The Activation of the Liver X Receptor Affects the Function and Differentiation of Osteoclasts

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Abstract

The nuclear Liver X Receptor (α, β) is mainly involved in cholesterol homeostasis, hepatic lipogenesis, the immune system and fertility; however, it may also affect the function of osteoclasts in bone replacement. The two osteoclast enzymes, TRAP and Cathepsin K, are responsible for dissolving old bone tissue to leave space for new bone tissue to form. The LXR may affect the amount of these enzymes in action. This study was completed on RAW 264.7 macrophage cells that were stimulated by RANKL to differentiate into osteoclasts. Both undifferentiated and differentiated cells were either treated with Tularik (synthetic LXR ligand) or DMSO (control). They were fixed and stained for TRAP to make sure that the enzymes were produced. Then RNA was isolated and run in gel electrophoresis to make sure that it has not degraded. Then cDNA was synthesised from the RNA and the TRAP and Cathepsin K gene expressions were examined using Real Time PCR. The graphs from the Real Time PCR show almost no TRAP/ Cathepsin K gene expression in the undifferentiated cells but in the differentiated cells it was expressed, as expected. The Tularik treatment did work as two LXR target genes were increased (ABCG1 and SREBP1c). The Tularik treated cells appeared to have even higher gene expression of TRAP and Cathepsin K. This implies that the activation of LXR increases the enzyme activity in osteoclasts and therefore can lead to the disease characterized by bone fragility: osteoporosis.

Introduction

The liver X receptor (LXR) is a nuclear receptor. Unlike membrane receptors, they are found in the nucleus and some are even already attached to the DNA. When activated by specific ligands they work as transcription factors to affect the transcription of genes. In the 1980s, nuclear receptors were cloned and found to have sequential similarities; therefore all are grouped into a Nuclear Receptor Superfamily [1, 2]. They all have a DNA-binding domain (DBD) and a ligand-binding domain (LBD). However, as their type of ligand varies, they are separated into 3 sub-families: (1) Endocrine Receptors that are activated by high-affinity hormones e.g. the estrogen receptor, (2) Adopted Orphan Receptors activated by low-affinity dietary lipids (e.g. cholesterol), i.e. LXR, and (3) Orphan Receptors, where the ligands are unknown.

Referring to *Diagram 1*, during nuclear receptor ligand binding, the receptors undergo chemical changes that dissociate corepressors and associate coactivator proteins that enables transcriptional activation. Unlike the endocrine receptors that bind to DNA as homodimers, adopted orphan receptors like LXR bind to DNA as heterodimers. They usually bind to their partner: the Retinoid X Receptor (RXR).

The LXR has 2 subtypes, α and β [3]. Both LXR types play an important role in reverse cholesterol transport, the pathway through which the body excretes excess cholesterol to maintain homeostasis. Firstly, the LXR regulates genes that inhibit *de novo* synthesis of cholesterol in the cell and prevents new cholesterol entering the cell via lipoproteins.

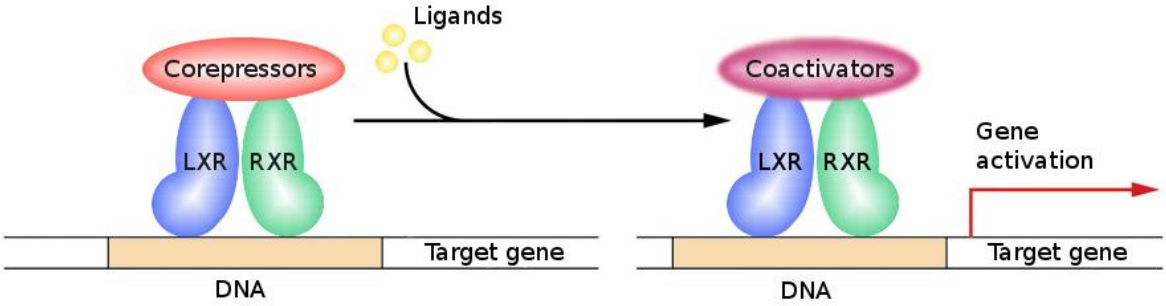

Diagram 1. A ligand binds to a nuclear receptor, i.e. LXR, which enables transcriptional activation. LXR binds to DNA as a heterodimer, with its partner the Retinoid X Receptor (RXR).

