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# Investigation of the Optimal Timing for Chondrogenic Priming of MSCs to Enhance Osteogenic Differentiation In Vitro as a Bone Tissue Engineering Strategy.

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**Abstract**

Recent in vitro tissue engineering approaches have shown that chondrogenic priming of human bone marrow MSCs can have a positive effect on osteogenesis in vivo. However, whether chondrogenic priming is an effective in vitro bone regeneration strategy is not yet known. In particular, the appropriate timing for chondrogenic priming in vitro is unknown albeit that in vivo cartilage formation persists for a specific period before bone formation. The objective of this study is to determine the optimum time for chondrogenic priming of MSCs to enhance osteogenic differentiation by MSCs in vitro. Pellets derived from murine and human MSCs were cultured in six different media groups; two control groups (chondrogenic and osteogenic) and four chondrogenic priming groups (10, 14, 21 and 28 days priming). Biochemical analyses (Hoechst, sGAG, ALP, Calcium), histology (Alcian Blue, Alizarin Red) and immunohistochemistry (Collagen type I, II, X) were performed on the samples at specific times. Our results show that after 49 days the highest amount of sGAG production occurred in MSCs chondrogenically primed for 21 and 28 days. Moreover we find that chondrogenic priming of MSCs in vitro for specific amount of time (14, 21 days) can have the optimum influence on their mineralisation capacity and can produce a construct that is mineralised throughout the core. Determining the optimum time for chondrogenic priming to enhance osteogenic differentiation in vitro provides information that might lead to a novel regenerative treatment for large bone defects, as well as addressing the major limitation of core degradation and construct failure.

## 1. Introduction

Current clinical treatments for bone defects and extensive fractures involve autologous tissue transplantation or implantation of vascularised bone grafts (2012, Cancedda R, *et al.*, 2007, Dawson JI, *et al.*, 2008, Rose FR, *et al.*, 2002), but these approaches are limited in their ability to ensure union of large bone defects and are also associated with high cost and risk of infection (2012, Cancedda R, *et al.*, 2007, Dawson JI, *et al.*, 2008, Rose FR, *et al.*, 2002). Bone tissue engineering with bone marrow derived mesenchymal stem cells (MSCs) is a promising strategy for treating bone diseases and reconstructing bone defects (Cancedda R, *et al.*, 2007, Dawson JI, *et al.*, 2008, Rose FR, *et al.*, 2002). Bone marrow derived mesenchymal stem cells can produce cartilage tissue when chondrogenically primed in vitro and implanted in animals (Hwang NS, *et al.*, 2011, Lin J, *et al.*, 2006, Pelttari K, *et al.*, 2008). Although MSC's have shown promise for producing a bone-like matrix (Bruder SP, *et al.*, 1998, Ohgushi H, *et al.*, 1989), existing approaches are limited as they are unable to produce functional bone tissue for clinical use in load bearing locations (Meijer GJ, *et al.*, 2008) and it has been shown that under certain conditions MSCs act as a barrier to healing in rodent cranial defects (Lyons FG, *et al.*, 2010). The use of MSCs for osteogenesis has not been fully optimised to regenerate bone tissue in vitro that can be used clinically to replace degenerated bone (Smith JO, *et al.*, 2011).

A possible solution to overcome such limitations and harness the osteogenic potential of MSCs is to mimic bone development during early fetal development and recreate it in vitro. In the embryo rudimentary bones are formed by two distinct mechanisms; endochondral ossification or intramembranous ossification. Endochondral ossification governs the formation of long bones and is often responsible for fracture repair in the adult skeleton, and as such is an essential biological mechanism governing bone formation. This process begins when mesenchymal stem cells condense to become clusters of cells

(Kronenberg HM, 2003, Mackie EJ, *et al.*, 2008, McNamara L, 2011), differentiate into chondroblasts, secrete a cartilaginous matrix and form a membrane around this cartilage template known as the perichondrium. Chondroblasts then differentiate to become chondrocytes and proliferate to grow the cartilage template (Kronenberg HM, 2003, Mackie EJ, *et al.*, 2008, McNamara L, 2011), after which they become hypertrophic. Perichondrial cells differentiate into osteoblasts and begin to form periochondrium and bone matrix, whilst hypertrophic chondrocytes form an extracellular matrix (ECM) that attracts blood vessels and eventually undergo apoptosis. Further growth occurs within the secondary ossification centres and is regulated by the epiphyseal plate (Kronenberg HM, 2003, Mackie EJ, *et al.*, 2008, McNamara L, 2011).

Recent studies have sought to mimic the initial stages of endochondral ossification (i.e. the production of the cartilage template) as a bone regeneration strategy to overcome issues with poor oxygen and nutrient supply in tissue engineered constructs (Checa S, *et al.*, 2010, Coyle CH, *et al.*, 2009, El-Serafi AT, *et al.*, 2011, Farrell E, *et al.*, 2009, Gawlitta D, *et al.*, 2010). Chondrogenic priming of MSCs in vitro prior to in vivo implantation promotes healing in an osteochondral defect model (Miot S, *et al.*, 2012), and it has been shown that chondrogenically primed constructs subsequently mineralised following subcutaneous implantation (Farrell E, *et al.*, 2011, Farrell E, *et al.*, 2009, Jukes JM, *et al.*, 2008). It has been shown that mineralisation occurred in constructs cultured for 21 days in chondrogenic media and 14 days in osteogenic media without TGF-β1 before implantation, but not in constructs primed for different durations (Scotti C, *et al.*, 2010). However these studies did not provide tissue appropriate for clinical use, due to issues with core degradation and an uneven distribution of bone mineral throughout the construct (Farrell E, *et al.*, 2011, Farrell E, *et al.*, 2009, Scotti C, *et al.*, 2010). Therefore, although chondrogenic priming may have potential as an in vitro bone tissue regeneration strategy, approaches must be further optimised to

overcome these limitations.

In vivo endochondral ossification is a tightly regulated process, which relies on the establishment of a cartilaginous template prior to vessel invasion and bone formation. It is likely that the timing of cartilage template formation is important for bone formation by endochondral ossification. Embryologists have estimated that cartilaginous limb buds are first seen in humans by the 26$^{th}$ day (Merz E, *et al.*, 2004, Smith A, 1968, Webster S, 2012), whereas in vivo studies show that bone formation begins in chick bones when the embryo is 9-18 days old (Hall BK, 1987, Hunziker EB, *et al.*, 1989, Nowlan NC, *et al.*, 2007, Roach HI, 1992, Roach HI, 1997). One study has shown that the timing of media supplementation is important for mineralisation of bone tissue constructs in vivo (Scotti C, *et al.*, 2010). However, to date, no study has investigated the role of timing of chondrogenic priming on the mineralisation capacity of MSCs in vitro. An in vitro approach that optimises the time for the chondrogenic priming process in vitro so as to mimic in vivo endochondral ossification might serve as an effective bone tissue regeneration strategy.

In this study we hypothesise that there is an optimum time for chondrogenic priming of MSCs in vitro that will enhance osteogenic differentiation. The specific objectives were to investigate the effects of treatment with chondrogenic and osteogenic media for various durations on (1) DNA content, (2) sGAG production, (3) Collagen content, (4) ALP production and (5) Calcium content of MSCs in pellet culture through biochemical and histological analyses.

