Expression of Sulf1 and Sulf2 in cartilage, bone and endochondral fracture healing

G. Zaman[1], K. Staines[3], C. Farquharson[3], P. T. Newton[3], J Dudhia[2], C. Chenu[1], A. A Pitsillides[1], G.K. Dhoot[1*]

[1]Department of Comparative Biomedical Sciences, RVC, University of London, NW1 OTU, UK

[2]Department of Clinical Sciences, RVC, University of London, NW1 OTU, UK

[3]The Roslin Institute and Royal (Dick) School of Veterinary Studies, The University of Edinburgh, Easter Bush, Midlothian, EH25 9RG, UK

*=the author for correspondence (tdhoot@rvc.ac.uk)

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Highlights:

- High Sulf1/Sulf2 expression in active osteoblasts with reduction in osteocytes.
- High variability in Sulf1/Sulf2 expression in articular chondrocytes.
- Hypertrophic chondrocytes in growing and healing bone express high levels of Sulf2 but not Sulf1.
- High Sulf2 expression in growing and healing bone closely correlates with Hedgehog signalling.

ABSTRACT: SULF1/SULF2 enzymes regulate cell signalling that impacts the growth and differentiation of many tissues. To determine their possible role in cartilage and bone growth or repair, their expression was examined during development and bone fracture healing using RT-PCR and immunochemical analyses. Examination of epiphyseal growth plates revealed differential, inverse patterns of SULF1 and SULF2 expression, with the former enriched in quiescent and the latter in hypertrophic chondrocyte zones. Markedly higher levels of both SULFs, however, were expressed in osteoblasts actively forming bone when compared with proliferating pre-osteoblasts in the periosteum or the entombed osteocytes which express the lowest levels. The increased Sulf1 and Sulf2 expression in differentiating osteoblasts was further confirmed by RT PCR analysis of mRNA levels in rat calvarial osteoblast cultures.

SULF1 and SULF2 were expressed in most fetal articular chondrocytes but down-regulated in a larger subset of cells in the postnatal articular cartilage. Unlike adult articular chondrocytes, SULF1/SULF2 expression varied markedly in postnatal hypertrophic chondrocytes in the growth plate, with very high SULF2 expression compared with SULF1 apparent during neonatal growth in both primary and secondary centres of ossification. Similarly, hypertrophic chondrocytes expressed greatly higher levels of SULF2 but not SULF1 during bone fracture healing. SULF2 expression unlike SULF1 also spread to the calcifying matrix around the hypertrophic chondrocytes indicating its possible ligand inhibiting role through HSPG desulfation. Higher levels of SULF2 in both developing and healing bone closely correlated with parallel increases in hedgehog signalling analysed by ptc1 receptor expression.

INTRODUCTION: SULF1/SULF2 enzymes are critical for modulating cell signalling pathways that require heparan sulfate proteoglycan (HSPG) as a co-factor facilitating ligand-receptor interactions (Dhoot et al. 2001; Morimoto-Tomita et al. 2002; Rosen and Lemjabbar-Alaoui 2010). HSPG function is dependent on the sulfation status of its heparan sulfate (HS) side chains which comprise of repeating disaccharide units of uronic acid linked to glucosamine (Lamanna et al. 2006). The disaccharide units are selectively sulfated at the N, 3-O, and 6-O positions of glucosamine and the 2-O position of iduronic acid residues by sulfotransferases in the Golgi apparatus (Merry and Gallagher 2002). HSPGs are secreted to the cell surface or the extracellular matrix (ECM) where they have signalling and matrix functions, respectively (Stringer and Gallagher 1997). The sulfation pattern of HS chains, however, is further fine-tuned extracellularly by the activities of SULF1 and SULF2 enzymes (Dhoot et al. 2001; Morimoto-Tomita et al. 2002). These are members of the sulfatase gene family that regulate the activities of many signalling pathways. The ability of SULFs to regulate such pathways relies on highly specific removal of 6-O-sulfates from HS chains. This leads to inhibition of 6-O sulfate requiring ligands, such as fibroblast growth factors (FGF), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) but also to promotion of other signalling pathways such as those involving Glial cell-derived neurotrophic factor (GDNF) and Wnts. Some further studies have also identified splice variants of both Sulf1 and Sulf2 genes in avian as well as human tissues (Sahota and Dhoot 2009; Gill et al. 2011; Gill et al. 2012). While tumour tissues express multiple splice variants of both Sulf1 and Sulf2, normal tissues so far have been described to express only a full length and a single shorter splice variant for both these genes (Gill et al. 2011; Gill et al. 2012).

