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AG Gow[a], R Muirhead[a], DC Hay[b], DJ Argyle[a]

[a]Royal (Dick) School of Veterinary Studies and The Roslin Institute, The University of Edinburgh, Roslin, Midlothian, United Kingdom.

[b]MRC Centre for Regenerative Medicine, The University of Edinburgh, Edinburgh, United Kingdom

Corresponding author:
Adam Gow,
Royal (Dick) School of Veterinary Studies and The Roslin Institute,
The University of Edinburgh,
Roslin,
Midlothian,
United Kingdom.
Tel +4413166507650, Fax +441316507652
Adam.gow@ed.ac.uk

Running title – LDL and AcLDL uptake in canine hepatocytes and MSCs

Abstract

Low-density lipoprotein (LDL) uptake is one of a number of tests used to demonstrate hepatocyte-like function after stem cell differentiation. Use of two compounds; LDL and acetylated LDL has been described despite each having different mechanisms of uptake. Three primary hepatocyte cultures and three sets of mesenchymal stromal cell (MSCs) cultures, derived from both adipose tissue and bone marrow, were harvested from dogs. Those cells were compared to commercially available human and mouse bone marrow derived MSCs. LDL receptor expression was demonstrated by gene expression and immunofluorescence in all primary hepatocyte cultures, undifferentiated canine bone marrow mesenchymal stromal cells (MSCs) and canine adipose MSCs. Undifferentiated human and mouse bone marrow MSCs also expressed the LDL receptor. In vitro, canine hepatocytes took up labelled LDL but not acetylated LDL. All undifferentiated MSCs took up LDL but not acetylated LDL. In conclusion, LDL and not acetylated LDL is a test of canine hepatocyte-like phenotype but this is not tissue or species specific and therefore is not informative assay when testing proof of MSC to hepatocyte differentiation.

Key Words: hepatic, canine, MSC, mesenchymal stromal cells

Abbreviations

AcLDL – Acetylated Low density lipoprotein

Ad-MSC – Adipose mesenchymal stromal cells

BM-MSC – Bone marrow mesenchymal stromal cells

DMEM – Dulbecco's modified Eagle's media

EBSS – Earle's balanced salt solution

EGTA - Ethylene glycol tetraacetic acid

FBS – Foetal bovine serum

LDL – Low density lipoprotein

MSCs – Mesenchymal stromal cells

PBS – phosphate buffered saline

WME – William's Media E

Introduction

There has been much interest in production of hepatocyte-like cells from stem cell sources [1]. In human medicine the resultant cells would be anticipated to have a variety of uses such as: transplantation, in bio-artificial liver devices to support patients until a transplant was available, or allow sufficient regeneration in cases of acute hepatic failure; as an in vitro model of liver disease; or as an in vitro system for modelling hepatic metabolism of candidate drugs [2]. In veterinary medicine, although all these potential uses are present, for many the expense and logistics may be prohibitive.

The pathophysiology of many spontaneous canine diseases has been shown to be similar to human clinical conditions for example, specifically in hepatology: hepatic encephalopathy [3] and fibrotic liver disease [4,5]. The ability to culture autologous stem cells, differentiate and re-implant into a large animal model with a comparable spontaneous disease niche to human cases is highly attractive. As a result, dogs are now recognised as a useful large animal translational model in the step between laboratory disease models and human clinical cases [6]. Use of animals with spontaneous disease also allows a reduction in experimental animal use.

An additional area that would be immediately attractive is *in vitro* drug modelling to reduce the cost of development, animal experimentation and failure at phase III clinical trials [7-9]. The ability to produce in vitro canine hepatocytes with species specific CYP profiles would allow rapid screening of candidate drugs for potential hepatotoxicity not only of the parent drug but also the hepatic metabolic products. This would greatly reduce the cost of drug development for the veterinary market but also contribute to reducing the number of animals required in pre-clinical testing. Furthermore, the ability to screen different breed phenotypes *in vitro* would hopefully avoid idiosyncratic breed reactions once the drug has been licensed, reducing the number of drugs withdrawn from the market and also reducing canine patients harmed.

Isolation and culture of primary hepatocytes was first described over 40 years ago, although culture conditions which allow mature hepatocyte division *in vitro* have not been achieved, and hepatocytes in culture do not multiply to any appreciable extent [10]. Furthermore, the majority of functions that would be desired of a hepatocyte e.g. protein production, CYP activity are lost rapidly in vitro [11]. A final problem with primary hepatocyte cultures is batch variability; depending on donor source and time between death and processing [12]. As a result, the continual sacrifice of animals to supply *in vitro* hepatocytes is not ideal, especially at a time when much effort is being directed towards the

mantra of the 3 R's (replace, reduce, refine) in animal research. Stem cells can be isolated without the need for animal sacrifice and have a high proliferative capacity. There has been much interest in differentiation of stem cells towards a hepatocyte phenotype [1]. This would allow stem cells to be isolated from a phenotypically diverse canine population and be differentiated towards hepatocytes as required.

Of the potential stem cell sources of hepatocyte-like cells, mesenchymal stromal cells (MSCs) hold the most immediate promise in veterinary medicine. As MSCs can be isolated from almost any tissue, differences in resultant functionality from different tissue sources can be assessed [13]. Most commonly adipose and bone marrow are used as sources and can be expanded to large numbers [14,15].

In producing hepatocyte-like cells, there is the question of how to characterise the resultant cells. Hepatocyte functionality is complex and as a result, a wide range of tests have been described including qualitative assays such as morphology and gene expression as well as functional assays including albumin production, urea synthesis and low-density lipoprotein (LDL) uptake [16]. One difficulty is that no one test is considered specific for hepatocytes, for example, although albumin is often used, gene expression has been demonstrated in pancreas, kidney, bone and microglial cells, synthesis in bone and microglia and human BM-MSC and human Ad-MSC [17-21]. A minimum panel required for differentiated cells to be classified as a hepatocyte has been defined by Hengstler et al [22]. As it is technically demanding to provide a complete panel of testing, demonstrating one each of storage, metabolic and synthetic function is commonly accepted as proof of hepatocyte-like phenotype [23].

One of these functional assays is low density lipoprotein uptake. LDL is a transport molecule for lipids, including cholesterol and triglycerides, in extracellular fluid. Specific LDL receptors on the cell membrane bind LDL and allow endocytosis. *In vivo*, most LDL receptors are present on hepatocytes to supply cholesterol for bile secretion, conversion to bile acids and production of *de novo* lipoproteins [24]. Commercially, two types of fluorescently tagged LDL are available, native LDL and acetylated LDL (AcLDL), which is a synthetic analogue of naturally occurring oxidised LDL. Acetylated LDL (and oxidised LDL) is not recognised by the LDL receptor but is taken up by scavenging receptors, present chiefly on macrophages and endothelial cells [25]. *In vivo*, oxidised LDL is responsible for endothelial dysfunction leading to atherosclerosis [26]. Nahhmias et al. showed that human and rat primary hepatocytes took up LDL but not AcLDL [27]. Harada-Shiba, et al. [28] demonstrated that

culture of wild-type mouse primary hepatocytes avidly took up fluorescently labelled LDL however hepatocytes from LDL receptor knock-out mice failed to do so. In stem cell differentiation to hepatocyte-like cells, LDL uptake is commonly used as part of confirmatory testing, yet despite this apparent clear-cut division, there appears to be confusion. There is variation in whether LDL or AcLDL is used, with many papers describing LDL use in the abstract yet stating use of AcLDL in the materials section[29-32].

The aims of this paper were to define if low density lipoprotein receptors were present on canine hepatocytes in culture by gene expression and immunofluorescence as well as comparing uptake of LDL and acetylated LDL by these canine hepatocytes to define which should be used to demonstrate a hepatocyte-like phenotype when differentiating canine stem cells. Gene expression of the LDL receptor was compared between canine hepatocytes and canine bone marrow and adipose-derived MSC's. Finally LDL receptor expression by immunofluorescence as well as LDL and AcLDL uptake of undifferentiated canine, murine and human BM-MSC and canine Ad-MSC's was examined. Canine hepatocytes were found to take up LDL and not acetylated LDL. Undifferentiated canine, human and murine MSC's also demonstrated specific LDL uptake and the presence of LDL receptors by gene expression and immunofluorescence.