## 2. Methods

### *2.1. Isolation and Characterisation of BALB/c and Human bone marrow derived Mesenchymal Stem Cells (MSCs)*

BALB/c primary Mesenchymal Stem Cell (MSCs) cultures were obtained and characterised according to protocols of Peister *et al.* as previously described (Birmingham E, *et al.*, 2012, Peister A, *et al.*, 2004). Briefly, 8 to 10 week old female and male mice were sacrificed under ethical approval from the Animal Care Research Ethics (ACREC) committee at the National University of Ireland, Galway. The femurs and tibiae of the mice were removed, and the ends of the bones were cut off. Bones were placed in RPMI-1640 media (Sigma Aldrich, Dublin, Ireland) supplemented with 9% foetal bovine serum (FBS, EU Thermo Scientific, Loughborough, UK), 9% horse serum (HS), 100 U/mL penicillin (Sigma Aldrich), 100 g/mL streptomycin (Sigma Aldrich), and 2 M L-glutamine (Sigma Aldrich) and centrifuged at 400 g for two minutes. The cell pellets were collected and resuspended in media in T175 flasks, and were then washed with sterile phosphate buffered solutions (PBS). After approximately 4 days, large colonies had formed, and were re-plated and cultured for a further 10 days.

Human bone marrow derived Mesenchymal Stem Cells (MSCs) were extracted from bone marrow aspirates. The bone marrow aspirates were obtained from the iliac crest of normal human donors under ethical approval, from the Research Ethics Committee of the National University of Ireland Galway and the Clinical Research Ethical Committee at University College Hospital, Galway, and following informed consent. Bone Marrow aspirates were obtained from 4 donors; 45, 48, 56 and 59 years of age. The MSCs were isolated on the basis of plastic adherence from bone marrow aspirates as previously described (Farrell E, *et al.*, 2009).

Once suitable colonies had formed in both BABLB/c and human MSCs the chondrogenic, osteogenic and adipogenic potential of these MSCs was confirmed as previously described (Birmingham E, *et al.*, 2012).

BALB/c MSC's for experiments were maintained in expansion medium which contains Iscoves MEM (Sigma Aldrich) supplemented with 10% FBS, 10% HS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin until confluent. Human Bone Marrow MSCs for experiments were maintained in expansion medium which contains Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS, EU Thermo Scientific, Loughborough, UK), 100 U/mL penicillin (Sigma Aldrich), 100 g/mL streptomycin (Sigma Aldrich), and 2 M L-glutamine (Sigma Aldrich).

### *2.2. Pellet Formation*

Once the cells reached a confluency of ~90% the cells were trypsinized, counted, and centrifuged at 650 g, 22°C for 5 minutes. The cells were then resuspended in expansion media so that there was $1x10^6$ cells/mL. This cell suspension was divided into 1.5 mL tubes so that there were 500,000 cells in each tube, and then centrifuged for 5 mins at 400 g to create cell pellets. Carefully avoiding the newly formed pellet, the media was removed from each of the pellets and 0.5 mL of either chondrogenic media or osteogenic media was added depending on experimental conditions (described in detail below). Chondrogenic medium consisted of a chemically defined medium which contained high glucose DMEM GlutaMAXTM (Gibco, Life Sciences, Dublin, Ireland), 10 ng/mL TGF-β3 (ProSpec-Tany TechnoGene Ltd., Ness-Ziona, Israel), 50 µg/mL Ascorbic Acid (Sigma Aldrich), 4.7 µg/mL Linoleic Acid (Sigma Aldrich), 100nM Dexamethasone (Sigma Aldrich) and 1x insulin-transferrin-selenium (ITS, BD Biosciences, Bedford, MA). Osteogenic medium consisted of the expansion medium supplemented with 100 nM of Dexamethasone, 50 µg/mL Ascorbic Acid and 10 mM β-Glycerol Phosphate (Sigma Aldrich). For all experiments pellet cultures

were fed twice per week by performing a 50% medium exchange. During each feed the pellets were agitated so as to prevent them from adhering to the micro-tube. This was achieved through aspirating the media beneath the pellet with a micro-pipette.

The BALB/c pellets were cultured under the following experimental conditions:

1. Osteogenic media for 49 days (Osteo).
2. Chondrogenic media for 49 days (Chondro).
3. 10 days chondrogenic priming and osteogenic media for 39 days.
4. 14 days chondrogenic priming and osteogenic media for 35 days.
5. 21 days chondrogenic priming and osteogenic media for 28 days
6. .28 days chondrogenic priming and osteogenic media for 21 days.

The human MSC pellets were cultured under the following experimental conditions:

1. Osteogenic media for 14 days (Osteo).
2. 14 days chondrogenic priming and osteogenic media for 14 days.
3. 21 days chondrogenic priming and osteogenic media for 14 days.

### *2.3. Pellet Harvesting*

Pellets from BALB/c MSCs were examined at 0, 10, 14, 21, 28, 35 and 49 days and were prepared for either histochemical analysis or biochemical analysis. Pellets from human MSCs were examined at 0# and prepared for biochemical analysis. At each of the time points, the culture medium from the pellets was collected; snap frozen and stored at -80°C until biochemical assays could be performed. The remaining pellets were washed with PBS and then treated in one of the following two ways; (1) snap frozen and stored at - 80°C for biochemical analysis or (2) fixed overnight in paraformaldehyde before being place in PBS and refrigerated for histochemical analysis. For the above study, two independent

experiments were carried out with at least two repeats in each experiment ($n$ = 2 for histological analysis and $n$ = 3 for biochemical analysis).

### *2.4. Quantitative Biochemical Analysis*

#### *2.4.1. DNA Content*

DNA content was analysed in the BALB/c and human derived MSC pellets. 500 μL of papain digest (100 mM Sodium Phosphate Buffer containing 10 mM L-cysteine (Sigma Aldrich), 125 μg/mL Papain (Sigma Aldrich) and 5 mM $Na_2EDTA$ (Sigma Aldrich) in $ddH_2O$ at pH 6.5) was added to the pellets and pellets were placed in an oven at 60°C overnight as previously described (Haugh MG, *et al.*, 2011). Once the pellets were digested the biochemical assays were performed straight away or stored at - 80°C until the assays could be performed. DNA content was performed using Hoechst 33258 DNA assay with calf thymus DNA (Sigma Aldrich) as a standard, following a previously published protocol (Haugh MG, *et al.*, 2011, Kim Y-J, *et al.*, 1988). Briefly, in minimal light 20 μL of papain digest of the sample/ standard is added to a 96-well plate in triplicate. To this 200 μL of working solution (assay buffer and 1 mg/mL Hoechst Dye solution (Sigma Aldrich)) was added. The plate was incubated away from light for ten minutes and then read on a microplate reader (Wallac Victor3 1420 Multilabel Counter) at excitation of 350 nm and emission of 450 nm as previously described (Birmingham E, *et al.*, 2012, Haugh MG, *et al.*, 2011).

#### *2.4.2. sGAG Content*

sGAG content was measured the BALB/c derived MSC pellets using the papain digested samples (prepared for the Hoechst assay) and dimethylmethylene blue (DMB) dye (26.25 μg/mL 1,9 Dimethylmethylene Blue, 0.625% ethanol and 2.5 μg/mL sodium formate (all Sigma Aldrich) in $ddH_2O$ at a pH 3) with 10 mg/mL bovine- derived chondroitin-4-sulfate (Sigma-Aldrich) as a standard, according to a previously published protocol (Estes BT, *et al.*,

2010). 40 μL of each sample and standard in triplicate was added to a 96-well plate and 125 μL of the DMB dye was added. The plate was read on the microplate reader at an optical density of 595 nm.

#### *2.4.3. ALP Production*

ALP production was determined in the BALB/c and human derived MSC pellets using a colorimetric assay of enzyme activity (SIGMAFAST *p*-NPP Kit, Sigma Aldrich) which uses *p*-nitrophenyl phosphate (*p*NPP) as a phosphatase substrate with ALP enzyme (Sigma Aldrich) as a standard. 40 μL of the medium was added to a 96-well plate in triplicate with a 50 μL of *p*NPP solution, which contains both *p*NPP and assay buffer. The samples were shielded from direct light at room temperature for one hour. After this, 20 μL of Stop Solution (3N NaOH) was added to the wells and the plate was read at 405 nm in a micro-plate reader as previously described (Birmingham E, *et al.*, 2012).