Many signalling pathways including BMPs, FGFs, Wnts, IGFs and Hedgehog are recognised to orchestrate important roles in bone development, bone remodelling and fracture repair (Kronenberg 2003). For example, canonical Wnt signalling has been shown to promote progenitor cell differentiation towards the osteoblast and chondrocyte lineage in developing skeletal elements whereas hedgehog signalling regulates chondrocyte hypertrophy during endochondral ossification. Many BMPs and their receptors are also expressed during bone formation although BMPs and FGFs have opposite effects on chondrocyte differentiation. The cartilage anlagen enlarge by chondrocyte proliferation and hypertrophy and matrix production which, in turn, drives bone growth; these processes are also recapitulated in growth plates during bone lengthening as well as during bone fracture repair. The pace of bone elongation is also driven by the orientation of the hypertrophic chondrocytes; a process regulated by hedgehog signalling (Choi et al. 2012). It is evident that growth factors play an important role in the rapid regeneration of bone and cartilage that takes place during fracture repair and that its complexity requires coordination of a number of the signalling pathways that regulate skeletal cell proliferation, matrix synthesis, and tissue differentiation.

The aim of this study was to examine the expression patterns of both SULF1 and SULF2 enzymes in skeletal tissues during development, growth and remodelling to investigate whether their expression patterns are consistent with regulatory roles in these processes. We also examined the bone healing process using an experimental rat femur fracture model to determine whether the patterns of expression of the extracellular SULF1/SULF2 enzymes may identify key events for accelerating this vital process by regulating their activities. Our data reveal elevated levels of SULF1 and 2 expression in fully differentiated osteoblasts and osteoclasts. We also observed high levels of SULF2, but not SULF1 in hypertrophic chondrocytes during development as well as fracture repair.

MATERIALS AND METHODS:

Fracture repair: Osteotomy, in accordance with UK government, Home Office regulations, was performed as previously described (Smitham et al. 2014). This work was conducted under a project licence granted by the UK Home Office and approved by the College's Ethics and Welfare Committee. On study Day 0, an osteotomy in the left femora of 12 week old male Wistar rat followed by stabilisation using external fixator pins attached to a mono-fixator frame. The external fixator system used in this protocol comprised two metal blocks, of either aluminium or titanium alloy, linked to two cylindrical stainless steel bars. The bars were anchored in one block, the other incorporating linear bearings allowing it to slide along the bars, thus permitting alteration of the osteotomy gap width. Briefly, the fixator was applied to the craniolateral aspect of the femur using four threaded M1.2 stainless steel pins. Surgery was performed under general anaesthesia (ketamine 75 mg/kg and xylazine 10 mg/kg) and or appropriate gaseous anaesthesia using aseptic techniques. Consistent positioning of the fixator pins was ensured using a drill locator template. A bone-fixator distance of 8 mm was created with a precision spacer. After pin placement, a transverse osteotomy was created midway between the proximal and distal pins using an oscillating diamond bone saw, with saline irrigation throughout. The bone fragments were distracted to leave an osteotomy gap of 0.5 mm, this being provided using a precision spacer, and was maintained by locking the fixator blocks

on to the connecting bars. Radiographs of the operation site were taken before recovery from anaesthesia.

Immunofluorescence and immunoperoxidase staining of paraffin sections: The tissues for sectioning were fixed in 4% paraformaldehyde for 2-5 days at room temperature before demineralisation in EDTA followed by xylene clearance and paraffin embedding. The specificities of the SULF1 antibody C for total SULF1 and SULF2 antibody D for total SULF2 have been described previously (Sahota and Dhoot 2009; Gill et al. 2011; Gill et al. 2012). Rabbit anti Ptc1 antibody was purchased from Millipore. Immunofluorescence was used to stain paraffin sections of normally developing and bone fracture tissues fixed in 4% PFA and decalcified in EDTA. Tissue sections were pre-treated with ProK for 30 minutes at 37^{o}C and incubation with permeabalisation buffer for 15 minutes at room temperature followed by incubation with 10% fetal calf serum (FCS) before incubation with primary antibodies against SULF1 or SULF2 or ptc1. The binding of rabbit primary antibodies to SULF1 and SULF2 and ptc1 was detected using streptavidin Alexa Fluor 594 or Alexa Fluor 488 fluorochrome bound to biotin-linked goat anti rabbit immunoglobulins. Sections treated with pre-immune rabbit sera were similarly incubated with fluorochrome-labelled secondary antibodies as controls. Primary antibodies, SULF1 antibody C was diluted 1/200, SULF2 antibody D as 1/100, while rabbit anti ptc1 was diluted 1/300. Goat anti rabbit immunoglobulins secondary antibodies and both fluorochromes were diluted 1/400. All primary antibody reactions were incubated overnight at 4°C followed by secondary antibody incubations for 1 hour each at room temperature. Following 4 PBS washes between and after each incubation, labelled tissue sections were mounted in polyvinyl alcohol mounting medium with DABCO and 2.5 µg/ml DAPI for nuclear visualisation and photographed using a Leica DM4000B fluorescent microscope. Most tissue sections were stained using immunofluorescent procedure but an immunoperoxidase staining was also used initially for some tissue sections (Figure 1) to confirm immunofluorescent staining that included blocking endogenous peroxidase activity by 3% H_2O_2 for 30 minutes before incubation with primary antibody following pre-incubation with 10% FCS as described previously

Cell culture and tissue samples:

