of this approach, information on sequence similarity and expression levels of the endogenous *TNFSF10* gene encoding TRAIL is necessary. Therefore, we cloned the porcine *TNFSF10* cDNA, determined *TNFSF10* mRNA levels in different tissues and at various stages of development, and mapped the porcine *TNFSF10* gene to SSC13q34→q36.

Materials and methods

Cloning and sequencing of the porcine TNFSF10 cDNA sequence

PCR primers (TRAIL#4/TRAIL867 and TRAIL#7/TRAIL#6; all primers used in this study are listed in Table 1) were designed based on the human, mouse and rat *TNFSF10/Tnfsf10* cDNA sequences (DDBJ/EMBL/GenBank database accession numbers U37518, U37522 and AY115578) and used to amplify partial sequences of the porcine *TNFSF10* cDNA. TRAIL#5 was used as gene-specific primer in 3′-RACE using the SMART™ RACE cDNA Amplification Kit (BD Clontech, Mountain View, CA) according to the manufacturer's instructions. 5′-RACE was performed using a standard protocol (Sambrook and Russell, 2001) and the gene-specific primers TRAIL#12 and TRAIL#13 as well as the adapter primers Ad and Ad20T. PCR products were cloned using the TOPO TA Cloning® Kit (Invitrogen, Karlsruhe, Germany) and sequenced.

Quantitative RT-PCR analysis of TNFSF10 expression

Total RNA of liver, lung and kidney of fetuses and pigs of different ages was extracted using TRIZOL reagent (Invitrogen) following the manufacturer's instructions. RNA quality was checked by gel electrophoresis before and after DNase digestion. Reverse transcription was performed using 400 ng total RNA, 3 µg hexamer primers, 10 mM DTT, 40 units RNaseOut (Invitrogen) and 200 units Superscript II reverse transcriptase (Invitrogen) at 42 °C for 50 min. After termination at 70 °C for 15 min, samples were stored at – 80 °C.

Primers for a housekeeping gene (H2A_se and H2A_as, Table 1) were designed from the bovine histone 2A (*H2A*) sequence (accession NM_174809) and used as internal control. Primers TRAIL#5 and TRAIL#6 were used for the amplification of a *TNFSF10* cDNA fragment (Table 1). Quantification of mRNA abundance was performed by real-time PCR detection using an ABI PRISM 7700 sequence detector (Applied Biosystems, Weiterstadt, Germany) and SybrGreen® as a double stranded DNA-specific fluorescent dye. Amplification mixes (25 µl) contained 2 µl cDNA, 12.5 µl SybrGreen PCR Mix, 0.25 µl IU AmpErase uracil *N*-glycosylase (all from Applied Biosystems), 1.5 µl of each primer (300 nM finally), and 7.25 µl water. The standard curve was performed using plasmids with the sequences of the selected genes, and six serial dilutions (1:5) were used for all selected genes ranging from 1 million to 320 copies. The abundance of *TNFSF10* mRNAs was calculated using the standard curve method, with determination of PCR amplification efficiency and normalization for H2A mRNA as the internal reference.

Table 1. Primers used in this study

Primer	Orientation	Sequence (5'-3')
TRAIL867	reverse	TAA AAA GGC $^{C}_{T}$CC $^{A}_{G}$AA $^{A}_{G}$AA $^{A}_{G}$CT GGC TTC
TRAIL #5	forward	TGG ACA AGC TAT CCT GAC CCT ATA C
TRAIL #6	reverse	CAC CTT GAT AGA TGG AAT AGA GTC C
TRAIL #12	reverse	TCA CAA ACT GAC GTA GCT GCC AC
TRAIL #13	reverse	TCA TCA GCA GTA TAG GGT CAG
AdT20	forward	ATT GAA TTC TCT AGA ACG CGT CTC GAG $(T)_{20}$
Ad	forward	ATT GAA TTC TCT AGA ACG CGT CTC GAG
H2A_se	forward	TGC TGG TGG TGG TGT CAT TC
H2A_as	reverse	CCA GGC ATC CTT TAG ACA GTC TTC T
TNFSF10_F	forward	TCA ACC CAA AGG CTC AAC
TNFSF10_R	reverse	CAG TGC TGC CCT TTT CTC

QPCR was started with 2 min at 50 °C for AmpErase activation and 10 min at 95 °C for denaturation. The program continued with 40 cycles of 15 s at 95 °C and 30 s at 60 °C and 30 s at 72 °C. Each assay included triplicates of cDNA.