#### *2.4.4. Calcium Content*

Calcium deposition within the pellets was measured in the BALB/c and human derived MSC pellets using the Calcium Liquicolour kit (Stanbio Laboratories) according to the manufacturer's protocol. Briefly pellets were digested by adding 1 mL of 0.5 M HCL and rotating the solution overnight in a cold room. 10 μL of each of the digested samples and assay standard was added to a 96 well plate and 200 μL of the working solution was added. The plate was read on a microplate reader at an absorbance of 550 nm as previously described (Curtin CM, *et al.*, 2012).

### ***2.5. Histology***

Pellets from BALB/c MSCs were examined by histological methods at days 0, 10, 14, 21, 28, 35 and 49. After each sample had been fixed overnight in paraformaldehyde, samples were dehydrated and embedded in paraffin using an automatic tissue processor (Leica ASP300,

Leica). All samples were sectioned with a thickness of 8 μm using a rotary microtome (Leica microtome, Leica). Sections were stained with 1% Alcian Blue 8GX solution for sGAG, as previously described (Haugh MG, *et al.*, 2011), and finally 2% Alizarin Red solution for mineralisation (all Sigma Aldrich).

### *2.6. Immunohistochemical Analysis*

Immunohistochemical analysis was used to test the samples from BALB/c derived MSCs for Collagen type I, II and X as previously described (Vinardell T, *et al.*, 2009). Briefly, sections were treated with chondroitinase ABC (Sigma Aldrich), rinsed with PBS, quenched of peroxidase activity, and blocked with goat serum (Sigma Aldrich) for 1 h. Sections were then incubated at room temperature with either rabbit polyclonal anti-collagen type I (1:400), rabbit polyclonal anti-collagen type II (1:100), or rabbit polyclonal anti-collagen type X (1:200) (All Abcam, UK) for 1 hour. After washing in PBS again the sections were incubated with secondary antibody for type I, type II and type X collagen (Anti-Rabbit IgG Biotin antibody produced in goat (1:200)) (Abcam UK) for 1 hour. Colour was developed using the Vectastain ABC reagent (Vectastain ABC kit, Vector Laboratories, UK) and exposure to Peroxydase DAB substrate kit (Vector laboratories, UK). Negative and positive controls for mice were included in the immunohistochemical staining protocol for each batch.

### *2.7. Statistical Analysis*

Results are expressed as mean ± standard deviation. For all the biochemical analysis two-way analysis of variance (ANOVA) with time and media type as the independent factors followed by pair-wise multiple comparison procedure (Tukey's HSD test). All analyses were performed with Minitab. For all comparisons, the level of significance was $p \leq 0.05$.

## 3. Results

### *3.1. Chondrogenic priming of BALB/c MSC's*

#### *3.1.1. DNA Content*

Although there were trends towards increased DNA content in the chondrogenically primed groups compared to the control groups, there was no statistical difference in the DNA content between any of the groups at any time point over the course of the 49 days, or within the groups at each time point, see Figure 2.

#### *3.1.2. Production of the Cartilage Template*

*sGAG Content*

After the initial surge in sGAG content present in the chondrogenic group at Day 14, there was no statistical difference in the accumulative sGAG content between the chondrogenic and osteogenic groups at any of the time points (see **Error! Reference source not found.** (a)). Furthermore, there was no significant difference in the accumulative sGAG content between the chondrogenic, osteogenic and the chondrogenic priming groups until Day 21. By Day 35, there was no statistical difference in sGAG content between the 10 days priming group and the chondrogenic group. In contrast, there was significantly higher sGAG content ($p<0.01$) in the 14, 21 and 28 days priming groups compared to the chondrogenic control. These differences persisted to Day 49. There was a significant increase in sGAG content from day 35 to 49 in the 21 and 28 days priming groups, and notably the sGAG content was significantly higher than the chondrogenic group ($p<0.01$) and the osteogenic group ($p<0.01$) at 49 days. On the other hand, the 14 days priming group decreased sGAG content between Day 35 and Day 49, albeit that the sGAG content was still significantly higher ($p<0.05$) than both the chondrogenic and the osteogenic groups.

*Alcian blue staining*

The sGAG results are supported by the alcian blue stained sections of each of the groups at Day 49; the 21 days priming group has the highest intensity of staining for sGAG (blue), see **Error! Reference source not found.** (b). Comparing each of the groups, the intensity of staining for sGAG throughout the entire pellet is higher in the 21 and 28 priming groups compared to the osteogenic, chondrogenic, and the 10 and 14 days priming groups.

### *3.1.3. Mineralisation of the Cartilage Template*

In order to effectively depict the influence of chondrogenic priming on ALP and calcium secretion by BALB/c MSCs the data was analysed in two ways; 1) the amount of ALP/calcium secreted over the entire duration of the experiment and compared with both control groups; Chondrogenic group (chondrogenic media alone for 49 days) and Osteogenic group (osteogenic media alone for 49 days) (Figure 4(a), 5(a)); and 2) the amount of ALP/calcium secreted after the switch from chondrogenic to osteogenic media, which was only compared with the osteogenic control group (Figure 4(b), 5(b)). These results are further analysed below.

*ALP secreted over the entire duration of the experiment*

ALP Production in the osteogenic group increased until Day 21, after which it began to plateau off, see **Error! Reference source not found.** (a). There was higher extracellular ALP production by the osteogenic group than the chondrogenic group at all of the time points, however these differences were only statistically different ($p<0.05$) at Day 21. In contrast all of the chondrogenic priming groups show an initial increase in ALP Production after changing the media from chondrogenic to osteogenic. By Day 49 most of the chondrogenic priming groups had statistically higher ALP Production ($p<0.01$) than both the osteogenic and chondrogenic groups

*ALP secreted by MSC's after the switch from chondrogenic to osteogenic media*

Comparing each of the chondrogenic priming groups after the media change revealed an initial increase in ALP production both 1 week and 2 weeks after the switch from chondrogenic to osteogenic media (see **Error! Reference source not found.** (b)). The 14 days priming groups showed the smallest increase in ALP Production 1 week after the initial media change. On the other hand, the 10, 21 and 28 days priming groups had a significant ($p<0.05$) increase in ALP Production 1 week after media change. By two weeks after the media change all of the chondrogenic priming groups had significantly higher ALP activity than the osteogenic group. The 21 days priming group also had significantly higher ALP activity compared to the 28 days priming group. Examining the chondrogenically primed groups 2 week post-media change the highest amount of ALP expressed is in the 14 and 21 days priming groups.

*Calcium production by MSCs over the entire duration of the experiment*

The osteogenic group increased calcium content throughout the time course of the experiment (see Figure 5 (a)), whereas there was no evidence of calcium production in the chondrogenic group at any time-point. The chondrogenic priming groups' calcium content increased throughout the time course of the experiment. By Day 35 both the osteogenic and 10 days priming group had similar calcium content whereas the 14 and 21 days priming groups had significantly ($p<0.05$) lower calcium content than the osteogenic group. By Day 49 both the 10 and 14 days priming groups had higher calcium content than the osteogenic group albeit that 10 days priming group was the only one with significantly higher calcium content ($p<0.05$). There was no difference in calcium content between the 21 days priming group and the osteogenic group, whereas the 28 days priming group had significantly ($p<0.05$) lower calcium content when compared to the osteogenic group.