The excess cholesterol leaves the cell via the ABC transporters (upregulated by LXR) and is transported to the liver via high density lipoproteins (HDL). The second pathway is at the whole body level, where the cholesterol is converted into bile acids for the body to excrete. The LXR also regulates the Cyp7a gene (which is responsible for making the enzyme 7 α hydroxylase) which converts cholesterol into bile acids, i.e. cholic acid.

It is known that other roles have been discovered for the LXR other than in cholesterol homeostasis, e.g. hepatic lipogenesis, glucose metabolism, fat storage and utilization, macrophage cholesterol metabolism, macrophage inflammatory signalling, immune system and fertility [4]. In the hepatic lipogenesis system, LXR increases the level of triglycerides in the liver and plasma through upregulation of SREBP1c and other lipogenic genes.

LXR also reduces the amount of glucose from the liver by repressing the hepatic gluconeogenic genes; this improves the tolerance of glucose intake. LXR also increases the glucose uptake into the tissues by inducing the glut4 transporters and thus favours the respiration process.

Macrophages play a main role in the development of the disease atherosclerosis, as they take up modified lipoproteins and release inflammatory mediators in the artery wall. In a macrophage full of lipids, the activation of LXR activates the ABC pathway to efflux this excess cholesterol, preventing lipid overload. Therefore LXR is essential in the macrophages to maintain cholesterol homeostasis and prevent cardiovascular diseases.

LXR also inhibits macrophage apoptosis which is essential for prevention of infection. Fat storage and utilization are also affected by LXR activation, since LXR knock-out mice are resistant to getting overweight when fed a high cholesterol and dietary fat diet because they are able to utilize dietary fat and increase their metabolic rate.

This study is exploring a new area of LXR: its role in the bone. The question as to whether LXR has a positive/negative effect on bone is in progress, but it appears to be important. A paper has recently been published [5] using KO mice ($LXR\alpha^{-/-}$, $LXR\beta^{-/-}$, $LXR\alpha^{-/-}\beta^{-/-}$). It concluded that LXRα is mainly involved in the cortical bone (the part that includes the compact bone and protects the bone marrow) and LXRβ is mainly involved in the trabecular bone (the part that includes the spongy bone and epiphyseal line).

The skeleton is always regenerated (for adults every 10 years) to maintain the bone, preventing it from becoming brittle and weak [6]. This involves two major cell types: reabsorption by osteoclasts and reformation by osteoblasts. They are located in the Basic Multicellular Unit (BMU). In the trabecular bone the BMU reabsorbs and replaces the bone on the trabecular surface. In the cortical bone this occurs through a tunnel in the bone. New cells are produced from the bone marrow, since the life span for the osteoclasts is 3 weeks and for the osteoblasts it is 3 months.

When remodelling bones, mononuclear precursor osteoclasts differentiate to form osteoclasts (containing multiple nuclei) and then attach to the bone. Enzymes like TRAP and Cathepsin K are released to dissolve bone tissue. The side of the osteoclast that is attached to the bone is wavy, in order to increase the surface area to favour the release of the enzymes. Then reformation of the bone is achieved by the attachment of osteoblasts.

Aim

The purpose of this investigation was to determine if the LXR directly affects the function of osteoclasts. Osteoclasts were differentiated from a mouse macrophage cell line (RAW 264.7) with RANKL and the expression of the main osteoclast enzyme genes (TRAP and Cathepsin K) were examined after treatment with the synthetic LXR ligand (Tularik).

Materials & Methods

Cell culture

To culture RAW 264.7 mouse macrophage cells they were grown in α-MEM (Gibco; including ribonucleosides and deoxyribonucleosides), 10% FCS (Fetal Calf Serum), 0.5% gentamicin (antibiotic) and 2mM L-glutamine. The cells were firstly grown in a small tissue culture flask.

Once cells were confluent, a cell scraper was used to scrape the cells that have attached to the plastic into media and they were transferred into a medium size flask. Once confluent they were again scraped and transferred into a large size flask. Cells were then scraped into 100ml medium and 2ml of each amount of cells pipetted into 4x 6 well plates (2 undifferentiated cells and 2 differentiated cells) and left to grow for 1 day.

Then 2µl Tularik (1µm) was added to one undifferentiated 6 well plate and 2µl DMSO (as a control, since Tularik is dissolved in the solution) to the other undifferentiated plate. These were left for 24 hours, then the RNA was extracted and the plates stained with an osteoclast marker.