Methods

The methods used in this study were approved by the R(D)SVS Veterinary Ethical Review Committee (Study 70:13).

Primary cell isolation and culture

Isolation of Canine hepatocytes

Hepatocytes were derived from liver tissue obtained post-mortem from dogs euthanased for reasons unrelated to this study. Liver tissue was acquired within 20 minutes of euthanasia. A wedge of hepatic tissue with one cut surface and intact Glisson's capsule was sectioned (between 20-100g), placed in William's Media E with 10% foetal bovine serum (FBS) and 100 U/mL penicillin G and 100 µg/mL streptomycin (WME, Invitrogen, UK) and transported to the laboratory on ice. The digestion protocol was based on the methods described by Seglen [33]. The tissue was rinsed with Earl's balanced salt solution (EBSS, Invitrogen, UK), placed in a Petri dish and 20-22g plastic catheters (Vygon, UK) inserted into the vessels on the cut surface. Chelating buffer (490ml EBSS, 10ml of pH7.4 25mM EGTA (Sigma)) at 37^{o}C was perfused at approximately 6mls/minute was using a 20ml

syringe (BD, UK) for 15 minutes. EBSS was then perfused at the same rate for 10 minutes. Finally collagenase buffer (50mg collagenase type II (Sigma), 200µl 1M$CaCl_2$, 100ml EBSS) was perfused for between 30-45 minutes until the tissue appeared spongy and digested. The liver capsule was then torn and the tissue gently agitated to release dissociated hepatocytes. The cell suspension was filtered through a 70µ cell strainer (BD, UK). The remaining liver tissue was rinsed with William's media E (WME) and this suspension also filtered. The resultant suspension was centrifuged at 350rpm for 3 minutes to pellet mature hepatocytes. The supernatant was removed, the pellet re-suspended and centrifugation repeated.

Hepatocytes were plated at a density of 1 x10^5/cm^2 in WME on collagen-coated wells. After 3 hours to allow attachment, the wells were gently rinsed with PBS at 37°C before Hepatocyte culture medium (Lonza, UK) was added. Hepatocytes were cultured for 24 hours before further experiments were performed.

Canine mesenchymal stromal cell isolation

For cells derived from bone marrow, the distal femoral epiphysis from dogs was removed post-mortem. A Jamshidi bone marrow biopsy needle (Baxter) was inserted into the medullary cavity via the trochanteric fossa and approximately 40mls of Dulbecco's modified Eagle's medium low glucose containing Glutamax-I (DMEM), with 10% FBS and 100 U/mL penicillin G and 100 µg/mL streptomycin (all Invitrogen, UK) (MSC media) injected with the resultant cell suspension collected from the distal segment. This was transported on ice to the laboratory.

The cell suspension was diluted 1:1 with phosphate buffered saline (PBS) and 20mls layered onto 15mls Ficoll Paque Premium (GE Life Sciences) in a 50ml Falcon tube. This was then centrifuged at 450g for 30 minutes without brake. The cell-containing interface was removed to a fresh Falcon tube, PBS added and pelleted at 150g for 5 minutes. The cells were then re-suspended in 30mls of MSC media, transferred to a T150 and incubated at 37°C and 5% CO_2. After 48 hours, media was removed, the flask washed with warmed PBS and fresh media added. Media was changed every 2-3 days and cells passaged once confluent.

For cells derived from adipose tissue, approximately 10g of falciform fat was excised from dogs post-mortem and placed in chilled MSC media. After transport to the laboratory, this was finely chopped into 2-4mm pieces, placed in a 50ml Falcon tube and warmed PBS containing 100 U/mL penicillin G, 100 µg/mL streptomycin and 1mg/ml collagenase Type II (Sigma-Aldrich). This was then incubated with constant shaking at 37°C for 2 hours. 10% FBS was then added to inactivate the enzymes and

the tube centrifuged at 300g for 5 minutes. The cell pellet was re-suspended in MSC media and filtered with 70µm cell strainer (BD Biosciences). The cells were then re-pelleted, suspended in 30ml MSC media transferred to a T150 and incubated at 37^{o}C and 5% CO_2. After 48 hours, media was removed, the flask washed with warmed PBS and fresh media added. Media was changed every 2-3 days and cells passaged once confluent.

Human and Murine mesenchymal stromal cell culture

Mouse and human bone marrow mesenchymal stem cells were purchased from Life technologies (Gibco Cat. No. S1502-100 and A15652 respectively). These were thawed and processed according to the suppliers instructions. 10ml of warm DMEM/F12 medium containing 10% FBS and 100 U/mL penicillin G and 100 µg/mL streptomycin (all Invitrogen, UK) was added to the mouse cells and 10mls of MesenPRO RS™ added to the human cells. Media was changed every 3 days and cells passaged when 80% confluent.

Canine MSC characterisation

Flow cytometry analysis

Cells were suspended in FACS buffer (PBS with 1% bovine serum albumin) at a concentration of 1 x10^{7} cells/ml and 100µl added to 5ml Falcon tubes (BD Biosciences). Primary antibodies and, where required, secondary antibodies are listed in Table 1. For CD11b staining 20µl blocking reagent: 2.4G2 (anti-Fc receptor [BD Pharmingen]) was added for 10 minutes at room temperature prior to antibody addition. After incubation at 4^{o}C in the dark with antibody, cells were washed in FACS buffer by centrifugation at 4^{o}C, 250g, for 5 minutes three times. Samples were re-suspended in 500µl of FACS buffer and kept on ice in the dark for analysis. 1µl of SYTOX–Red (ThermoFisher) was added prior to analysis to allow gating of dead cells. Samples were run on a FACSCalibur and results acquired with CellQuestPro (both BD Biosciences). Post-acquisition analysis was performed using FlowJo (Treestar, USA). Three adipose-derived MSC's, three bone marrow-derived MSC's, all at passage 4 were tested. No antibody and isotype controls were run for each cell type and experiment. Canine peripheral blood mononuclear cells were used as positive control for MHCII, CD45 and CD19 and canine bone marrow-derived macrophages as a positive control for CD11b.

Canine mesenchymal stromal cell trilineage differentiation

Induction of Osteogenesis and Adipogenesis

Three adipose-derived MSC's, three bone marrow-derived MSC's, all at passage 3 were tested. Once 60-70% confluence was achieved, commercially available media was used for adipogenesis and osteogenesis according to the manufacturer's instructions (STEMPRO® Adipogenesis Differentiation Kit and STEMPRO® Osteogenesis Differentiation Kit, Invitrogen, respectively). Control cells were maintained in standard MSC media. Media was aspirated from the wells and the cells rinsed in PBS before being fixed in 10% formaldehyde for 30 minutes. For lipid staining, the wells were then gently rinsed with distilled water then 60% isopropanol added for 5 minutes. This was then aspirated and the Oil Red O solution added. The wells were then incubated for 5 minutes before the solution rinsed with distilled water until the rinse became clear. For osteogenesis, media was aspirated from the wells and the cells rinsed with PBS. Absolute ethanol was added to the wells for 30 minutes then aspirated and the wells allowed to dry. Alizarin red solution was then added for 5 minutes, removed and the wells carefully rinsed three times with distilled water. Wells were examined grossly for staining as well as microscopically.