Isolation and characterization of a genomic TNFSF10 clone

A genomic DNA clone (RP44-472G15) of approximately 165 kb was isolated from the porcine RPCI-44 BAC library after screening high density BAC filters according to the RPCI protocols (http://www.chori.org/bacpac/). A ^{32}P-labeled 279-bp PCR product from the porcine *TNSF10* gene was used as probe. The PCR primers for this product were TNFSF10_F and TNFSF10_R (Table 1). DNA of the BAC clone RP44#472G15 was isolated using the Qiagen plasmid midi kit (Qiagen, Hilden, Germany). BAC ends were sequenced with the ThermoSequenase kit (Amersham Biosciences, Freiburg, Germany) on a LI-COR 4200L-2 automated sequencer (MWG Biotech, Ebersberg, Germany). The end sequences were deposited under accessions AJ786143 and AJ786144 in the EMBL nucleotide database.

Fluorescence in situ hybridization (FISH) analysis

The porcine genomic BAC clone RP44-472G15 containing the porcine *TNFSF10* gene was labeled with digoxigenin by nick translation using a Nick-Translations-Mix (Roche, Mannheim, Germany). FISH on GTG-banded pig chromosomes was performed using 750 ng of digoxigenin-labeled BAC DNA. In this experiment 24 µg sheared total porcine DNA and 10 µg salmon sperm were used as competitors. After hybridization over night, signal detection was performed using a digoxigenin-FITC detection kit (Quantum Appligene, Heidelberg, Germany). The chromosomes were counterstained with DAPI and propidium iodide and embedded in antifade solution. Thirty metaphases that had previously been photographed were re-examined after hybridization with a Zeiss Axioplan 2 microscope equipped for fluorescence.

Probe description:
Probe name: RP44-472G15
Probe type: porcine genomic BAC clone
Insert size: 165 kb
Vector: pTARBAC2
Proof of authenticity: DNA sequencing and gene-specific PCR
Gene reference of human TNFSF10: Wiley et al., 1995

Radiation hybrid (RH) mapping

The primer pair TNFSF10_F and TNFSF10_R developed for the amplification of the *TNFSF10* probe was also used for RH mapping. Amplification of the marker was done on the INRA-Minnesota Porcine Radiation Hybrid (IMpRH) panel (Yerle et al., 1998; Hawken et al., 1999) (http://imprh.toulouse.inra.fr/) as described previously (Leeb and Rohrer, 2002). The mapping tool at the IMpRH Server (Milan et al., 2000) was used for the mapping of this new marker relatively to markers previously mapped on the IMpRH panel. The order of markers already mapped on the first generation map is considered as known. The two point option of the RHMAP3.0 package (Lange et al., 1995) reveals two-point distances with flanking markers.

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pig     MAVMQTPGGPSPGQ----TCVLILIFTVLLQALCVALTYVYFTNELKQMQDKYSKSGIAC    56
human   MAMMEVQGGPSLGQ----TCVLIVIFTVLLQSLCVAVTYVYFTNELKQMQDKYSKSGIAC    56
rat     MASTGNLKGPSFSQHFTMTVICIVLLQVLLQALTVAVTYMYFNNEVKQLQDNYSKIGLAC    60
mouse   MPSSGALKDLSFSQHFRMMVICIVLLQVLLQAVSVAVTYMYFTNEMKQLQDNYSKIGLAC    60
        *       ::*  *    :   *:   ****:: **:** **:** ** ** *** * **

pig     FLKEDDSFWDPTDDERMLSPCWQVKWQLRQFVRKMILRTYEETISTVSEKQQGIPHLERE   116
human   FLKEDDSYWDPNDEESMNSPCWQVKWQLRQLVRKMILRTSEETISTVQEKQQNISPLVRE   116
rat     FSKEDGDFWDSTDEGILNRPCLQVKRQLYQLIEEVTLRTFEKTISTVPEKQLSTPPLPRG   120
mouse   FSKTDEDFWDSTDGEILNRPCLQVKRQLYQLIEEVTLRTFQDTISTVPEKQLSTPPLPRG   120
        * *:*  :** :*    : ** *** ** *:     *** : ***** ***   ::: *