Rat calvaria cultures: Primary osteoblasts from rat were prepared by sequential enzyme digestion of rat calvarial bones from 2-day-old Sprague-Dawley rats, as previously described (Shah et al. 2010). Following the first two digests that were discarded, the cells from the 3rd digest were resuspended in DMEM (Staines et al. 2012) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin as previously described (Shah M et al 2010). Cells were cultured for 2–4 days at 37°C, 5% CO_2 in 75 cm^2 flasks until they reached confluence. Such rat primary cells were then sub-cultured into 6-well plates in DMEM supplemented with 2 mM β-glycerophosphate, 50 mg/ml ascorbic acid and 10nM dexamethasone with half medium changes every 3 days until day 21. Osteoblast differentiation was measured by quantification of 1) mineralised bone nodules stained with alizarin red (1% solution in water) for 5 min and 2) alkaline phosphatase (ALP) activity which is greatly enhanced during *in vitro* bone formation. ALP activity was determined by Fast-Blue staining after fixing the cells with neutral

buffered formalin. Cells were incubated in ALP staining solution for 30 min and washed with deionized water before imaging.

ATDC5 cell culture: Chondrogenic ATDC5 cells (Riken Cell Bank, Ibaraki, Japan) were cultured in a differentiation medium [DMEM/F-12 (1:1) with GlutaMAX I containing 5% fetal bovine serum (FBS), 1% insulin transferrin and selenium, 1% sodium pyruvate and 0.5% gentamicin (Invitrogen, Paisley, UK)] at a density of 6,000 cells/cm^2 in multi-well plates (Iwaki Cell Biology; Sterilin, Feltham, UK) as described previously (Newton et al. 2012; Staines et al. 2012). Cells were left for 6 days to reach confluency at which point the medium was supplemented with 10 mM βGP and 50μg/ml L-ascorbate-2-phosphate (ascorbic acid). Cells were incubated in a humidified atmosphere (37°C, 5% CO_2) for up to 34 days and the medium was changed every second or third day.

Human skeletal tissues: Primary human osteoblast cell cultures were prepared from bone segments taken during routine shoulder operations at the Hospital of St. John and St. Elizabeth, London following patient consent before each operation as previously described (Clarkin et al. 2008). Samples of human articular cartilage from different age groups were also obtained following patient consent before each operation within 6 h of surgery from weight-bearing regions of femoral condyles from patients undergoing amputation or joint replacement for osteosarcoma or chondrosarcoma distant from the joint, and in all cases, joints were not involved in the tumor pathology as previously described (Hickery et al. 2003).

RT PCR and qPCR: RNA from ATDC5 cell cultures and cell cultures of rat calvaria was prepared using an RNeasy mini kit (Invitrogen) according to the manufacturer's instructions. The RNA from different skeletal elements was prepared following the removal of muscle, and the cartilaginous ends of the bones and centrifugation of the bone shafts for 5000rpm for 2 minutes to remove the marrow before snap-freezing in liquid nitrogen. Such frozen tissues were pulverized under liquid nitrogen using a mortar and pestle and lysed in Qiazol lysis reagent (Qiagen Ltd., Crawley, UK). Total RNA was extracted from lysed samples using RNeasy mini kit (QIAGEN, Crawley, UK). RNA integrity of samples was assessed by electrophoresis using ethidium bromide staining and by OD260/OD280 nm absorption ratio (>1.95). Total RNA was reverse transcribed with SuperScript II RNase reverse transcriptase (Invitrogen), using random primers (Invitrogen, Paisley, UK) for RT PCR and qPCR analysis. Real-time qPCR was carried out as previously described (Zaman G 2012) using QuantiTect SYBR green PCR kit and Opticon 2 LightCycler (MJ Research, Waltham, MA, USA). The expression levels of Sulf1 and Sulf2 were normalised to the reference gene 18s rRNA. RT PCR was carried out using Sigma PCR kit. PCR Primers used in this study were:

Human Sulf1 (catalytic domain)
=5’-CGAGGTTCAGAGGACGGATA-3’
=5’-GCCTCTCCACAGAATCATCC-3’

Human Sulf1 (hydrophilic domain)
=5’-TTGTCCATACTCGGCAGACA-3’
=5’-TCCCATCCATCCCATAACTG-3’

Human Sulf2 (catalytic domain)
=5′-CAACTGTGTTCTCCCTGCTGGGT-3′
=5′-CTGGAGCATGTTGGTGAATTCC-3’

Human Sulf2 (hydrophilic domain)
= 5’-GCCAACCCCATTAAAGTGAC-3’
=5’-TGCATTCATCAGCTGGTAGG-3’

Mouse Sulf1 (catalytic domain)
=5′-ATGAAGTATTCCCTCTGGGCTCTG-3′;
=5′-CAATGTGGTAGCCGTGGTCC-3′

Mouse Sulf 2 (catalytic domain)
=5′-ATGGCACCCCCTGGCCTGCCACTAT-3′
=5′-CATAGACTTGCCCTTCACCAGCCC-3′

Human β- actin
=5’-CTATGAGCTGCCTGACGGTC-3’
=5’-AGTTTCATGGATGCCACAGG-3’