Induction of Chondrogenesis

Three adipose-derived MSC's, three bone marrow-derived MSC's, all at passage 3 were tested. 2.5 x 10^5 MSC were aliquoted into 1.5ml polypropylene 1.5ml microcentrifuge tubes (Fischer Scientific, UK). These were then spun at 500g for 5 minutes to pellet the cells. MSC media was then aspirated and either fresh MSC media or chondrogenic media added. 5 tubes of each cell and media type were run. After 12 hours the cell pellet was gently detached from the bottom to become free-floating by pipetting. Media was then changed every 2-3 days for 21 days. RNA extraction was performed on 2 pellets in each experiment. The three remaining pellets were fixed in 4% formaldehyde and Toluidine blue staining performed. Pellets were embedded in paraffin wax blocks and sections cut using a microtome. Dewaxing was performed using xylene for 15 minutes, descending concentrations of ethanol (100, 95, 90, 70, and 0%) for 10 minutes each. The sections were rinsed in distilled water and submerged in 1% aqueous Toluidine blue solution (Sigma Aldrich) for 30 minutes at room temperature. The slides were then rinsed in 1 part distilled water and 4 parts 1% HCl in 70% ethanol for 5 seconds. The slides were then rinsed in distilled water, dried and mounted using DPX mountant (VWR, UK).

Analysis of Gene expression

Two adipose-derived MSC's, two bone marrow-derived MSC's all passage three were tested along with 3 sets of freshly dissociated primary hepatocytes cultured for 24 hours. RNeasy mini kit (Qiagen, UK) was used according to the manufacturer's instructions. Cell pellets were disrupted in 350µl of lysis buffer using a Qiashredder (Qiagen, UK). DNA digestion was performed using DNase I (Qiagen, UK) at the recommended point in the RNA extraction protocol. Total RNA was quantified and purity checked using absorbance spectrophotometry at 260 and 280nm (Nanodrop 1000, Thermo, UK).

RNA was converted to cDNA using Omniscript reverse transcription kit (Qiagen, UK) according to the manufacturer's instructions using random nanomers (Sigma-Aldrich, UK) and RNase inhibitor (Promega, UK). 1µg of RNA was added per reaction.

Platinum Sybr Green Qpcr Kit (Invitrogen, UK) was used of all qPCR reactions. Reactions were performed on the Stratagene MX3000P (Agilent, UK). Relative gene expression was performed using cDNA, diluted 1:20 and 9.5µl of this added to each well. Each reaction was performed in triplicate and three no template controls were also performed for each primer using 9.5 µl of nuclease free water. Primers are summarised in Table 2. Cycling conditions were as follows: 2 minutes at 50^{o}C, 2 minutes at 95^{o}C, then 40 cycles of 95^{o}C x 15secs and 60^{o}C x 30secs. The final cycle was 95^{o}C for 1 minute then cooling to 60^{o}C before monitoring for dissociation to 95^{o}C. The dissociation curve produced and lack of amplification of the no template controls were checked using MXPro Software (Agilent, UK).Data was analysed using Microsoft Office Excel 2003 program using the method described by Pfaffl [34] to calculate relative gene expression.

LDL receptor immunofluorescence

Three canine adipose-derived MSC's, three canine bone marrow-derived MSC's as well as human and mouse MSC's were tested along with a canine transitional cell carcinoma cell line. Cells were cultured in four chambered slides (BD Biosciences) until they were approximately 50% confluent. Three sets of canine primary hepatocytes were cultured on collagen type I coated plastic at a seeding density of 1x10^{5}/cm^{2} for 48 hours prior to staining. Media was removed, the cells washed with PBS and then fixed with 4% paraformaldehyde for 30 minutes. The cells were washed with PBS three times, permiabilised with 100% ethanol for 5 minutes before repeating the wash step. Blocking buffer (PBS containing 10% goat serum (Invitrogen) and 0.1% Tween 20 (Sigma) was added for 1 hour at room temperature. This was then aspirated and rabbit anti-human LDL receptor antibody with known cross-reactivity with the dog (ABIN672111, antibodies-online) was diluted to 1:500 with

PBS containing 1% goat serum, 0.1% Tween added. This was then incubated at 4^{o}C overnight and then washed three times in PBST. Fluorescently tagged goat anti-rabbit secondary antibody (Alexa Fluor 594 goat anti-rabbit IgG, Life Technologies), diluted 1:1000 in PBS containing 1% goat serum and 0.1% Tween was added and incubated at room temperature in the dark for 1 hour. Nuclear staining with DAPI was performed as described previously. Secondary only and no antibody controls were also performed for each cell type. For each sample tested, 500 cells were examined and the percentage positively staining was calculated.

DiI-LDL and DiI-AcLDL uptake

Sets of three canine adipose-derived MSC's, three canine bone marrow-derived MSC's, human and mouse MSC's, three canine primary hepatocytes cultured for 48 hours were tested along with a canine transitional cell carcinoma cell line. DiI-LDL and acetylated DiI-LDL (both Invitrogen, UK) were diluted to 20µg/ml in appropriate culture media and added to cell culture wells for 3 hours. Wells were then washed with PBS three times and fixed with 4% paraformaldehyde for 30 minutes. Nuclear staining with DAPI was performed as described previously. The slides were then washed with PBST and examined using fluorescent microscopy. No LDL controls were also performed. For each sample tested, 500 cells were examined and the percentage positively taking up the compound was calculated.

Results

Canine MSC can be derived from bone marrow and adipose tissue and demonstrate trilineage differentiation.

Both canine Ad-MSC and BM-MSC had a similar level of positive staining for CD44 (23.6% and 26.0% respectively (and CD90 (44.6% and 40.0% respectively) (Figure 1 and Table 3). Both cell types were negative for MHCII, CD11b, CD19 and CD45 by flow cytometry (Figure 1 and Table 3). On flow cytometry, both Ad-MSC and BM-MSC were negative for CD105 and STRO-1, which have previously been reported to be expressed by canine BM-MSC using immunocytochemistry [37]. Both Ad-MSC and BM-MSC's appeared strongly positive for both these markers with immunocytochemistry with a negative control demonstrating no staining (Supplementary information 1).

Multiple Oil red-O positively-staining vacuoles were detected in the cytoplasm of both Ad-MSC and BM-MSC after adipocyte differentiation (Figure 2 A&B). Alizarin red staining demonstrated calcification osteogenic differentiation in both cell types (Figure 2 C&D). Metachromatic staining suggestive of cartilage formation was shown after chondrogenic differentiation (Figure 2 E&F). Gene expression analysis revealed upregulation in aggregan but not Sox9 expression (Supplementary information 2).

Canine primary hepatocytes and MSC demonstrate low density lipoprotein receptor expression by real-time PCR and immunofluorescence

Gene expression relative to the three reference genes demonstrated LDL-receptor expression in canine BM-MSC and Ad-MSC of a similar magnitude to canine primary hepatocytes (Figure 3). Of the three primary hepatocyte cultures, relative expression varied from 0.03-0.3. These were hepatocytes in the first 24 hours of culture

LDL receptor expression was documented by immunofluorescence in canine hepatocytes, canine, murine and human BM-MSC's as well as the canine Ad-MSC's (Figure 4). All cell types demonstrated immunofluorescence. The three primary canine hepatocyte cultures median percentage of positive cells was 96.8% (range 94.8- 98.2%). Canine Ad-MSC's median percentage positive was 98.8% (range 98.2 – 99.2%). The canine, human and murine BM-MSC's percentage positive cells were: 98.6% (range 97.8-99.9%), 99.5% (range 99.4-99.8%) and 99.6% (range 99.4-99.8%) respectively. Negative controls demonstrated a low level of background immunofluorescence in the canine primary hepatocytes and canine BM-MSC's. The transitional cell line demonstrated no binding.

Hepatocytes and MSC's take up LDL and not AcLDL

After incubation with DiI-LDL, all canine hepatocytes, BM-MSC and Ad-MSC as well as murine and human MSC demonstrated uptake of this compound whilst the transitional cell carcinoma cells demonstrated no uptake (Figure 5). After incubation with AcLDL, none of the canine, murine or human MSC's were positive although some fluorescent extracellular debris was noted (Figure 6). Within the canine hepatocyte cultures although the vast majority of cells showed no uptake, there were sporadic individual cells which demonstrated avid uptake of AcLDL (Figure 6A demonstrates a cluster of these cells). Overall, a median of 4.5% of the canine primary hepatocyte culture cells were positive for AcLDL uptake (range 3.2-6.7%).