*Calcium production by MSCs after the switch from chondrogenic to osteogenic media*

Comparing the primed groups with the osteogenic group directly the later chondrogenic priming groups (14, 21, and 28 Days priming groups) only had an increase in calcium content two weeks after the media switch. The 10 days priming groups had a small increase in calcium content one week post media switch. However, two weeks post media switch all of the chondrogenic priming groups had significantly higher (all $p<0.01$) calcium content than the osteogenic group alone, which had no calcium content until 3 weeks in osteogenic media. The 28 days priming group had significantly higher calcium content than all other chondrogenic priming groups as well as the osteogenic group, and the 10 days priming group has the lowest calcium content when compared to the other chondrogenically primed groups

*Alizarin Red staining*

The mineralisation differences observed are further verified through alizarin red staining (see Figure 6). Two weeks after the media switch, mineralisation is only seen in the chondrogenic priming groups and not in the osteogenic group. In particular, mineralisation is seen throughout the pellet in the 14 and 21 days priming groups, but only in the periphery in the other chondrogenic priming groups. This correlates with the results of the calcium assay, where the highest amount of mineralisation occurs in the later chondrogenic priming groups.

*Immunohistochemical Analysis*

The results from the calcium content assay are further verified in the immunohistochemical staining of the groups two weeks after the media switch. The highest amount of positive staining for Collagen type I is seen in the 28 days priming group and the lowest amount is seen in the osteogenic group. The highest amount of Collagen type II is seen in the later chondrogenic priming groups (i.e. 14-28 days priming), therefore correlating with the findings of the alcian blue staining and sGAG assay. Finally, Collagen type X staining was

positive on the periphery of all of the pellets two weeks after the media switch with the highest amount of ($p<0.05$) staining in 28 days priming group (see Figure **6**).

### *3.2. Chondrogenic priming of Human MSC's*

#### *3.2.1. Mineralisation of the Cartilage Template*

In order to effectively depict the influence of chondrogenic priming on ALP and calcium secretion by human MSCs the data was analysed by the amount of ALP/calcium secreted after the switch from chondrogenic to osteogenic media, which was only compared with the osteogenic control group (Figure 7). These results are further analysed below.

*ALP secreted by human MSC's after the switch from chondrogenic to osteogenic media*

Examining the ALP production/DNA content for the human cells both the 14 and 21 days priming groups have significantly higher ALP production than the osteogenic group at both 1 week and 2 week post media switch. The 21 days priming group also had significantly higher ALP activity compared to the 14 days priming group 1 week post media change.

*Calcium production by MSCs after the switch from chondrogenic to osteogenic media*

Examining the Calcium content/ DNA content for human cells both the 14 and 21 days priming groups have higher Calcium content than the osteogenic group at all time points. However, the 21 days priming has significantly higher Calcium content than both the 14 days priming and the osteogenic group at both 1 week and 2 weeks post media switch.

## 4. Discussion

The results of this study show for the first time that chondrogenic priming for specific durations (14, 21 days), prior to being exposed to osteogenic factors, allows BALB/c and human derived MSCs to differentiate and produce a chondrogenic template and can enhance osteogenic differentiation by MSCs (as indicated by ALP, Calcium content and Collagen type I and Alizarin Red staining). Most importantly chondrogenic priming induced more mineralisation by BALB/c and human MSCs compared to growth in osteogenic media alone. Furthermore chondrogenic priming for a period of 21 days enhanced the distribution of mineral through the entire pellet, whereas other durations led to mineralisation on the periphery alone. Interestingly chondrogenic differentiation was also enhanced in MSCs that were chondrogenically primed for longer durations, and this was significantly higher than the MSC's cultured in chondrogenic media for the entire experiment. Taken together, these results indicate for the first that time there is an optimum time, approximately 21 days, for chondrogenic priming of MSCs to enhance both osteogenic and chondrogenic differentiation in vitro.

One limitation of this study was that osteogenic and chondrogenic factors (ascorbic acid dexamethasone, β-glycerol and TGF-β3) were introduced into the culture media of MSCs to encourage MSC differentiation down specific pathways. It is unlikely that these factors are present in the combinations used here during endochondral ossification in vivo and these may alter cellular viability in long term in vitro studies. However previous research studies have exposed MSCs to both chondrogenic and osteogenic differentiating agents for long durations (14 days to 5 weeks) and have demonstrated long term viability and matrix production by MSC's (Cheng NC, *et al.*, 2009, Coleman CM, *et al.*, 2013, Estes BT, *et al.*, 2010, Farrell E, *et al.*, 2011, Farrell E, *et al.*, 2009, Haugh MG, *et al.*, 2011, Jaiswal N, *et al.*, 1997, Mauck RL, *et al.*, 2003, Mauck RL, *et al.*, 2006, Thorpe SD, *et al.*, 2010, Vinardell T, *et al.*, 2012).

Furthermore control groups were exposed to the same osteogenic or chondrogenic factors so the differences observed in osteogenesis due to the duration of priming cannot be explained by differences in cell viability. Another limitation is the fact that human MSCs were only investigated for a subset of conditions and that BALB/c cells rather than human mesenchymal stem cells were used for histological and immunohistochemical analyses. It was not feasible to obtain a large enough stock of human bone marrow MSCs to conduct all of the necessary experiments, involving numerous treatment groups and time points, to address this question. However, BALB/c cells provide a suitable model to test the hypothesis(Baddoo M, *et al.*, 2003, Peister A, *et al.*, 2004, Tropel P, *et al.*, 2004) and can overcome limitations of intraspecies variability, due to the ability to obtain relatively large litters of animals from the same parents, and can provide the large volume of bone marrow required for undertaking large studies without having to expand MSCs beyond passage 3. Furthermore our studies have confirmed the BALB/c ALP and calcium results in a subset of conditions using human derived MSCs, thus supporting the use of BALB/c cells to test the hypothesis there is an optimum time for chondrogenic priming of MSCs in vitro that will enhance osteogenic differentiation.

The first stage of endochondral ossification involves MSCs condensing and aligning to become clusters of cells, and subsequently these MSCs differentiate down the chondrogenic pathway (Kronenberg HM, 2003, Mackie EJ, *et al.*, 2008, McNamara L, 2011). In order to investigate the optimum timing for production of a cartilage template, we performed biochemical and histological analyses at various time points. In this study there is an increase in sGAG content from Day 21 to Day 49. These results are in keeping with those of previous studies, which cultured human MSCs cultured in pellet form (Angele P, *et al.*, 2003) or in agarose scaffolds (Haugh MG, *et al.*, 2011). Unlike other studies our study reports a significant drop in sGAG content in the chondrogenic group from Day 14 to 21.

This initial drop in sGAG content and subsequent gradual increase in sGAG content was also seen in all of the chondrogenic priming groups. Our histological analyses confirmed the formation of a cartilage-like tissue, which was similar to the histological staining reported in previous cartilage tissue engineering studies using MSCs seeded on agarose hydrogel scaffolds (Erickson IE, *et al.*, 2009, Mauck RL, *et al.*, 2006, Vinardell T, *et al.*, 2009). However, our study shows for the first time that there is an optimum timing for chondrogenic priming that enhances chondrogenic differentiation, as is demonstrated by elevated sGAG production in MSCs that were chondrogenically primed for 21 and 28 days.

Previous in vitro studies have shown that constructs that were chondrogenically primed in vitro for 21 days can mineralise when subsequently implanted into an animal model in vivo (Farrell E, *et al.*, 2011, Farrell E, *et al.*, 2009, Jukes JM, *et al.*, 2008, Miot S, *et al.*, 2012, Scotti C, *et al.*, 2010). Other studies, have also reported that chondrogenic priming of MSCs can enhance chondrogenic and osteogenic differentiation of the cells (Farrell E, *et al.*, 2011, Farrell E, *et al.*, 2009, Jukes JM, *et al.*, 2008), but to date no study has identified the beneficial effect of chondrogenic priming in vitro for production of bone tissue mineral. The most interesting observation of this study is that chondrogenic priming of BALB/c MSCs and human bone marrow MSCs for specific time periods (14 and 21 days) can enhance ALP Production, calcium content, collagen type I production and mineralisation when compared to the osteogenic group and other chondrogenic priming groups. Moreover, histology results from the BALB/c MSCs show that Collagen type I and Alizarin Red staining were present throughout the pellet for both groups, whereas the 28 days chondrogenic priming group had the highest amount of calcium but also had the highest amount of Collagen type X which is known to be a marker for the expression of cartilage hypertrophy (Mueller MB, *et al.*, 2008). Alizarin Red staining was also limited to the periphery indicating that the centre of these constructs may have become hypertrophic. Previous chondrogenic priming studies have been

limited by the occurrence of core degradation, arising from lack of nutrient delivery and waste removal when implanted in vivo, and mineralisation occurring around the periphery or in the core alone, but not throughout the pellet (Farrell E, *et al.*, 2011, Farrell E, *et al.*, 2009, Scotti C, *et al.*, 2010).. Our study shows that mineralisation throughout a pellet is possible in vitro if chondrogenically primed for a specific amount of time (14, 21 days).