Human Cyclin D1
=5’- TCATGGCTGAAGTCACCTCTTGGT-3’
=5’-TCCACTGGATGGTTTGTCACTGGA-3

Human BMPR2
=5’- AAAGCCCAGAAGAGCACAGA-3’
5’-AGCGATTCAGTGGAGATGAC-3’

Human FGFR2
= 5’-GGATCAAGCACGTGGAAAAGAAC-3’
=5’-GGCGATTAAGAAGACCCCTATGC-3’

Human FGFR3
= 5’-ACGTTACCGTGCTCAAGACGGC-3’
=5’-AGGAAGAAGCCCACCCCG-3’

RESULTS:

SULF1/SULF2 expression in skeletal cells of adult rat bone: Qualitative analysis of immunochemical labelling of adult (16 week) bone sections showed high level of SULF1 (Figure 1.A, B) and SULF2 (Figure 1.C) expression in osteoblasts that was particularly pronounced in the active osteoblasts laying down the bone adjacent to bone fracture (Figure 1.B). The level of both SULFS was lower in osteocytes when compared with osteoblasts (Figure 1A, C) as both these antibodies have been demonstrated to exhibit similar staining intensities (Sahota and Dhoot 2009; Gill et al. 2011; Gill et al. 2012). The specificity of SULF antibodies was confirmed by staining of the adjacent bone sections with pre-immune SULF2 (not shown) and pre-immune SULF1 serum showing no reaction (Figure 1.D) but positive reaction for SULF1 (Figure 1.E). SULF1 and SULF2 expression was also observed in

osteoclasts (Figure 1.F,J) identified from their multinuclear morphology and TRAP staining (Figure 1.G,H,I). Figure H at low magnification highlights the abundance of osteoclasts at the junction of remodelling cartilage between the hypertrophic chondrocytes and spongy bone, and on trabecular bone surface that express both SULF1 and SULF2.

The levels of *Sulf1* and *Sulf2* gene expression were further examined in osteoblasts prepared from rat calvaria during their differentiation *in vitro.* RNA prepared from such cultures at 3, 7, 10, 13 and 17 days showed gradual increases in both *Sulf1* and *Sulf2* mRNA levels using real-time quantitative PCR (Figure 2.A). Figure 2B illustrates differentiation with time of rat calvarial-derived osteoblasts reacted for alkaline phosphatase activity with fast blue stain at day 7, 14 and 21.

Differential expression of *Sulf1* and *Sulf2* in differentiating mouse chondrogenic ATDC5 cells: To determine if levels of SULFS are modified during chondrocyte differentiation, we examined the expression of both *Sulf1* and *Sulf2* mRNA levels during prolonged growth of ATDC5 cells undergoing differentiation (Figure 2C). Unlike the immunolabelling analysis of human and rat articular cartilage showing comparable levels of both SULF1 and SULF2 in most samples as shown in later figures, these two transcripts changed in opposite direction in ATDC5 cells concomitant with the onset of matrix mineralisation (Newton et al. 2012). *Sulf1* under these conditions thus showed very low level expression up to day 8 after which it gradually increased up to day 34. In contrast, *Sulf2* was highly expressed from day 6 after which its expression gradually decreased up to day 34 (Figure 2C).

***Sulf1/Sulf2* expression patterns in human cancellous and sub-chondral bone**: Our recent studies have demonstrated the existence of functionally distinct *Sulf1* and *Sulf2* splice variants in some normal fetal and tumour tissues (Gill et al. 2011; Gill et al. 2012). To determine if the levels or nature of *Sulf1* or *Sulf2* variants varied in cancellous and sub-chondral bone-derived osteoblasts, primers specific for each *Sulf* variant mRNA were used for RT PCR analysis of prostaglandin E2 (PGE2) treated cancellous and sub-chondral bone-derived osteoblasts since the full length SULFs are known to inhibit VEGF activity and PGE2 treatment has been shown to induce VEGF production (Clarkin et al. 2008). Both *Sulf1* and *Sulf2* variants were expressed by all osteoblasts and PGE2 treatment produced no effect on their relative expression levels examined by this procedure (Figure 3). The differences in relative levels of *Sulf1* expression between some samples were more commonly observed with primers to catalytic region than to the hydrophilic region of *Sulf1*. All samples showed the presence of a single major band representing full length *Sulf1* but some samples also showed low-abundance of a shorter band with 240bp deletion (indicated by arrow heads and arrows) for *Sulf2* in the catalytic domain (Figure 3). The sequence analysis of this band confirmed it as a deletion of 240bp exon 6. The level of expression of this band, however, was too low to identify differences between samples. These *in-vitro* data are in agreement with the immunolabelling of bone and support an increase in SULF1 and SULF2 expression with osteoblast differentiation.