Discussion

From these results, canine primary hepatocytes in culture have LDL receptor expression as demonstrated by gene expression and also immunofluorescence. Comparing uptake of LDL and AcLDL in the hepatocyte culture demonstrates that LDL uptake is mediated by this receptor as only LDL and not AcLDL is taken up by the hepatocytes. Therefore, uptake of LDL and not AcLDL should be used as part of the demonstration of hepatocyte-like function for canine cells. This is consistent with the previously reported function of human and mouse primary hepatocytes [27,28]. It is of note that sporadic cells in the hepatocytes culture plates took up AcLDL avidly and it is likely that these are either Kuppfer cells or endothelial cells which were isolated along with hepatocytes from the liver digestion process. Both these cell types are reported to contain the scavenging receptors which endocytose acetylated or oxidised LDL [25,35]. This is consistent with the report by Babaev, et al. [36] who found that human primary hepatocyte cultures almost uniformly took up LDL with only 5% of cells in culture taking up modified LDL. These were identified as macrophages or endothelial cells based on their ability to take up tagged formaldehyde-treated albumin and carboxylates microspheres. In the present study only 4.5% of cells in the primary hepatocyte cultures took up AcLDL, a similar number to the previous study.

The isolated and cultured canine BM-MSC and Ad-MSC's demonstrated adipogenic and osteogenic potential. We have previously demonstrated isolation and characterisation of canine BM-MSC, including the demonstration of chondrogenic differentiation was based on toluidine blue staining, increased collagen type II gene expression and Sox9 immunostaining [37]. In the present study, Toludine blue metachromatic staining of cartilage pellets, although present, was less dramatic than that demonstrated by Requicha et al. [38]. Both cell types also demonstrated significant increases in aggrecan gene expression, the major proteoglycan in articular cartilage produced by chondrocytes [39]. Interestingly, both cell types showed no increase in Sox9 expression, which is the master-regulator of chondrogenesis. As one of the functions of Sox9 is to bind to the aggrecan promoter and upregulate aggrecan expression, this appears an unusual result [40]. One possible explanation for this apparent paradox is that gene expression during differentiation is dynamic and Sox9 gene expression is shut-off during final maturation of chondrocytes into hypertrophic chondrocytes[41]. Therefore it is possible up-regulation of Sox9 was missed in these samples. Another possibility is

that chondrogenesis is dysregulated and stimulation of gene expression is down-stream of Sox9 expression. From the literature there appears to be variation in Sox9 expression. Reich et al. detected an increase in Sox9 with BM-MSC but a decrease with Ad-MSC [42]. This corroborated with more convincing cartilage formation in BM-MSC by histology and up-regulation of Collagen 2A1 only in BM-MSC. Other authors have demonstrated convincing chondrogenic differentiation of both canine Ad-MSC and BM-MSC with Sox9 expression and also convincing chondrogenic differentiation with no Sox9 up-regulation [38,43,44]. However other studies have failed to demonstrate chondrogenesis in canine MSC's [45,46]. These disparate results need to be viewed with knowledge that a donor age-related reduction in differentiation ability of MSCs has been demonstrated, along with variation in ability according to donor site and passage number [43,47-49].

In this study, both Ad-MSC and BM-MSc demonstrated no expression of CD105 or STRO-1 by flow-cytometry yet appeared positive on immunocytochemistry. Canine BM-MSC has previously reported to be positive for CD105 and STRO-1 by immunocytochemistry [37], however Screven et al. found both canine Ad-MSC and BM-MSC to be negative on flow-cytometry but positive for CD105 gene expression by real-time PCR [50]. It is possible that the antibody is not suitable for flow-cytometry however further investigation will be required to define if CD105 expression is a useful marker for canine MSC's.

Both undifferentiated canine BM-MSC and Ad-MSC demonstrated LDL receptor gene expression of a comparable magnitude to canine primary hepatocytes. Furthermore, undifferentiated canine, mouse and human BM-MSCs and also undifferentiated canine AD-MSCs stained for the presence of the LDL receptor by immunocytochemistry. Functionality of the receptor was confirmed by LDL uptake and not AcLDL uptake. Therefore it would appear that for differentiation studies producing hepatocyte-like cells, confirming LDL uptake is not specific for a hepatocyte phenotype and in addition it should not be used for MSC to hepatocyte differentiation studies.

It has been demonstrated that mouse BM-MSC express LDL-oxidised receptors (LOX-1) and take up DiI-oxidised-LDL (the biological analogue to acetylated-LDL) and also express LOX-1 by gene expression and Western blot, which is in contrast to the present study, where all MSC's tested (canine Ad-MSC and canine , murine and human BM-MSC) were found not to take up AcLDL [51,52]. These apparent differences may be an artefact of culture conditions rather than a fundamental cellular difference as Liesveld et al. showed that human BM-MSC took up DiI-LDL in standard McCoy's media with 10% FCS, whilst culture in McCoy's' with 25% serum and 1µmol/L hydrocortisone, this reduced, and DiI-AcLDL uptake increased [53]. Both the mouse and human BM-

MSC's were cultured under the conditions recommended by the supplier and produced identical results to the canine MSC's i.e. no AcLDL uptake but avid LDL uptake.

In line with the theory that MSC are fibroblast populations[54], it has been demonstrated that fibroblasts express the LDL receptor, take up LDL but not modified LDL[55-58]. Therefore in this respect, the presence of LDL receptors and specific uptake of LDL on MSC's is not surprising.

In summary, LDL receptor is present on canine primary hepatocytes in culture. LDL and not AcLDL uptake is a function of canine primary hepatocytes. LDL receptor expression and LDL uptake is not specific to a hepatocyte phenotype and undifferentiated MSC's express the LDL receptor and endocytose LDL. Assessing the effect of culture conditions on LDL receptor expression and the cells ability to take up LDL and AcLDL would be useful to assess if canine MSC demonstrate the same plasticity as human BM-MSC in regard to LDL and AcLDL uptake.

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Author Disclosure Statement

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Table Legends

Marker	Primary antibody	Dilution	Isotype	Flurophore /secondary
MHCII	MCA1044GA, AbD Serotec	1:50	IgG2a	FITC
CD11b	MCA1777S, AbD Serotec	1:10	IgG1	Goat anti mouse Alexa Fluor 488 A-11001, Life technologies
CD19	ab24936, Abcam	1:100	IgA	FITC
CD44	MCA1041GA	1:10	IgG2a	Goat anti rat Alexa Fluor 488 A-11006, Life Technologies
CD45	MCA1042G, AbD Serotec	1:10	IgG2	Phycoerythrin
CD90	MCA1036G, AbD Serotec	1:20	1Gg2b	Goat anti rat Alexa Fluor 488 A-11006, Life Technologies
CD105	orb10285 Biorbyt	1:50	Polyclonal rabbit	Goat anti rabbit Alexa Fluor 488, Life Technologies
STRO1	MAB1038 R&D Systems	1:100	IgM	Goat anti-mouse IgG A-11001 (Life technologies)

Table 1 *Cell surface markers and antibodies used for flow cytometry*

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Gene	Primer Sequences	PCR Amplicon length (base pairs)	Genebank Accession number	Primer Sequence Reference
HPRT	Forward: AGCTTGCTGGTGAAAAGGAC Reverse: TTATAGTCAAGGGCATATCC	104	XM_005625362	Brinkhof et al. (2006)
B2MG	Forward: TCCTCATCC TCCTCGCT Reverse: TTCTCTGCTGGGTGTCG	85	XM_535458	Brinkhof et al. (2006)
RPL8	Forward: CCATGAATCCTGTGGAGC Reverse: GTAGAGGGTTTGCCGAT	64	XM_532360	Brinkhof et al. (2006)
LDL-Receptor	Forward: ATTGTGGTGGATCCCGTGC Reverse: GAAAGATCCAGGGTGATGCCATT	149	XM_00632869.1	---