It is clear from our results that the amount of time the cells are chondrogenically primed for can have a positive or negative effect on both the osteogenic and chondrogenic differentiation potential of MSCs in vitro. This may be due to the fact that allowing the cells to form a chondrogenic phenotype and then an osteogenic phenotype, thus recreating the in vivo endochondral ossification process, can significantly increase the mineralisation potential of MSCs. However, our results also demonstrate that a mature cartilage template needs to be formed to significantly increase the mineralisation potential of the MSCs. However, if the template is allowed to form for too long the core of the construct can become hypertrophic, and the periphery of the construct will only mineralise and may lead to complications in vivo. We propose that chondrogenic priming of MSC's for specific periods of time (14, 21 days) enhances in vitro osteogenesis due to the fact that it more closely mimics the endochondral ossification in vivo, which begins after cartilage differentiation has occurred. Very little is known regarding the exact timing of endochondral ossification during human development. Embryologists have estimated through ultrasounds that the first sign of limb buds is seen by approximately the 26th day of gestation but the exact day in which the endochondral ossification process begins is still unknown (Merz E, *et al.*, 2004, Smith A, 1968, Webster S, 2012). Further biochemical and histological analysis of the cartilage template during early fetal development are required to fully understand these findings and studies might corroborate our findings that chondrogenic priming for longer periods of time mimics endochondral ossification in vivo.

## 5. Conclusions

The results of this study show that chondrogenic priming of BALB/c derived MSCs and human bone marrow MSCs can significantly increase their potential for osteogenic differentiation and mineralisation, even more so than culturing the cells in osteogenic growth factors alone. Moreover this study shows for the first time that chondrogenic priming for specific durations (14, 21 days), can enhance osteogenic differentiation by MSCs in vitro and can produce a construct that is mineralised throughout the core. Determining the optimum time for chondrogenic priming to enhance osteogenic differentiation in vitro provides information that might lead to a novel regenerative treatment for large bone defects, as well as addressing the major limitation of core degradation and construct failure.

## 6. Acknowledgements

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## AUTHOR DISCLOSURE STATEMENT

There are no financial and personal relationships between the authors and others that might bias their work. All authors state that they have no conflicts of interest.

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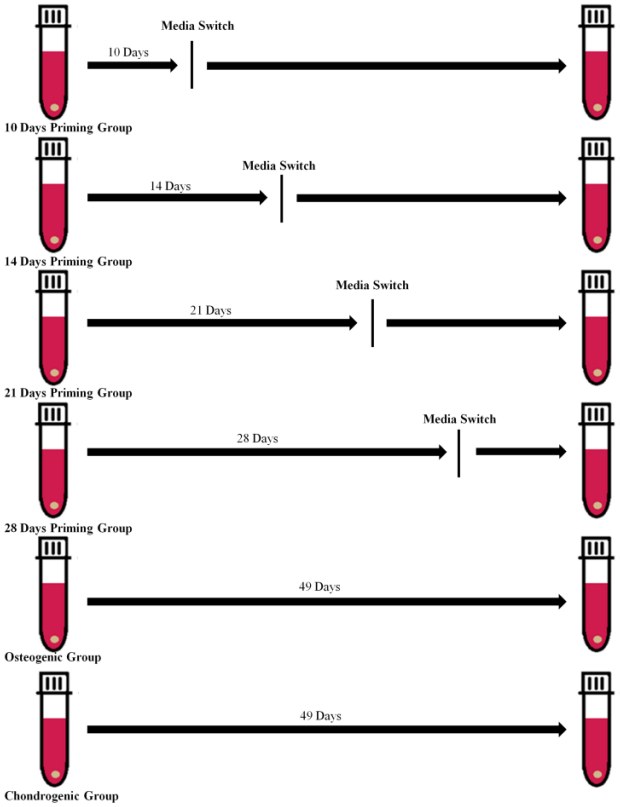



**Figure 1**: Schematic of the experimental groups harvested samples at each time point.

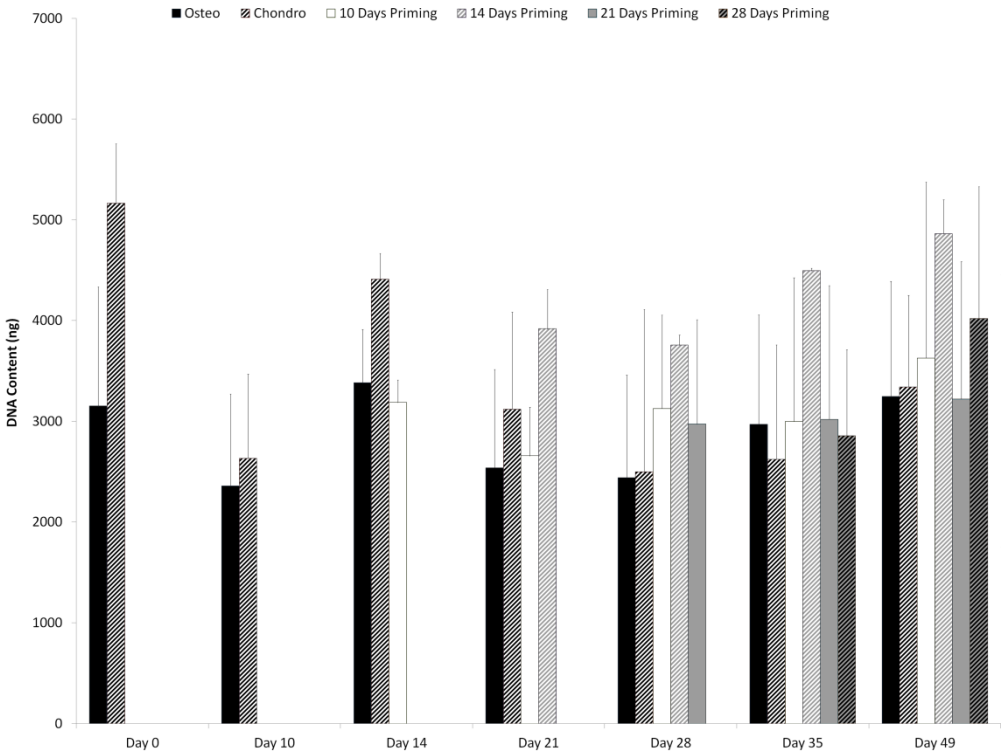



**Figure 2:** DNA content of the BALB/c groups at Day 0, 10, 21, 28, 35 and 49. *n*=6 samples per group per time point. Error bars denote standard deviation.