Then differentiated media was added (same as above but also 30ng/ml sRANKL Nordic Biosite). This differentiates the cells over 4 days. Also, 2µl Tularik was added to one differentiated 6 well plate and 2µl DMSO to the other differentiated plate. After 4 days the RNA was extracted and the plates were stained for mature osteoclasts.

Staining osteoclasts

To stain the osteoclasts the Acid Phosphatase Leukocyte Kit (Sigma) was used. This kit stains the TRAP protein brown, and was used as a marker to see if the differentiation was successful. Firstly, the medium from the 6 well plates was removed, and the cells were washed with PBS (Phosphate Buffered Saline) to remove traces of media. Then the cells were fixed with 2ml of a formaldehyde solution (25ml citrate solution, 65ml acetone and 8ml 37% formaldehyde) for 30 seconds. Then the solution was removed and the plates were washed with PBS.

To make the stain, 125µl Fast garnet GBC Base solution was mixed with 125µl $Na\text{-}NO_3$ solution and left for 2 minutes. Then 11.3ml H_2O, 125µl naphthol AS-B1 phosphate solution, 500µl acetate solution and 250µl tartrate solution were added. The staining solution was mixed and added into each well of the plates and left for 1 hour at 37C in darkness. The cells were rinsed again with PBS and left for 24 hours to dry (*Fig. 1*).

RNA isolation

Another way of observing the expression of TRAP (other than staining for the TRAP protein) is by isolating the RNA, and looking at the gene expression of TRAP. To isolate RNA the RNeasy kit (QIAGEN) was used. In the fume hood, the media was

removed and the cells rinsed with PBS. The cells were lysed directly in the culture dish by adding 350µl buffer RLT with 10µl/ml β-mercaptoethanol. The cells were left for 2 minutes to lyse, and then scraped off with a cell scraper. Then they were frozen on dry ice and thawed at room temperature to completely lyse the cells.

Then 350µl 70% ethanol was added and pipetted up and down until the solution was clear and mixed. This was pipetted into a spin column and centrifuged for 15 seconds at 8000x g. The flow through was discarded and 700µl buffer RW1 was added and centrifuged for 15 seconds at 8000x g. The flow through was discarded again and the column transferred to new 2ml tube and 500µl buffer RPE added. Then it was centrifuged again for 15 seconds at 8000x g and the flow through discarded. Another 500µl buffer RPE was added and centrifuge for 2 min at 8000x g to dry membrane. Then it was transferred into a new 1.5ml eppendorf, and 30µl RNase free water was added, and then centrifuged for 1 min at 8000x g.

To quantify the RNA in the samples a spectrophotometer was used. This measures the absorbance of UV light as DNA/RNA and the proteins have different absorbance curves. The peak of absorbance for DNA is at 260nm and for protein at 280nm. Therefore less than 1.8 means there is more protein. The aim was to get a 260/280 ratio between 1.8 and 2.0 and values of 100-300µg/ml.

To visualise the RNA (to see if it is degraded) gel electrophoresis was used. To make the gel, 1g agarose powder was added into 100ml 1xTBE buffer (108 g Tris base, 55 g Boric acid, 9.3 Na_4 EDTA) and 1 drop ethidium bromide/50ml (a non-radioactive marker for DNA/RNA that fluoresces when exposed to UV light). After the gel has solidified the 10µl 1kb ladder was added first (sizes the RNA from 500bp to 12kb) and then the samples (5µl RNA and 3µl loading buffer). The electrophoresis was run at 100V for 45min. Then the 28S and 18S rRNA bands were visualised in UV light (as mRNA and tRNA fragments are too small and too few to see, therefore we assume that if rRNA is not degraded then all RNA is not degraded). The RNA was stored at minus 80C.

cDNA synthesis

As RNA is single stranded it is unstable, therefore to look at gene expression it needs to be double stranded. Thus complementary DNA (cDNA) was synthesized from RNA using the enzyme reverse transcriptase (superscript II).

Firstly, any contaminating DNA was removed using the DNase enzyme by incubating 8µl of the RNA with 1µl DNase buffer 10X and 1µl DNase I for 15min at room temperature.