Table 2 *Primers used in quantitative polymerase chain reaction*

Table 2 *Primers used in quantitative polymerase chain reaction*

Cell Type	Marker	Mean Percent positive	Standard Deviation
Ad-MSC	CD90	43.3	2.6
	CD44	21.6	2.2
	CD105	0.34	0.17
	CD45	0.19	0.02
	STRO-1	0.44	0.22
	CD11b	0.48	0.22
	CD19	0.51	0.29
	MHCII	0.55	0.51
BM-MSC	CD90	39.5	3.4
	CD44	27.3	3.8
	CD105	0.8	0.18
	CD45	0.27	0.13
	STRO-1	0.64	0.32
	CD11b	0.88	0.30
	CD19	0.81	0.02
	MHCII	0.66	0.37

Table 3 Results of flow cytometry for cell surface markers. *The mean percent positive from 3 sets of Ad-MSC and BM-MSC with standard deviation. Both MSC cells types were positive for CD90 and CD44 and negative for CD11b, CD19, CD45, MHCII, STRO-1 and CD105.*

Table 3 Results of flow cytometry for cell surface markers. *The mean percent positive from 3 sets of Ad-MSC and BM-MSC with standard deviation. Both MSC cells types were positive for CD90 and CD44 and negative for CD11b, CD19, CD45, MHCII, STRO-1 and CD105.*

Figure Legends

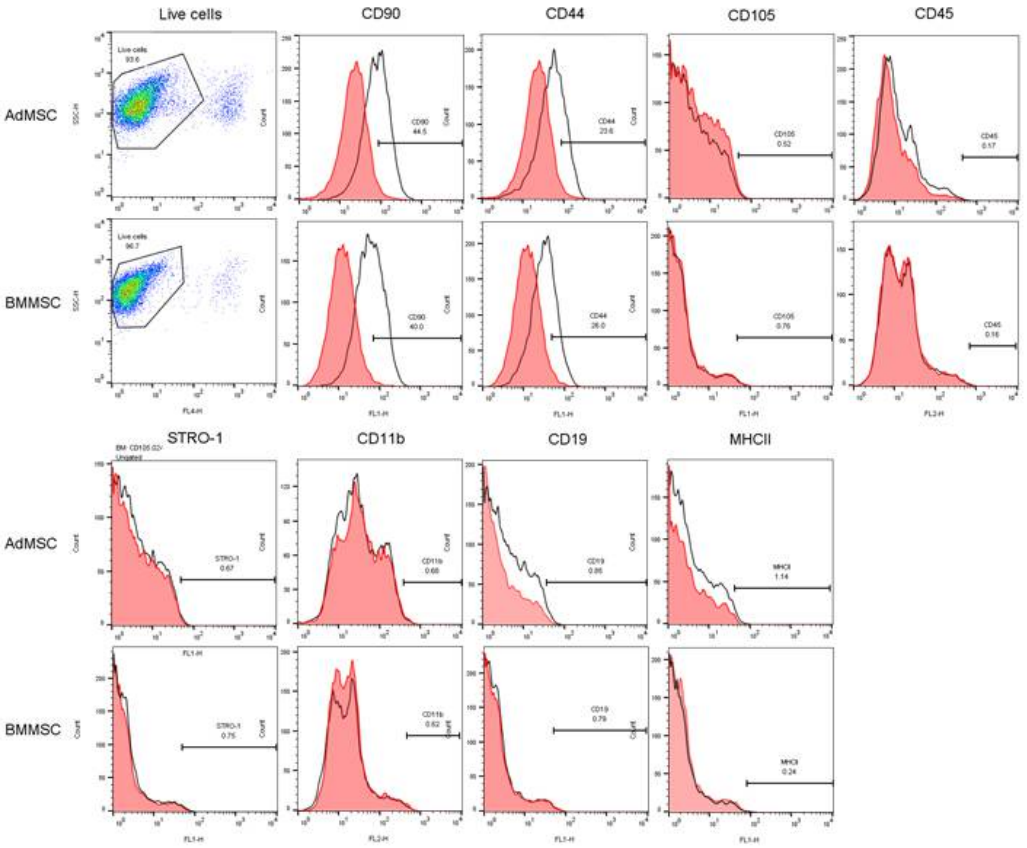
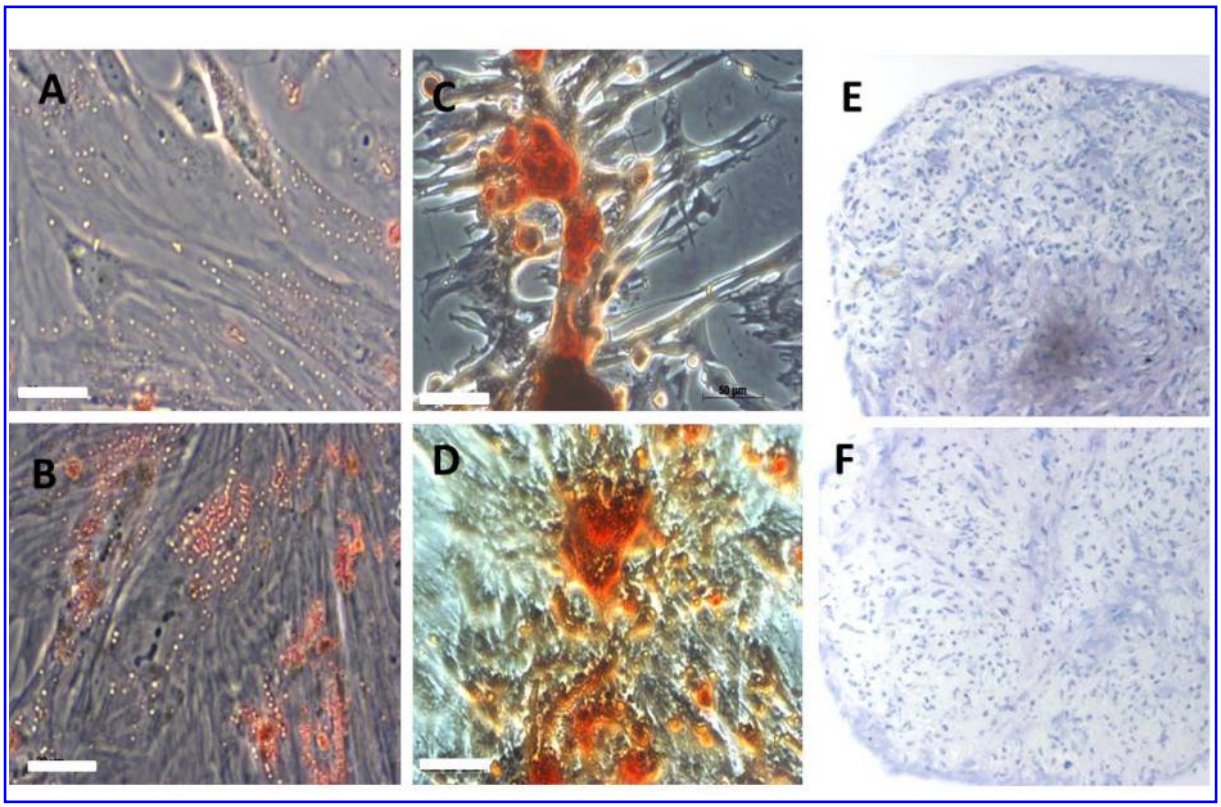


Figure 1: Expression of cell surface molecules in canine MSC's by flow cytometry
Showing live cell gating and representative histogram demonstrating canine adipose and bone marrow MSC cell surface molecule expression. The shaded area represents the negative isotype control and open line represent sample labelled with the indicated cell surface molecule.