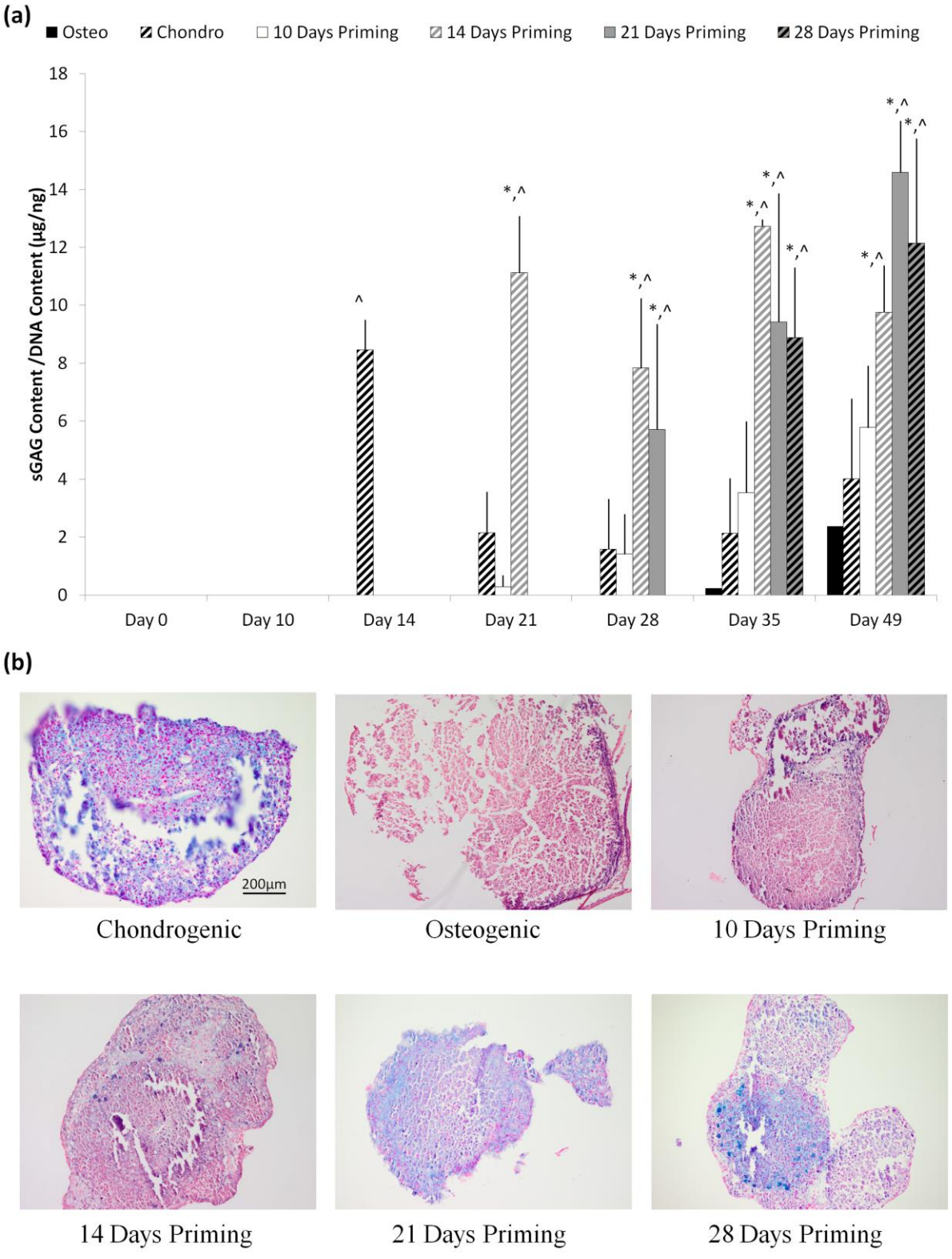



**Figure 3**: **(a)** sGAG Content of the BALB/c groups at Day 0, 10, 14, 21, 28, 35 and 49. *n*=6 samples per group per time point. * p<0.05 versus Chondro Group, & ^ p<0.05 versus Osteo Group. Error bars denote standard deviation. **(b)** Alcian Blue Staining of each of the groups at Day 49. Each of the images was taken at a magnification of 10X.

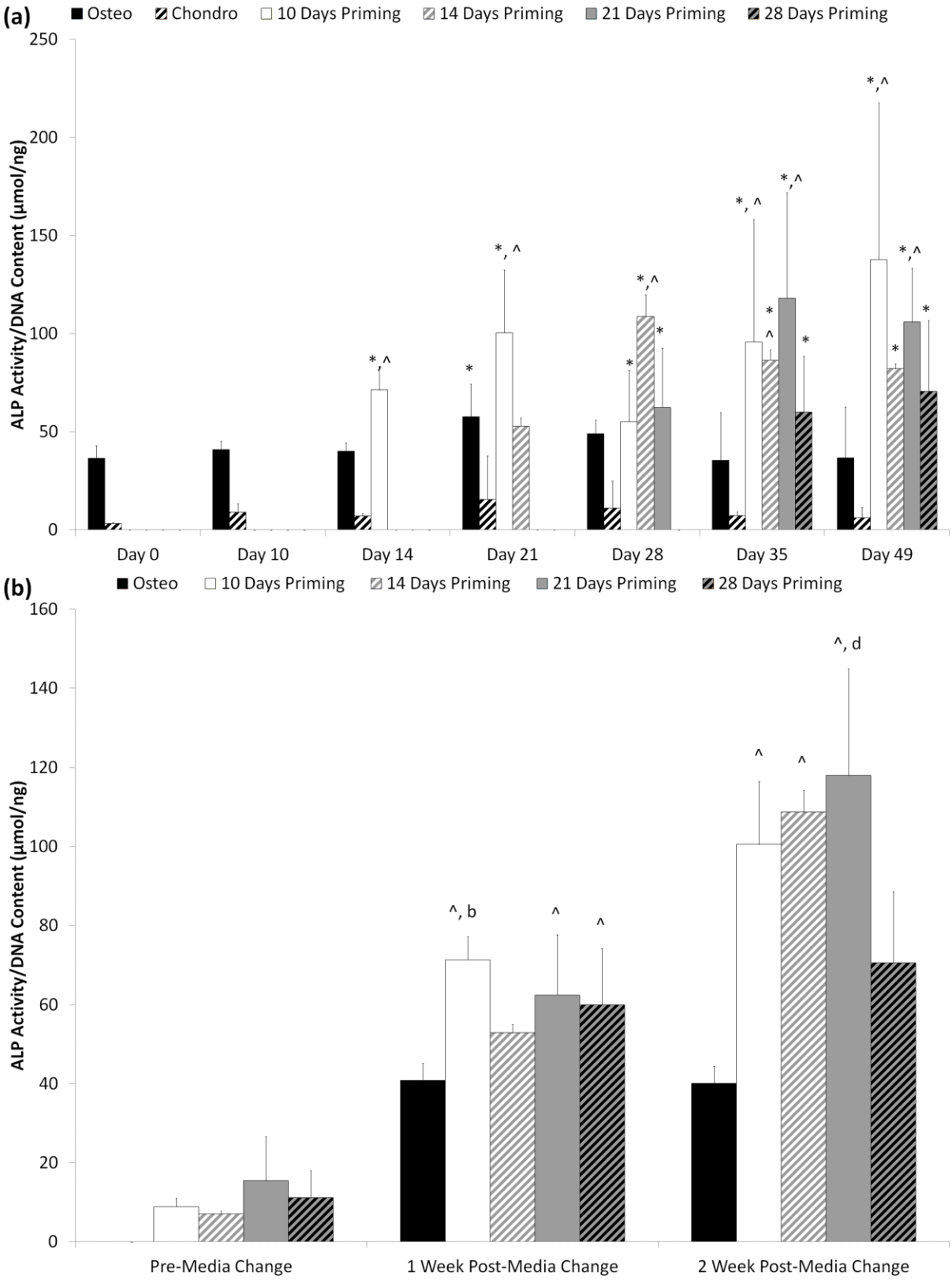



**Figure 4**: **(a)** ALP content/DNA content of the of the BALB/c groups at Day 0, 10, 14, 21, 28, 35 and 49. *n*=6 samples per group per time point. **(b)** ALP content/DNA content of the BALB/c groups and compared to the osteogenic group, 1 week and 2 week post-media

change. * $p<0.05$ versus Chondro Group, ^ $p<0.05$ versus Osteo Group, [a] $p<0.05$ versus 10 Days Priming group, [b] $p<0.05$ versus 14 Days Priming group, [c] $p<0.05$ versus 21 Days Priming group & [d] $p<0.05$ versus 28 Days priming group. Error bars denote standard deviation.