After the incubation, 1µl EDTA 25mM was added to inactivate DNase. The sample was then incubated in a PCR machine at 65C for 15 min. Then 10µl of the superscript master mix was added (4µl superscript buffer 5X, 2µl superscript DDT, 1µl random primer [100Ng], 1µl dNTP [10mM], 1.5µl water and 0.5µl superscript II enzyme) and then incubated at 22ºC for 10min, 42ºC for 60min, followed by 70ºC for 15min to inactivate the enzyme.

Real Time PCR

Real Time PCR (Polymerase Chain Reaction) was used to amplify the genes of interest, i.e. TRAP & Cathepsin K. Two primers (short artificial DNA strands) were designed that specifically recognize a short part of the gene (usually 50bp). In the PCR reaction 1.5µl forward primer was added, along with 1.5µl reverse primer, 8.5µl water and 12.5µl SYBR green master mix. Then 1µl cDNA was added. Then 10µl of the sample was pipetted into each well of a 96 well plate. In these reactions the two osteoclast genes (TRAP & Cathepsin K) and two LXR target genes (ABCG1 & SREBP1c) were amplified, along with one house keeping gene (β-actin). The reason for having the house keeping gene is

because they exist in every cell type and therefore this normalises the RNA levels.

Results

As we can see by *Fig. 1 C*, the differentiated cells show more brown stain compared with the undifferentiated cells (*Fig. 1A*), thus more expression of TRAP. This means that the differentiation of the osteoclasts was completed successfully. There appears to be a difference in the amount of TRAP positive cells after Tularik treatment with fewer osteoclasts (Fig 1D).

Fig. 2 clearly shows both the 28S and 18S rRNA bands from both the undifferentiated and the differentiated cells indicating that the RNA has not degraded, whether it is treated with Tularik or not.

In *Fig. 3* the gene expression of the osteoclast enzymes TRAP (C) and Cathepsin K (D) is expressed in the differentiated cells, but not undifferentiated, which was predicted. Also the treatment of Tularik shows an increase of the gene expressions.

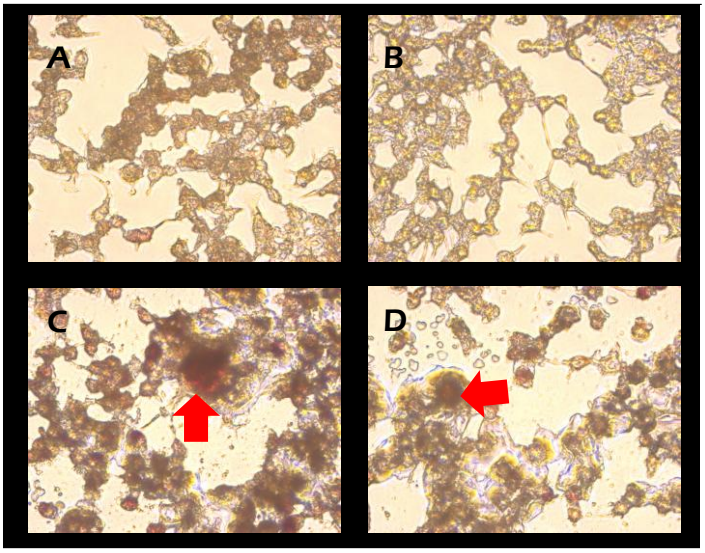

Fig. 1 Undifferentiated cells treated with DMSO (A) or Tularik (B) for 24 hrs. Differentiated cells treated with DMSO (C) or Tularik (D) for 4 days. All cells were fixed and stained for TRAP with the Acid Phosphatase Leukocyte Kit (Sigma). Arrow indicates a mature osteoclast.

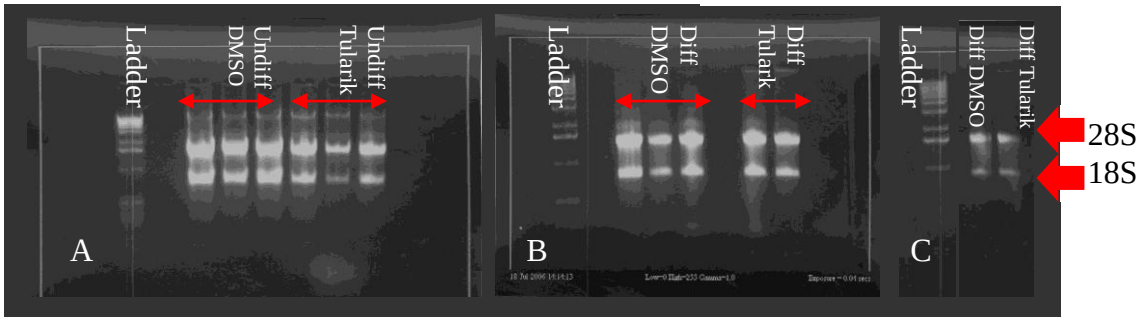

Fig. 2 Gel electrophoresis showing RNA extracted from the undifferentiated cells (A) and from the differentiated cells (B) and (C), [an experimental miscalculation occurred and therefore the 4th sample was repeated].