pig     KGPQRVAAHITGTSRKRSTFPSLSSKYEKALGQKINSWESSRKGHSFLNNFHLRNGELVI   176
human   RGPQRVAAHITGTRGRSNTLSSPNSKNEKALGRKINSWESSRSGHSFLSNLHLRNGELVI   176
rat     RRPQRVAAHITGITRRSNLALIPISKDGKTLGQKIETWESSRRGHSFLNHVHLRNGELVI   180
mouse   GRPQKVAAHITGITRRSNSALIPISKDGKTLGQKIESWESSRKGHSFLNHVLFRNGELVI   180
          **:*******  ::::    : **  * **:** :***** *****:  ::*******

pig     HQTGFYYIYSQTYFRFQEPEEILGTVSTEGNRKKNRQMIQYIYKWTSYPDPILLMKSARN   236
human   HEKGFYYIYSQTYFRFQE--------EIKENTKNDKQMVQYIYKYTSYPDPILLMKSARN   228
rat     QEEGLYYIYSQTYYRFKEAKEAS--KTVSKDGGRIKQMVQYIYKYTSYPDPILLMKSARN   238
mouse   EQEGLYYIYSQTYFRFQEAEDAS--KMVSKDKVRTKQLVQYIYKYTSYPDPIVLMKSARN   238
           * ********:**:*                 :*::*****:*******:*******
         ◆
pig     SCWSKDSEYGLYSIYQGGIFELKEDDRIFVSVTNEQLIDMDQEASFFGAFLIG          289
human   SCWSKDAEYGLYSIYQGGIFELKENDRIFVSVTNEHLIDMDHEASFFGAFLVG          281
rat     SCWSREAEYGLYSIYQGGLFELKENDRIFVSVTNEHLMDLDHEASFFGA              287
mouse   SCWSRDAEYGLYSIYQGGLFELKKNDRIFVSVTNEHLMDLDQEASFFGAFLIN          291
        **** ::*********** ****::**********:* * * *******
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Fig. 1. Deduced amino acid sequence of porcine TNFSF10 and alignment to the human, rat, and mouse TNFSF10 proteins, respectively. The amino acid sequences for the human, rat, and mouse TNFSF10 were obtained from nucleotide sequences of the DDBJ/EMBL/GenBank database (human: U37518; rat: AY115578; mouse: U37522). Asterisks and colons under the aligned sequences indicate complete match and match among three species of four, respectively. The putative transmembrane anchor is underlined in the human sequence. A conserved cysteine residue that is assumed to mediate zinc binding is indicated by a rhombus.

Results and discussion

Porcine TNFSF10 cDNA sequence

To isolate the complete coding region of the porcine *TNFSF10* gene, RACE experiments and PCR were performed. The cDNA has an ORF of 870 nucleotides. The sequence has been deposited in DDBJ/EMBL/GenBank under accession AY639873. The ORF has 85% identity to the *TNFSF10* nucleotide sequences of human *TNFSF10*, 75 and 72% identity to the nucleotide sequences of rat and mouse *Tnfsf10*, respectively. The deduced porcine TNFSF10 protein consists of 289 amino acids with a calculated molecular mass of 33.5 kDa and a predicted pI of 8.15. At the amino acid level the porcine TNFSF10 protein shows 86% similarity to human TNFSF10 and 72 or 70% to rat or mouse TNFSF10 amino acid sequences, respectively (Fig. 1). The putative transmembrane anchor for the porcine TNFSF10 protein comprises amino acids 18–28 similar to human. Amino acids 172–188 match the consensus sequence for the TNF type II membrane protein family. The domain for metal binding is highly conserved among pig, human, rat and mouse, with cysteine 230 (human) as the putative zinc binding amino acid, required for activity and trimerization of the TNFSF10 protein (Bodmer et al., 2000; Hymowitz et al., 2000). The high degree of amino acid identity between pig and human, especially in the C-terminal domain of the TNFSF10 protein, suggests that the porcine protein might be able to interact with human TRAIL receptors.