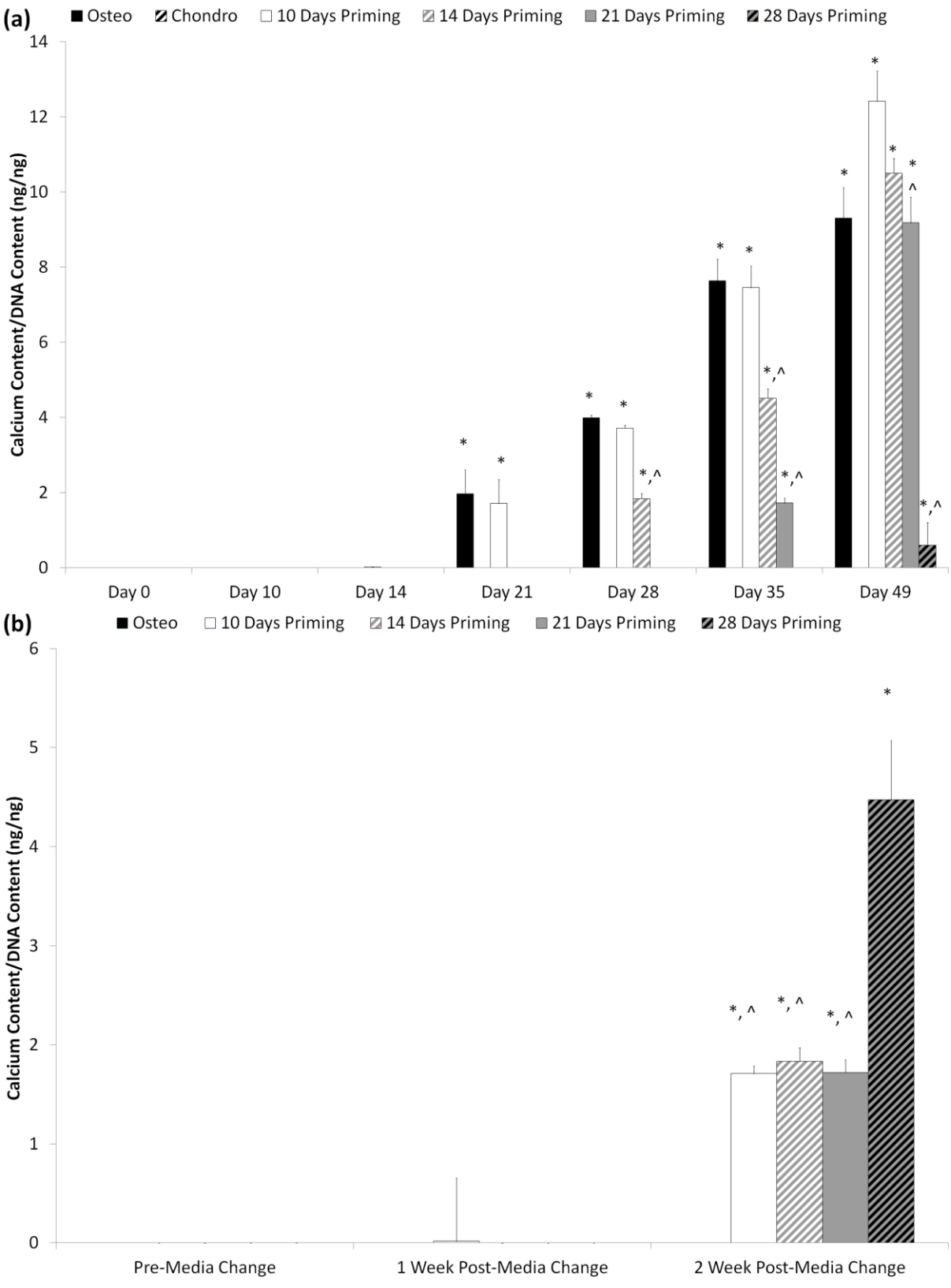



**Figure 5**: **(a)** Calcium Content of the BALB/c groups at Day 0, 10, 14, 21, 28, 35 and 49. n=6 samples per group per time point. **(b)** Calcium content of the BALB/c groups compared to the osteogenic group, 1 week and 2 week post-media change. * $p<0.05$ versus Chondro

Group, ^ $p<0.05$ versus Osteo Group, [a] $p<0.05$ versus 10 Days Priming group, [b] $p<0.05$ versus 14 Days Priming group, [c] $p<0.05$ versus 21 Days Priming group & [d] $p<0.05$ versus 28 Days priming group. Error bars denote standard deviation.