***Sulf1/Sulf2* expression in human cartilage:** *Sulf1/Sulf2* expression was also examined in normal human articular cartilage using splice variant specific primers. Full length *Sulf1* and *Sulf2* transcripts in human articular cartilage samples were expressed at variable abundance whereas the splice variants were undetectable

(Figure 4). The variable expression of *Sulf1* and *Sulf2* was not related to the age of the individuals but interestingly qualitative evaluation indicated that high levels of both *Sulf1* and *Sulf2* were generally found in samples that also showed high levels of cyclin D1 and the cell signalling components, FGFR2, FGFR3 and BMPR2-receptors (Figure 4). The reason for non-detection of both *Sulf1/Sulf2* and signalling molecules in two samples (18 & 44) is not clear but may relate to the quiescent nature and lack of activity in such cells.

Heterogeneous expression of SULF1 and SULF2 in articular chondrocytes: Since the RT PCR analysis of human cartilage demonstrated highly variable levels of both *Sulf1* and *Sulf2*, we further examined such expression at the cellular level using immunohistochemical labelling in rat articular cartilage. Both SULF1 and SULF2 were expressed by the majority of chondrocytes during development and in adult articular cartilage. Some chondrocytes, however, failed to show either SULF1 or SULF2 labelling and the proportion of these double negative chondrocytes was higher postnatally compared to fetal cartilage (Figure 5). While the expression of both enzymes was evenly distributed throughout the developing cartilage, their expression was more pronounced in the superficial and mid- zone chondrocytes in the adult cartilage.

In contrast to SULF1, SULF2 is highly expressed in hypertrophic chondrocytes of the developing bone: Immunocytochemical staining of longitudinal sections of fetal, early postnatal and adult rat limb bones through growth plates demonstrated marked differences in the expression patterns of SULF1 and SULF2 proteins. SULF1 and SULF2 were expressed at relatively high levels in fetal and adult cartilage with SULF2 expression in greater abundance compared to SULF1 (Figure 6). This difference was more marked at postnatal days 7 and 10 where SULF1 was low and restricted to punctate localization on the cell membrane in hypertrophic chondrocytes whereas the levels of SULF2 associated with these cells were high. SULF2 expression, however, was not particularly evident in the hypertrophic cell cytoplasm but was rather restricted to the ECM surrounding the cell membrane, particularly around the most mature hypertrophic chondrocytes towards the metaphysis. The relative expression of SULF1 (low) and SULF2 (high) was also observed in hypertrophic chondrocytes within secondary ossification centres (data not shown).

SULF2 but not SULF1 expression in hypertrophic chondrocytes correlates with Hedgehog signalling in both growth plate and bone fracture healing: To determine if healing bone recapitulates SULF1 and SULF2 expression patterns observed in developing bone, we examined their localisation along with markers of Hedgehog signalling during post fracture bone healing (Figure 7A, A1). Examination of bone fracture sites 9 days post injury demonstrated some changes similar to developing bone but also identified the presence of some cellular structures that often appeared to be present in the centres of hypertrophic chondrocytes. While the hypertrophic chondrocytes and/or their remnants only stained for SULF2 and ptc1 but barely for SULF1, the cellular structures located in the hypertrophic cells stained for all three including SULF1 (Figure 7.B-D). It was not clear if such “cells” represented remnants of hypertrophic chondrocytes destined to undergo apoptosis or some other emerging cells in this area. Further examination of other hypertrophic areas in the same and some other healing bones demonstrated morphology of such cells more akin to healthy cells and not apoptotic cells (Figure 7 E1, E2, E3). SULF1

staining intensity and relative proportion of this non-hypertrophic set of cells positive for not only SULF2 and ptc1 but also SULF1 varied in number as is apparent from the higher proportion of such cells in Figure 7. E1, E2 and E3. SULF1 and SULF2 expression in the adjacent healing bone in contrast was essentially identical as shown by their comparable staining intensities (not shown).

To determine if the high levels of SULF2 expression in hypertrophic chondrocytes correlate with Hedhehog cell signaling, we also examined the expression of ptc1 receptor as a marker of this signaling pathway in both bone fracture and the growth plate. The immunocytochemical staining of hypertrophic chondrocytes in the growth plate as well as changes in hypertrophic cells during bone fracture showed the expression of SULF2 spreading from cell membrane to ECM closely correlating with ptc1 receptor but not SULF1 (Figure 7.C1, D1, E2, E3 & Figure 8). The SULF2 and ptc1 staining in the areas of hypertrophy clearly extended beyond cell membrane into ECM (Figure 7 & 8).

DISCUSSION:

Most cells exhibit a marked down regulation of SULF1 and SULF2 expression postnatally and seem only to re-activate expression upon injury (Sahota and Dhoot 2009; Dhoot 2012). Our studies show that cartilage and bone cells do not follow this trend but instead retain an easily detectable level of both SULF1 and SULF2 expression in postnatal and adult skeletal elements that also agrees with previous studies demonstrating some Sulf1/Sulf2 expression in fetal and postnatal tissues (Zhao et al. 2006; Otsuki et al. 2008; Ratzka et al. 2008). Previously, only adult neuronal tissues have been found to express high levels of these enzymes (Joy et al. 2015); some low level expression in endothelial cells has been documented where they have been shown to act as cell signalling inhibitors (Sahota and Dhoot 2009). Continued expression of SULF1/SULF2 by chondrocytes in postnatal cartilage may also relate to some inhibitory role although SULF expression was not observed in all chondrocytes. The detection of some SULF-positive and SULF-negative articular chondrocytes, however, indicates the varying levels of chondrocytic activity or quiescence. In neuronal tissue, SULFs persist in the adult to inhibit cell proliferation and neurite outgrowth (Joy et al. 2015). It is not clear if the expression and non-detection of SULFs in a subset of cells represents activity versus quiescence since the chondrocytes are not known to undergo significant cell proliferation. The change in the levels of SULF1/SULF2 does not only relate to the relative proportions of positive and negative cells expressing these enzymes but also the expansion of the negative ECM area between these cells. Nevertheless, both SULF1 and SULF2 expression was detected in a sub-population of articular chondrocytes. Unlike increasing expression of SULF1/SULF2 with age reported by Ratzka et al (Ratzka et al. 2008), we found the levels of these enzymes in adult human as well as rodent articular cartilage to be much lower than that observed during fetal development. The *in vivo* expression, however, was in marked contrast to the prolonged *in vitro* differentiation of chondrocytic cell line ATDC5 showing an increase in *Sulf1* but a decrease in *Sulf2*. SULF1 and SULF2 enzymes during *in vitro* differentiation of this chondrocytic cell line could thus have differential functions requiring activation or inhibition of specific signalling pathways. This differed from the *in vivo* articular chondrocytes expressing fairly similar levels of both SULFs

detected by immunocytochemistry. The differential changes in SULF1 and SULF2 expression levels were also identified in hypertrophic chondrocytes that unlike ATDC5 showed up-regulation of SULF2 but down-regulation of SULF1. Not all chondrocytes, however, showed differential SULF1 and SULF2 changes like hypertrophic chondrocytes since not only articular chondrocytes but also some other cells observed in hypertrophic chondrocyte rich areas following fracture repair showed generally similar expression patterns.

Persistent expression of SULFs in osteoblasts could relate to prolonged postnatal period of growth as well as bone remodelling post-growth period. This is supported by the high SULF levels in osteoblasts laying down the bone but with reduced levels in the osteocytes. The up-regulation of SULFs with differentiation is also supported by the RT PCR analysis of osteoblast cultures prepared from rat calvariae that also demonstrated comparable increases in both *Sulf1* and *Sulf2* during *in vitro* differentiation indicating possibly similar roles of these enzymes in bone cell signalling. High SULF levels in active osteoblasts were particularly apparent during bone fracture healing where the SULFs could enhance specific cell signalling pathways such as BMP and/or Wnt signalling.

To examine if sub-chondral osteoblasts near the vascularisation area expressed different levels of *Sulf1* and *Sulf2* or their shorter variants that could promote angiogenesis, we compared these cultures with cancellous bone derived osteoblasts in the presence and absence of PGE2 known to promote VEGF expression by such cells (Clarkin et al. 2008). The osteoblast cultures prepared from both cancellous and subchondral bone as representatives of *in vivo* endochondral ossification also demonstrated generally similar changes in *Sulf1* and *Sulf2* except with a slightly higher level expression of *Sulf2* relative to *Sulf1*, indicating similar roles of *Sulf1* and *Sulf2* during *in vitro* differentiation of osteoblasts. Osteoblasts prepared from cancellous and subchondral bone showed mainly full length *Sulf1* and *Sulf2* although a very low level expression of *Sulf2* with 240bp exon 6 deletion was also detected in a number of these samples that was not significantly different in cancellous versus subchondral or during 3 hour incubation with 1µM PGE2. This is compatible with little or no expression of such variants in most postnatal tissues except during acute injury compared with fetal (Gill et al. 2010; Hitchins et al. 2013) and particularly tumour tissues (Gill et al. 2014). The requirement of only a very subtle change in the levels of shorter variants, not quantifiable by this method, or proximity to the hypertrophic chondrocytes expressing high levels of full length SULF2, which has been described to have some pro-angiogenic function (Morimoto-Tomita et al. 2005), may be sufficient to promote angiogenesis in sub-chondral bone.

Dynamic and variable SULF1/SULF2 expression profiles highlight shared as well as unique roles of these enzymes. Hypertrophic chondrocytes showed the presence of mainly SULF2 with only low level SULF1 expression. The presence of a mixture of cells following bone fracture that were positive for SULF2 but larger hypertrophic cells showing little or no SULF1 expression indicates that while SULF1 and SULF2 were segregated in hypertrophic cells, some other cells that may be remnants of necrotic hypertrophic chondrocytes expressed both SULF1 and SULF2. This suggests that some functions may be unique to SULF1 and SULF2 but other functions are shared in which they could even compensate for each other.

It is possible that hypertrophic chondrocytes that are believed to be in hypoxic environment at the chondro-vascular border promote angiogenesis since VEGF is believed to be expressed in and around hypertrophic chondrocytes of the growth plate (Petersen et al. 2002). Differential SULF1/SULF2 expression in hypertrophic chondrocytes may thus indicate their differential roles in vascularisation as has been suggested by the antiangiogenic activities of SULF1 (Sahota and Dhoot 2009; Wang et al. 2004) but a pro-angiogenic activity of SULF2 in some mammary tumours (Morimoto-Tomita et al. 2005).