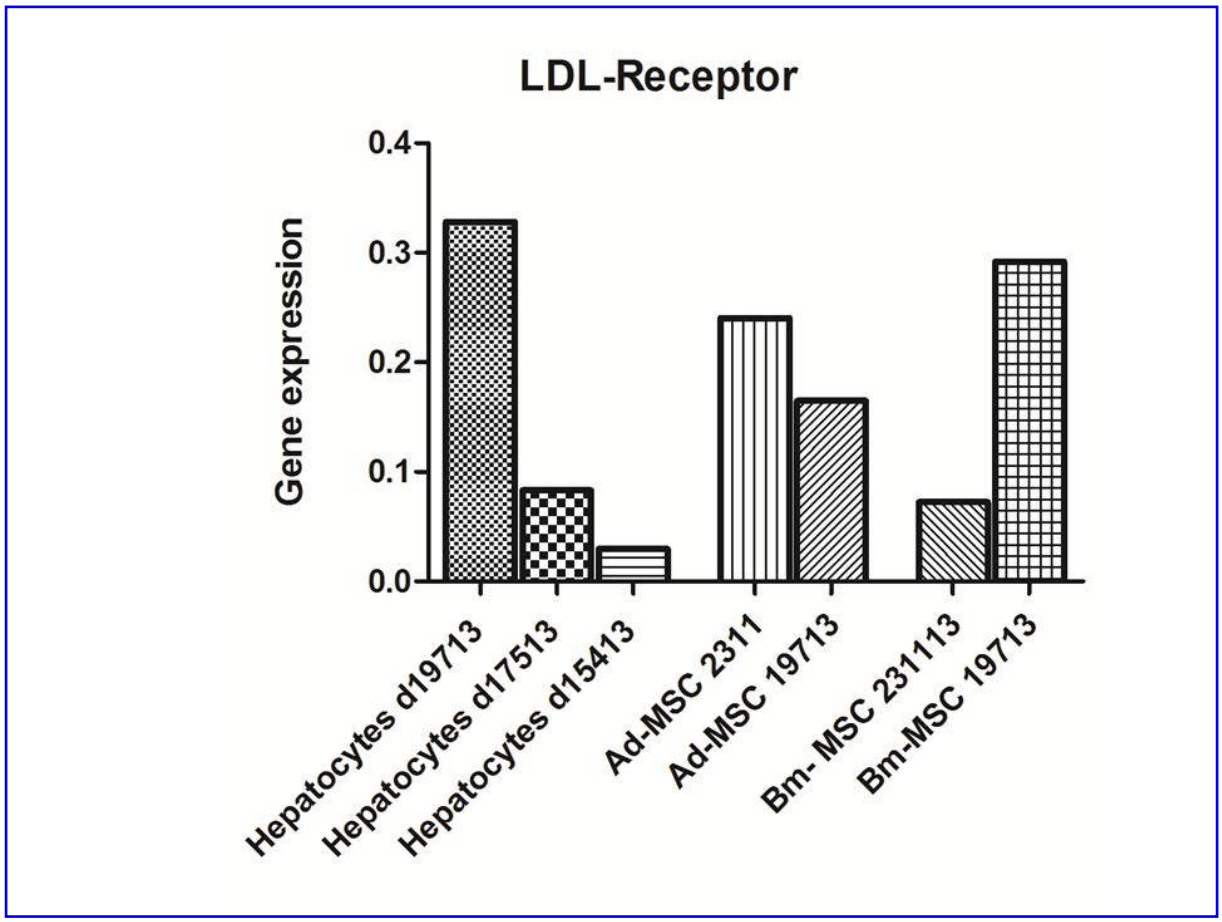


Figure 2 Adipogenic, osteogenic and chrondrogenic differentiation of canine MSC's
Oil red-O after adipogenic differentiation BM-MSC (A), Ad-MSC (B). Alizarin red staining after osteogenic differentiation BM-MSC (C), Ad-MSC (D). Toludine blue staining after chondrogenic differentiation BM-MSC (E), Ad-MSC (F). White bar represents 25µM.

Figure 3: LDL-receptor gene expression in canine primary hepatocytes and Ad-MSC and BM-MSC
Relative gene expression of LDL receptor normalised to three reference genes (B2MG, RPL8, HPRT). Three sets of fresh cultured canine primary hepatocytes and two each of Ad-MSC and BM-MSC cultures. Three replicates per cell type were performed.

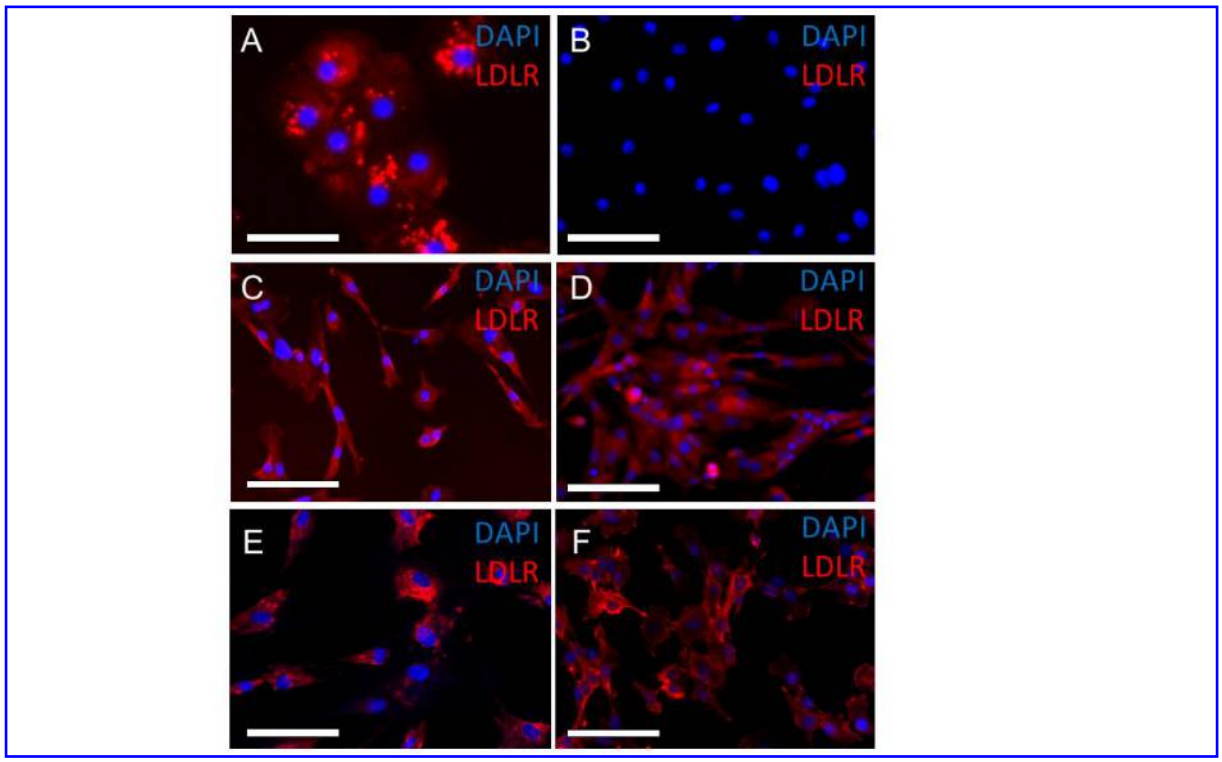


Figure 4: LDL receptor immunofluorescence
Canine hepatocytes (A), Canine transitional cell carcinoma (B), Ad-MSC (C), BM-MSC (D), human BM-MSC (E) and murine BM-MSC (F). Nuclei stained with DAPI. White bar represents 100µm. Canine hepatocytes showed membrane binding and intense fluorescent intra-cytoplasmic accumulations. All MSC's demonstrated strong binding of LDL receptor antibody. Secondary antibody negative controls showed no fluorescence (not shown). Canine transitional cell carcinoma cells demonstrated no antibody binding.