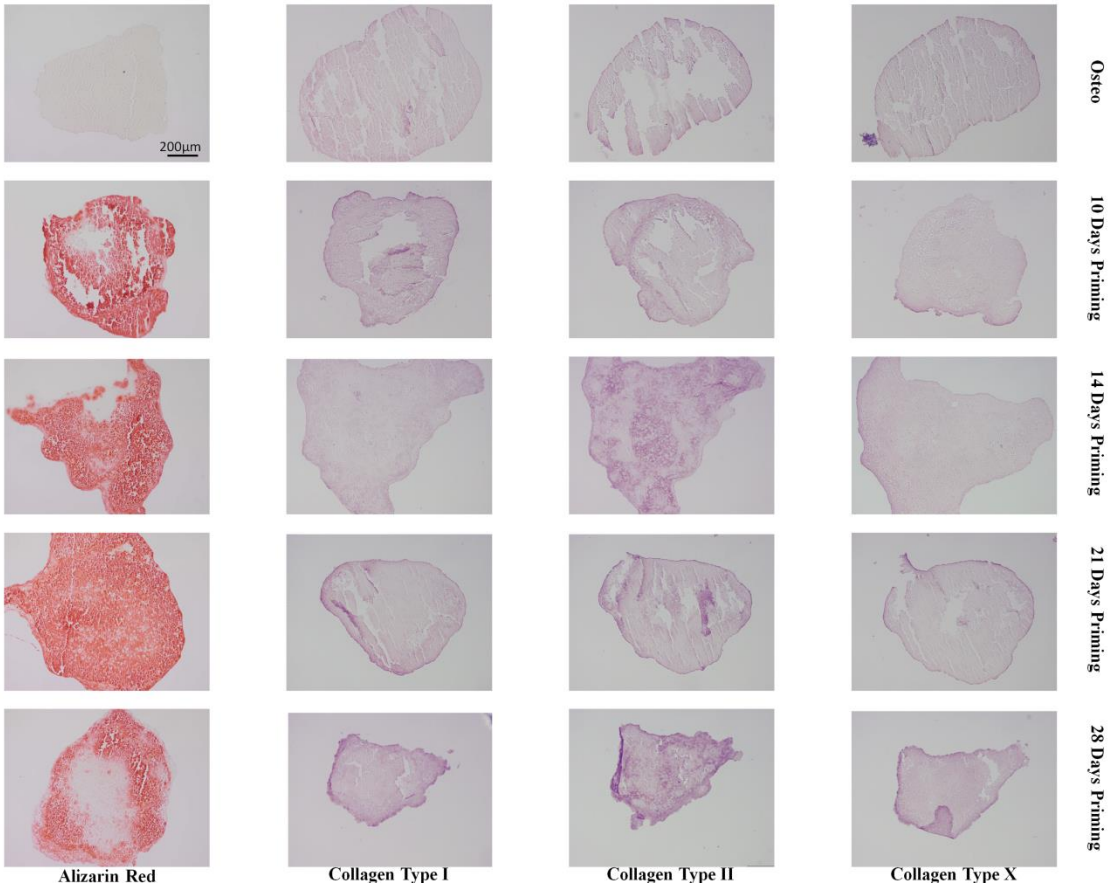
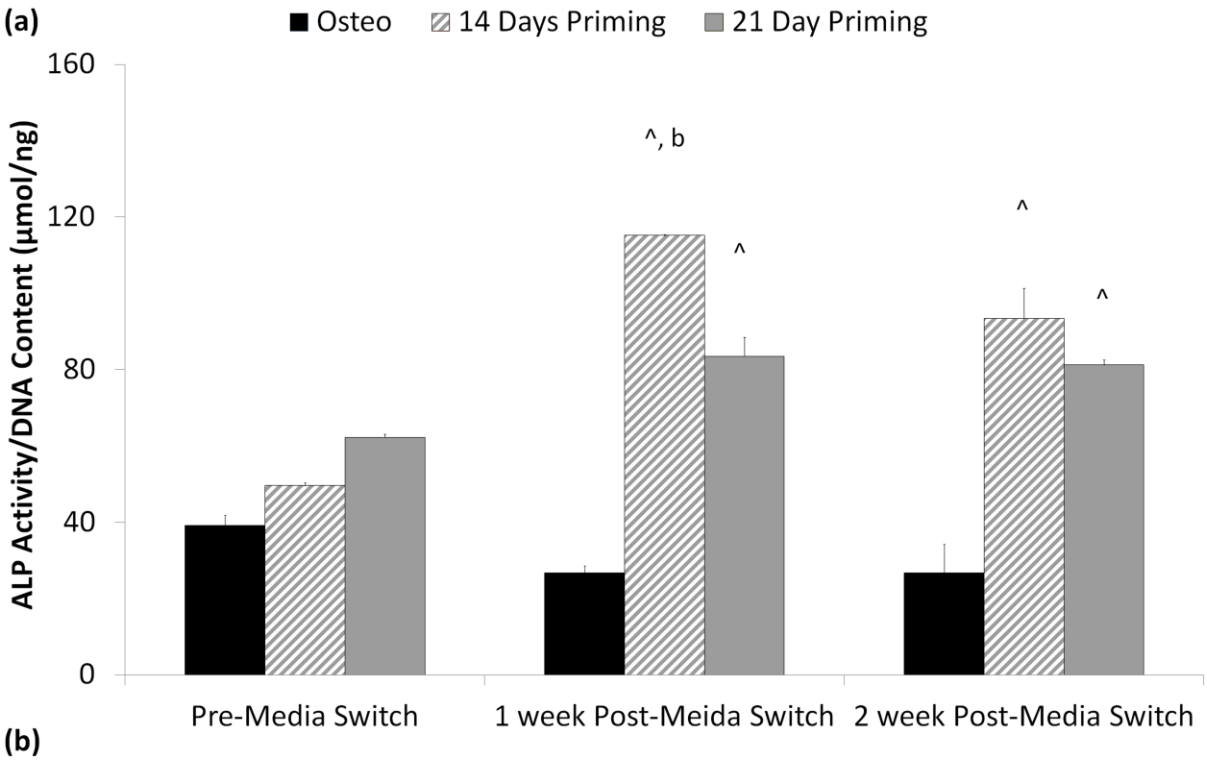



**Figure 6**: Alizarin Red & Immunohistochemical staining (Col Type I, Col Type II and Col Type X) of the BALB/c chondrogenically primed groups and osteogenic group at 2 weeks post media switch. Each of the images was taken at a magnification of 10X.



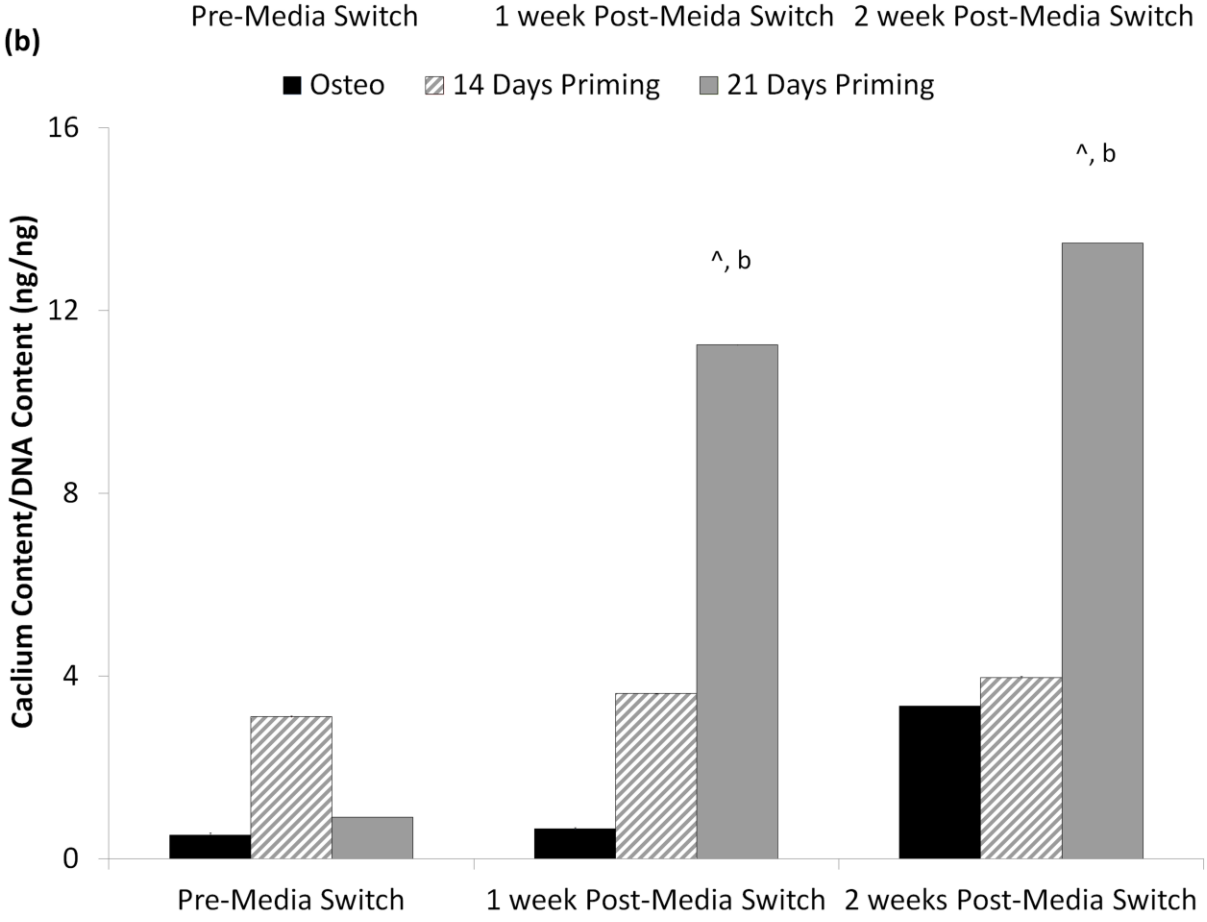




**Figure 7: (a)**: ALP content/DNA content of the human MSCs priming groups and compared to the osteogenic group, 1 week and 2 week post-media change. **(b)**: Calcium content of the human MSCs priming groups compared to the osteogenic group, 1 week and 2 week post-media change. * $p<0.05$ versus Chondro Group, ^ $p<0.05$ versus Osteo Group, [a] $p<0.05$

versus 10 Days Priming group, [b] $p<0.05$ versus 14 Days Priming group, [c] $p<0.05$ versus 21 Days Priming group & [d] $p<0.05$ versus 28 Days priming group. Error bars denote standard deviation.