Expression of the porcine TNFSF10 gene

The human gene for TNFSF10 is known to be ubiquitously expressed in a large variety of adult tissues (Wiley et al., 1995). To investigate expression of the porcine homolog, RT-PCR and Northern blot experiments were performed using RNA isolated from various tissues of a 10-week-old piglet. We detected TRAIL transcripts of approximately 1.7 kb in spleen, lung, liver, kidney, heart, testis, muscle and skin (data not shown). This suggested ubiquitous expression of *TNFSF10* mRNA in pig. Highest expression levels were detected in spleen, liver and lung. In addition, we used quantitative RT-PCR to investigate *TNFSF10* gene expression at two time points during fetal development (day 73 and 98) and postnatally in 2-week-old piglets and adult pigs (8 months). *TNFSF10* transcript abundance in liver, lung and kidney was monitored in relation to histone 2A transcripts, considering histone 2A to be a constitu-

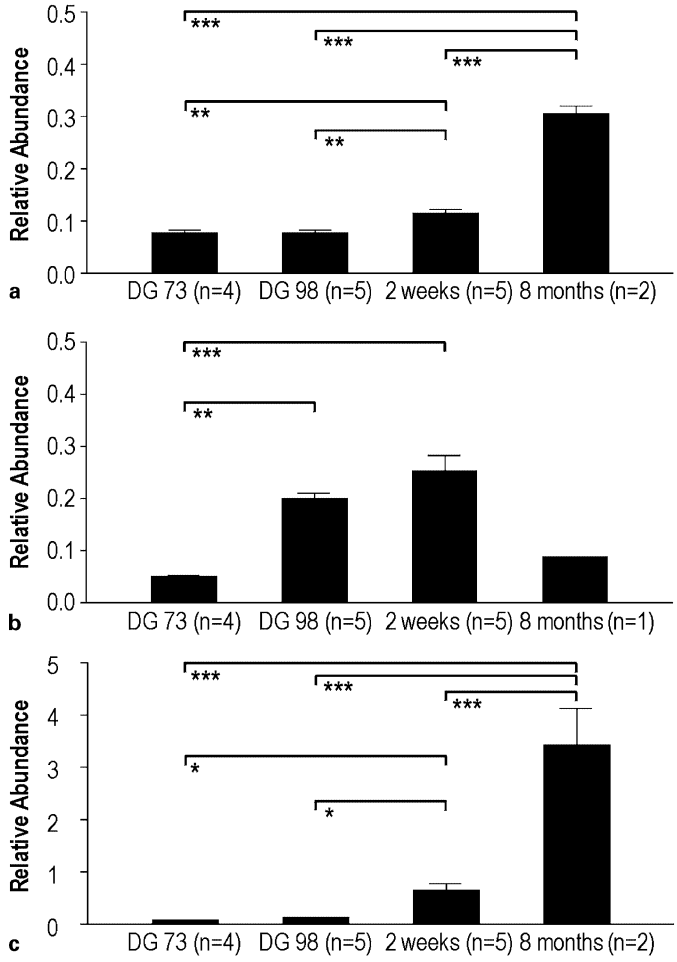

Fig. 2. Expression analysis of the porcine *TNFSF10* gene in fetal and adult tissues. Relative abundances were calculated using the standard curve method by dividing the copy numbers of *TNFSF10* mRNA by the copy numbers of *H2A* mRNA. Bars represent means and standard deviations of the different developmental ages. DG: day of gestation; n: number of animals tested. (**a**) Kidney; (**b**) lung; (**c**) liver; *P* values: * < 0.05, ** < 0.01, *** < 0.0001.

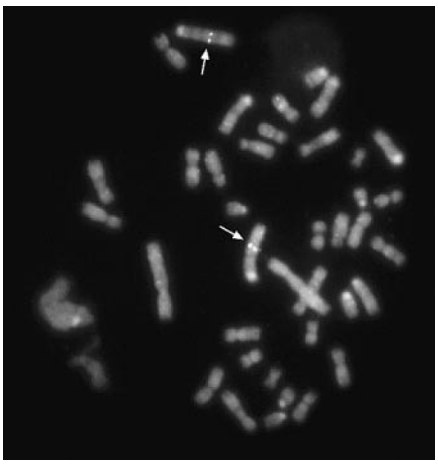

Fig. 3. Chromosome assignment of the porcine *TNFSF10* gene by FISH analysis on a porcine metaphase spread. The digoxigenin labeled BAC clone RP44-472HG15 containing the porcine *TNFSF10* gene was hybridized to GTG-banded metaphase chromosomes of a normal pig. Double signals indicated by arrows are visible on both chromosomes 13q34 → q36. The chromosomes were counterstained with propidium iodide and subsequently identified by DAPI staining.