The spread of SULF2 enzyme to the ECM around the hypertrophic chondrocytes also agrees with our early report of SULF2 association with the stromal compartment in some tumours (Gill et al. 2014) although SULF2 detected in stromal compartment in such cases was produced by the inflammatory cells whereas SULF2 in hypertrophic chondrocyte rich areas is generated by the chondrocytes themselves diffusing into the surrounding ECM since no interstitial cells are observed in this area. This is also compatible with SULF2 secretion into the culture medium indicating that although both these enzymes are docked to the cell membrane they can also detach to diffuse into the extracellular milieu (Uchimura et al. 2006). It is not clear if both SULF1 and SULF2 influence certain ligand gradients such as Shh. For example, Ramsbottom et al (Ramsbottom et al. 2014) recently reported SULF1 influencing the Shh morphogen gradient during the dorsal ventral patterning of the neural tube in Xenopus by restricting ligand diffusion whereas *Sulf1* knockdown resulted in a more diffuse distribution of Shh ligand. SULF2 thus could also modify the sulfation of heparan sulfate of HSPGs extracellularly to regulate the bio-availability of Hedgehog ligand for Hedgehog/ptc1 cell signalling or indeed any other signalling ligand such as Wnt. This could also modulate the ligand interaction with glypicans to facilitate Hedgehog signalling as has been reported for Wnt signalling (Ai et al. 2003). As was the case with SULF2, ptc1, a hedgehog receptor was detected not only on the cell membrane but also in the ECM. Cell surface receptors are usually embedded in the cell membrane and therefore restricted to the site of cell signalling only. It is possible that ptc1 could diffuse into ECM if it is processed in this compartment to be used as an inhibitor, as, for example, has been described for endoglin. Indeed, ptc1 has been reported to sequester hedgehog ligand and restrict its movement to generate hedgehog morphogen gradients (Dessaud et al. 2007). These data suggest possible roles for SULFs in controlling Hedgehog signalling and unique functional roles for secreted SULF2 in the ECM. Since a number of growth factors are expressed during different phases of fracture-healing, the role of SULF1 and SULF2 molecules could be important as potential therapeutic agents to enhance their signalling, bone repair or to accelerate fracture-healing.

FIGURE LEGENDS:

Figure 1: *SULF1/SULF2 cellular expression in some rat skeletal elements*: Sections of normal adult rat bone (A, C, F, J) and post-fracture healing bone tissues (B, D, E) stained with antibodies to SULF1, SULF2 and preimmune serum using immunofluorescence (A,B,D,E,F) and immunoperoxidase (C, J) procedures as well as TRAP2 staining for osteoclasts (F,H,I) to confirm Sulf expression in such cells; ob = osteoblasts, ocy =osteocytes. Figure H at low magnification indicates the location of higher concentration of osteoclasts at the junction of remodelling cartilage near

the hypertrophic chondrocytes and at the surface of trabecular bone while higher magnification in I & J confirms the location of SULF1/SULF2 in such multinuclear cells.

Figure 2: A & B-: Osteoblasts prepared from rat calvaria grown *in vitro* up to 17days for qPCR analysis (A) and cells grown for 1, 2 and 3 weeks for differentiation staining for alkaline phosphatase activity with fast blue stain (B). C: qPCR analysis of ATDC5 mouse cells grown *in vitro* for upto 34 days for changes in Sulf1 and Sulf2 mRNA levels during differentiation.

Figure 3: Osteoblasts prepared from human cancellous and sub-chondral bone cultured in the absence and presence of PGE2 for 3 hours before RT PCR analysis for Sulf1 and Sulf2 expression with normalisation using β-actin levels. For Sulf2, a full length main band and a faint shorter band with exon 6 deletion are pointed by the arrows or arrow heads.

Figure 4: The use of RNA from growing, adult and ageing human articular cartilage used for RT PCR analysis of Sulf1 and Sulf2 representing both catalytic and hydrophilic domains and their correlation with BMPR2, CyclinD1, FGFR2 and FGFR3 with normalisation using β-actin primers. The numbers in the top lane refer to age in years.

Figure 5: The immunofluorescence staining of paraffin sections through articular cartilage region of 17 day fetal, 7d postnatal and 3 month old rat with antibodies to SULF1 and SULF2. Also included is a magnified region in the bottom left hand corner of each image with some negative chondrocytes pointed out by arrows. Blue DAPI stains all nuclei.

Figure 6: The immunofluorescence staining of paraffin sections through the growth plate of 17.5 day fetal, 7d and 10d postnatal and 3-month old rat limbs with antibodies to SULF1 and SULF2. Also included is a magnified region through the hypertrophic chondrocyte area in the bottom left hand corner of each image. Blue DAPI stains all nuclei.