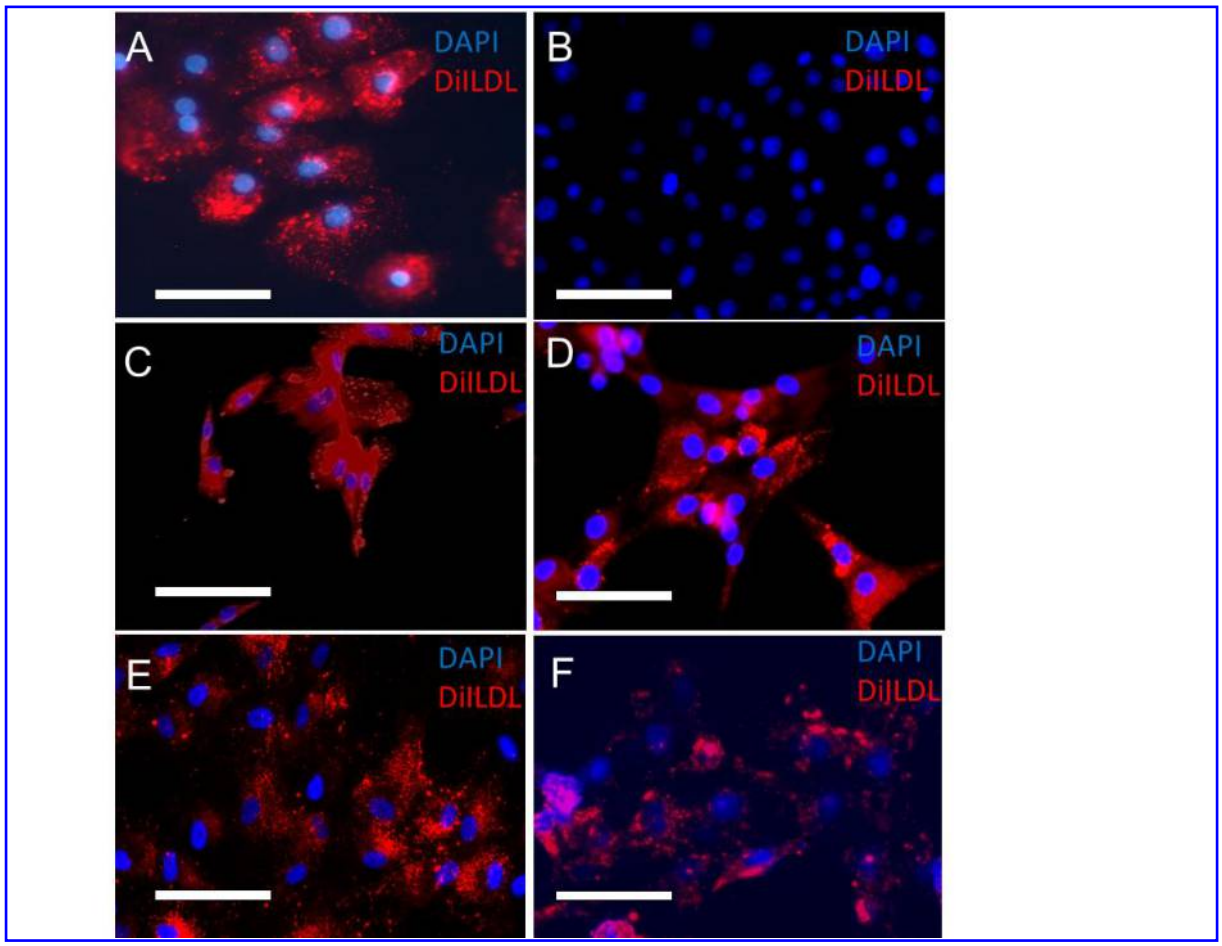


Figure 5: DiI-LDL uptake in canine primary hepatocytes and MSC's
Canine hepatocytes (A), Canine transitional cell carcinoma (B), Ad-MSC (C), BM-MSC (D), human BM-MSC (E) and murine BM-MSC (F). Nuclei stained with DAPI. White bar represents 100µm. Canine hepatocytes and all MSC's demonstrated strong uptake of DiI-LDL. Canine transitional cell carcinoma cells showed no uptake.

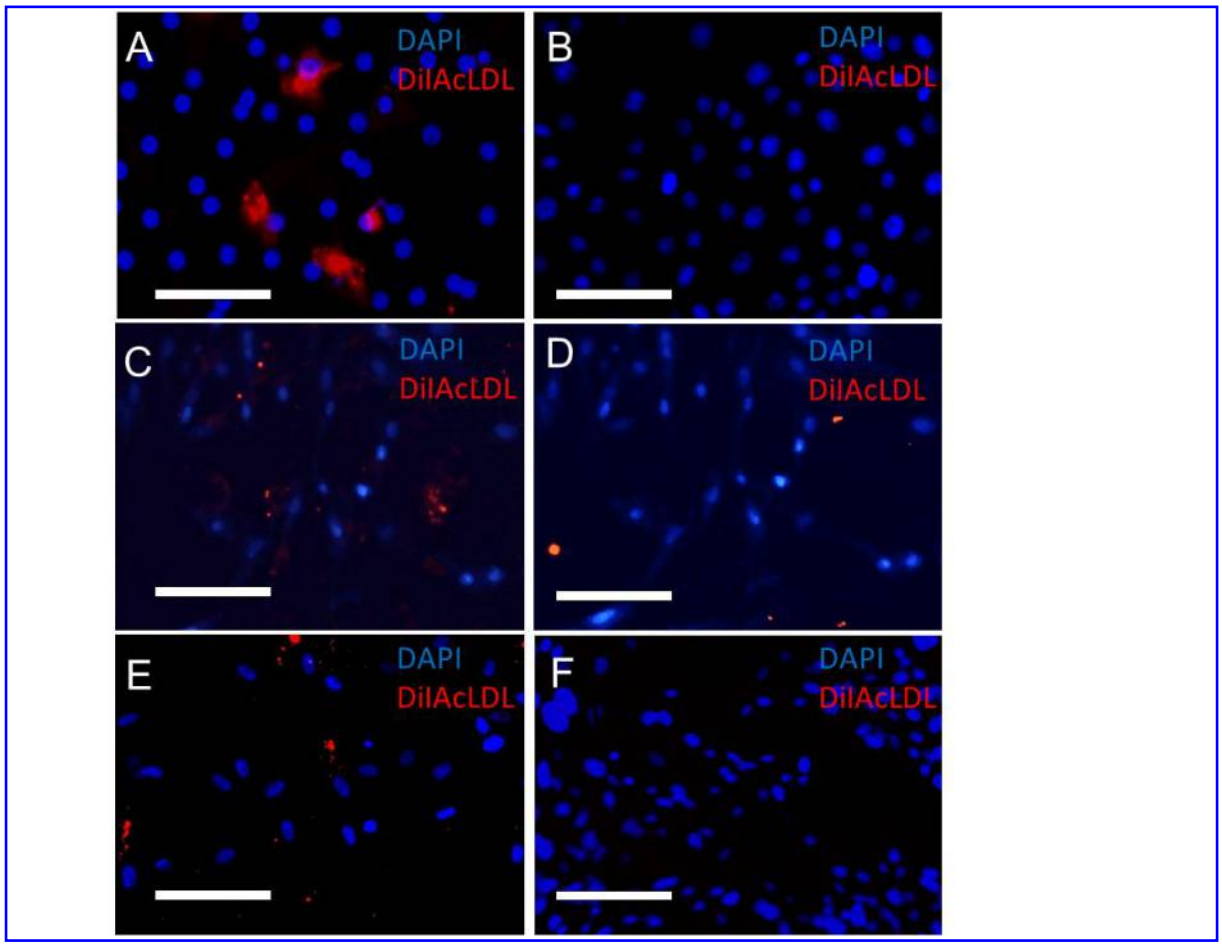


Figure 6: DiI-AcLDL uptake in canine primary hepatocytes and MSC's
Canine hepatocytes (A), Canine transitional cell carcinoma (B), Ad-MSC (C), BM-MSC (D), human BM-MSC (E) and murine BM-MSC (F). Nuclei stained with DAPI. White bar represents 100µm. In the canine hepatocyte cultures, a few sporadic cells demonstrated avid AcLDL uptake however the vast majority of cells showed no uptake. No uptake was seen in all MSC's or transitional cell cultures with only some extracellular fluorescent debris seen.