tively expressed gene. We detected *TNFSF10* transcripts in all tissue samples analyzed showing that expression was not restricted to postnatal life (Fig. 2). Whereas in kidneys and liver, normalized *TNFSF10* expression did not change during fetal development and showed only moderate changes after birth, we observed an increase of *TNFSF10* mRNA levels in kidneys and liver of 8-month-old pigs (Fig. 2a, c). In contrast, expression levels in lungs seemed to be regulated during fetal development, showing a clear increase between mid- and late pregnancy. Postnatally, expression levels in lungs increased slightly above the high levels reached in late pregnancy, but declined in adult animals (Fig. 2b). The explicit increase of expression levels in fetal lung correlates with the saccular phase of lung development in pig (Rüsse and Sinowatz, 1991). Parallel to differentiation and growth of the bronchiolar and alveolar system, outgrowth of the vascular system in lung takes place. Since it was suggested that TNFSF10 plays an important role in endothelial cell migration, vessel tube formation and extracellular matrix synthesis in lung fibroblasts (Yurovsky, 2003; Secchiero et al., 2004), TNFSF10 might be involved in lung tissue differentiation. Moreover, *TNFSF10* expression was located in the medial smooth cell layers of pulmonary arteries of mouse, rat and human (Gochuico et al., 2000; Spierings et al., 2004), as well as in the bronchial epithelium and alveolar septa in human (Spierings et al., 2004). Probably increased expression levels of TRAIL mRNA in pig are correlated with lung growth and differentiation. Accordingly, this might explain the low expression levels in fully grown animals observed in our analyses.

In liver, we observed continuous increase of *TNFSF10* transcript levels after birth as well as in adult animals (Fig. 2c). In mice up to 30–40% of liver natural killer (NK) cells were shown to express *Tnfsf10* constitutively (Ochi et al., 2004). If this holds true also in swine, increasing *TNFSF10* transcript levels might be due to a continuous raise in the *TNFSF10* expressing cell population of the growing liver. Moreover, TNFSF10 expression on liver NK cells is upregulated by interferon γ (Smyth et al., 2001). This cytokine is secreted among others by macrophages and mononuclear cells of the liver in response to infectious agents and bacterial endotoxins cleared from portal blood. Microbial colonization of the gut starts shortly after birth, so we suggest that *TNFSF10* expression levels rise strongly after activation of *TNFSF10* expressing liver NK cells early in postnatal life. Later on, the immune status of the animals might be reflected in *TNFSF10* expression levels.

Chromosomal assignment of the porcine TNFSF10 gene

The porcine BAC clone RP44-472G15 has been retrieved from the RPCI-44 BAC library with a porcine *TNSF10* cDNA probe. Colony PCR indicated that the clone indeed contained the desired insert. Additional support for the correct identity of the BAC clone was gained by the analysis of the BAC end

sequences. The T7 end sequence had a BLAST hit to the human genome (build 34.3) at 173.48 Mb on HSA3 (E = $5 \cdot 10^{-13}$) while the SP6 end sequence had a BLAST hit at 173.68 Mb on HSA3 (E = $5 \cdot 10^{-13}$). Thus the BLAST hits of the two porcine BAC end sequences bracketed the human *TNFSF10* gene at 173.54–173.56 Mb on HSA3. The chromosomal location of the porcine *TNFSF10* gene was determined to be at SSC13q34→q36 by FISH of the BAC clone to metaphase chromosomes (Fig. 3). Additionally, the localization of the *TNFSF10* gene was verified by RH mapping on the porcine IMpRH panel. The RH results were submitted to the IMpRH database (http://imprh.toulouse.inra.fr). Two-point analysis (Wiley et al., 1995) revealed that *TNFSF10* is linked to the marker IMpRH00891 on SSC13 with a distance of 18 cR and a two-point Lod score of 14.89. The RH results were thus consistent with the cytogenetic localization of *TNFSF10* at SSC13q34→q36. The human *TNFSF10* ortholog is located on HSA3q26 (Wiley et al., 1995). The chromosomal locations of the rodent *Tnfsf10* genes are MMU3A3 for the mouse and RNO2q24 for the rat (http://www.ncbi.nlm.nih.gov/mapview). These localizations correspond well to the synteny data of the porcine RH map (Rink et al., 2002).

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