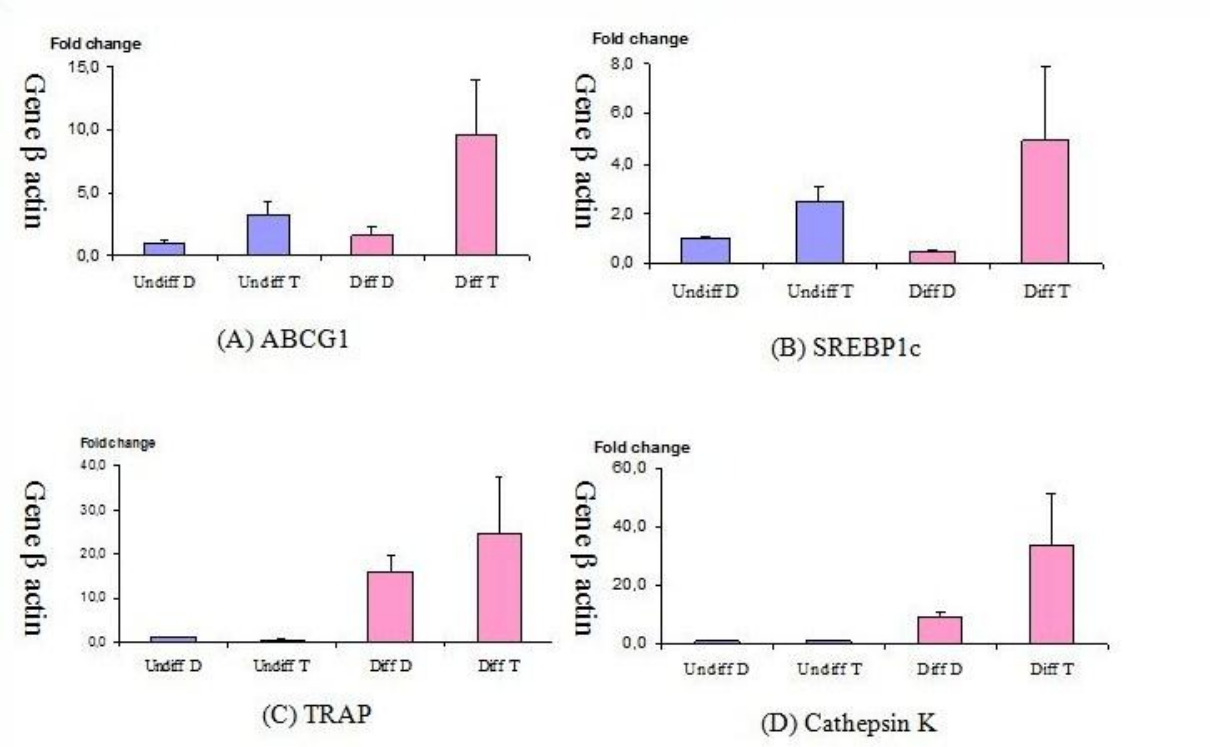

Fig. 3 The levels of the gene expression of two LXR target genes ABCG1 (A) and SREBP1c (B) and two osteoclast genes TRAP (C) and Cathepsin K (D) compared with the housekeeping gene β-actin (unit 1) in undifferentiated/differentiated RAW 264.7 cells treated with DMSO (D) and Tularik (T).

Discussion

ABCG1 (ATP-binding cassette) is involved in the reverse cholesterol transport system to maintain low cholesterol levels as it transports excess cholesterol from macrophages to the liver. In the liver LXR regulates the sterol regulatory element binding proteins (SREBP) to esterify and store excess cholesterol. The LXR treatment worked well since the addition of Tularik increased the expression of the LXR target genes ABCG1 and SREBP1c (*Fig. 3A, B*) as expected.