Supplementary information 1

Immunocytochemistry of canine MSC for STRO-1 and CD105

Antibody information

Marker	Primary antibody	Dilution	Secondary antibody	Dilution	Flurophore
STRO1	IgM mouse anti-human MAB1038 (R&D Systems)	1:100	Goat anti-mouse IgG A-11001 (Life technologies)	1:500	Alexa Fluor® 488
CD105	Polyclonal rabbit orb10285 (Biorbyt)	1:50	Goat anti-rabbit IgG A-11008 (Life technologies)	1:500	Alexa Fluor® 488

Protocol

Canine Ad-MSC, BM-MSC and canine transitional cell carcinoma cells were used and experiments were performed in triplicate. Cells were cultured in four chambered slides (BD Biosciences) until they were approximately 50% confluent. Media was removed, the cells washed with PBS and then fixed with 4% paraformaldehyde for 30 minutes. The cells were washed with PBS three times, permiabilised with 100% ethanol for 5 minutes before repeating the wash step. Blocking buffer (PBS containing 10% goat serum (Invitrogen) and 0.1% Tween 20 (Sigma-Aldrich, UK) was added for 1 hour at room temperature. This was then aspirated and the appropriate dilution of antibody in PBS containing 1% goat serum, 0.1% Tween added (Table 2). Slides were incubated at 4^{o}C overnight and then washed three times. The secondary antibody, again diluted in PBS containing 1% goat serum and 0.1% Tween was added and incubated at room temperature in the dark for 1 hour. Slides were then rinsed three times with PBST and 4', 6-diamidino-2-phenylindole (DAPI) at a concentration of 200ng/ml for 30 minutes added. The slides were then washed with PBST and examined using fluorescent microscopy (Zeiss, Aviovert 40) and Zeiss Aviovision 4.7 software. Secondary only and no antibody controls were also performed for each cell type and antibody.

Results

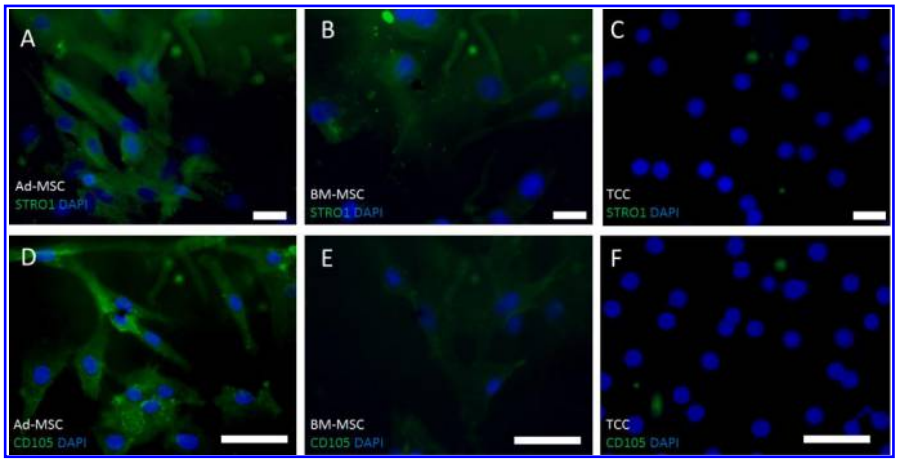


Figure Legend: STRO1 and CD105 immunocytochemistry of canine MSC's

Representative images of canine Ad-MSC and BM-MSC demonstrating positive staining for STRO1 and CD105. TCC – canine transitional cell carcinoma cell line, demonstrating no staining for both markers.

Supplementary Information 2

Chondrocyte gene expression of chondrogenic differentiation of canine MSC's

Primer sequences

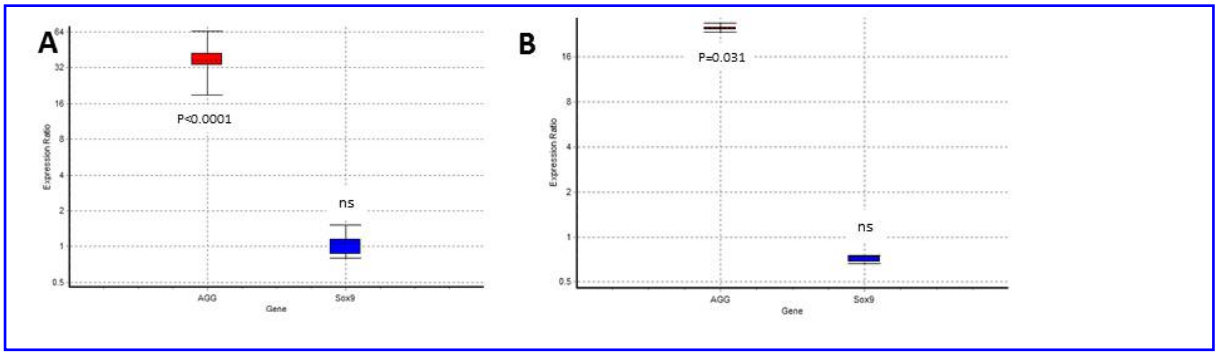
Gene	Primer Sequences	PCR Amplicon length (base pairs)	Genebank Accession number
HPRT	Forward: AGCTTGCTGGTGAAAAGGAC Reverse: TTATAGTCAAGGGCATATCC	104	XM_005625362
B2MG	Forward: TCCTCATCC TCCTCGCT Reverse: TTCTCTGCTGGGTGTCG	85	XM_535458
RPL8	Forward: CCATGAATCCTGTGGAGC Reverse: GTAGAGGGTTTGCCGAT	64	XM_532360
Aggrecan	Forward: ATCAACAGTGCTTACCAAGACA Reverse: ATAACCTCACAGCGATAGATCC	122	XM_005618252.1
Sox9	Forward: GCTCGCAGTACGACTACACTGAC Reverse: GTTCATGTAGGTGAAGGTGGAG	101	NM_001002978.1

RNeasy mini kit (Qiagen, UK) was used according to the manufacturer's instructions. Cell pellets were disrupted in 350µl of lysis buffer using a Qiashredder (Qiagen, UK). DNA digestion was performed using DNase I (Qiagen, UK) at the recommended point in the RNA extraction protocol. Total RNA was quantified and purity checked using absorbance spectrophotometry at 260 and 280nm (Nanodrop 1000, Thermo, UK).

RNA was converted to cDNA using Omniscript reverse transcription kit (Qiagen, UK) according to the manufacturer's instructions using random nanomers (Sigma-Aldrich, UK) and RNase inhibitor (Promega, UK). 1µg of RNA was added per reaction.

Platinum Sybr Green Qpcr Kit (Invitrogen, UK) was used of all qPCR reactions. Reactions were performed on the Stratagene MX3000P (Agilent, UK). Relative gene expression was performed using cDNA, diluted 1:20 and 9.5µl of this added to each well. Each reaction was performed in triplicate and three no template controls were also performed for each primer using 9.5 µl of nuclease free water. Primers are summarised in Table 3. Cycling conditions were as follows: 2 minutes at 50^{o}C, 2 minutes at 95^{o}C, then 40 cycles of 95^{o}C x 15secs and 60^{o}C x 30secs. The final cycle was 95^{o}C for 1 minute then cooling to 60^{o}C before monitoring for dissociation to 95^{o}C. The dissociation curve produced and lack of amplification of the no template controls were checked using MXPro Software (Agilent, UK).Relative gene expression compared undifferentiated MSC and Chondrocyte differentiated MSC. Rest 2009 gene expression software was used for analysis (http://rest-2009.gene-quantification.info).

Agrecan and Sox9 were compared to the three reference genes.



A – AD-MSC chondrocyte differentiation compared to undifferentied AD-MSC.
B – BM-MSC chondrocyte differentiation compared to undifferentied BM-MSC
AGG - Aggrecan