In this study the results show that LXR may play a role in the expression of the two osteoclast genes: TRAP and Cathepsin K. As expected, *Fig. 3 C* and *D* show that there is almost no expression in the undifferentiated cells. However, the differentiated cells (osteoclasts) show a possible increase in the expression of the enzymes TRAP and Cathepsin K after the addition of Tularik. However, the error bars are too high and this experiment has to be repeated to confirm the results.

These results indicate that activating LXR could lead to more osteoclast activation (through increasing the TRAP and Cathepsin K enzymes) and thus more bone resorption. This could lead to the development of osteoporosis, a common disease in elderly women that results from increased osteoclast activity which leads to a more porous and fragile bone. However, in future studies it would be necessary to investigate the effect of activation of LXR on osteoblast function. If it induces increased osteoblast activity too, then there would still be a balance in bone remodelling. The end result would just be increased bone replacement, instead of a potential development of osteoporosis.

New drugs for cardiovascular diseases, which bind to LXR in order to increase the excretion of cholesterol, could soon be on the market. Therefore, the ideal situation

would be that LXR has no detrimental effects on bone replacement, especially when cardiovascular diseases affect many elders, and osteoporosis is already so widespread in elderly women.

Conclusion

There are mainly three things to conclude: (1) both undifferentiated and differentiated cells are responding to Tularik because the level of the well known target genes of LXR (ABCG1 and SREB1c) have increased, (2) the RAW 264.7 cells have differentiated as both TRAP and Cathepsin K expressions have increased, (3) it is possible that the amount of TRAP and Cathepsin K has increased after activating LXR with Tularik. Increased osteoclast activity could potentially lead to the development of osteoporosis. However, the study needs to be repeated to conclude these results, and a similar study should be done on osteoblasts as well.

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Glossary

ABCG1: An enzyme that is controlled by LXR in the macrophage efflux & transport of cholesterol to the liver i.e. ABC pathway in the reverse cholesterol transport system.

α-MEM: Cell growth medium.

Apoptosis: Programmed cell death.

Bile acids: Steroid acids found in the bile in mammals, produced in the liver through oxidation of cholesterol. They are stored in the gallbladder to be then released into the intestine, and then excreted.

BMU (Basic Multicellular Unit): An area in the trabecular and cortical bone where osteoclasts and osteoblasts are kept.

Cathepsin K: An osteoclast enzyme that dissolves bone.

cDNA: Complementary DNA is the synthesized DNA from mRNA.

Cyp7a: An enzyme that converts cholesterol into bile acids.

DMSO: Dimethyl sulfoxide $(CH_3)_2SO$ is used in PCR to inhibit secondary structure in the DNA template or primers. It also protects cells/tissues at low temperature for storage.

Electrophoresis: Running DNA/RNA/protein (using high voltage) on an agarose gel (microscopic network) to split segments apart based on their sizes.

Epiphyseal line: The borderline between trabecular and cortical bone.

FCS (Fetal Calf Serum): The blood serum (no bloodcells included) taken from a fetal calf. It includes antibiotics and nutrients needed for cell growth.

Macrophage: White blood cell that is involved in the immune system. It "eats up" foreign pathogenic material i.e. viruses and bacteria.

Osteoblast: A cell in the bone replacement system that builds up new bone tissue.

Osteoclast: A cell in the bone replacement system that breaks down old bone tissue.

PCR (Polymerase Chain Reaction): A method of amplifying short parts of DNA.

RANKL: Stands for Receptor Activator of Nuclear Factor K Ligand. The RANK ligand attaches to the RANK receptor on the osteoclast precursor membrane to stimulate the differentiation into the osteoclast phenotype.

Real Time PCR: This machine replaces the PCR and semi-quantitative (run the PCR products on a gel and then quantify the intensity of the bands) methods. It uses the SYBR green molecule to bind to the amplified cDNA. Then by detecting the intensity of fluorescence the amount of the sample is revealed.

Spectrophometer: Determines the concentration and purity (as few proteins as possible) of the DNA/RNA solution through the difference of DNA/RNA UV absorbance curve with the protein one.

SREBP1c: An enzyme that is controlled by LXR for storage of cholesterol in the liver in the reverse cholesterol transport system.

TRAP: An osteoclast enzyme that dissolves bone.

Tularik: A synthetic LXR binding ligand.