Figure 7: The H & E (A) staining and immunofluorescence staining of paraffin sections through healing bone 9 day post fracture with antibodies to SULF1 (B), SULF2 (C) and ptc1 (D) at low and high magnification (A1, B1, C1, D1). The rectangle shown in A-D indicates the selected area magnified in A1-D1. To highlight cell morphology, also included is a small section of further magnification shown in the bottom right hand corner of A1, B1, C1 and D1. White lines in B, C and D indicate the morphological landmarks in different sections to highlight the differences in SULF1, SULF2 and ptc1 staining. Blue DAPI stains all nuclei. White arrows point to “cells” stained for not only SULF2 and ptc1 but also SULF1. Green lines point to the hypertrophic chondrocytes stained for SULF2 and ptc1 but not SULF1. E1, E2 and E3 are sections through another region of bone healing 9d post fracture stained for SULF1, SULF2 and ptc1 to highlight the presence of a number of “cells” staining (pointed out by some arrows) with all three antibodies.

Figure 8: Sections through growth plate of 7d postnatal rat tibia and 14 day post fracture healing femur stained for SULF1, SULF2 and ptc1 to highlight the correlation of SULF2 staining of hypertrophic chondrocytes with ptc1 in both growth plate and healing bone.

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Conflict of Interest: None

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Figure 1

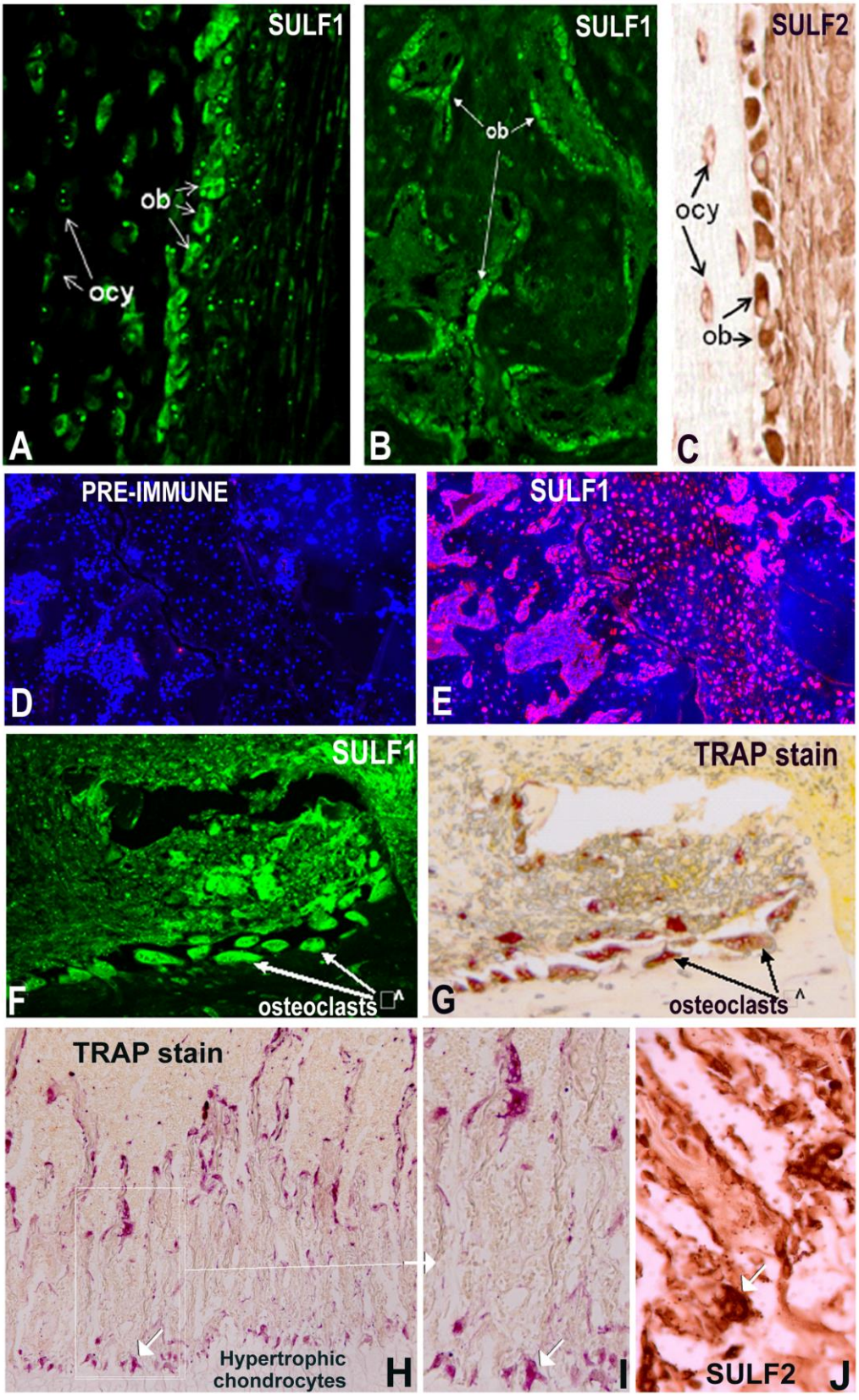
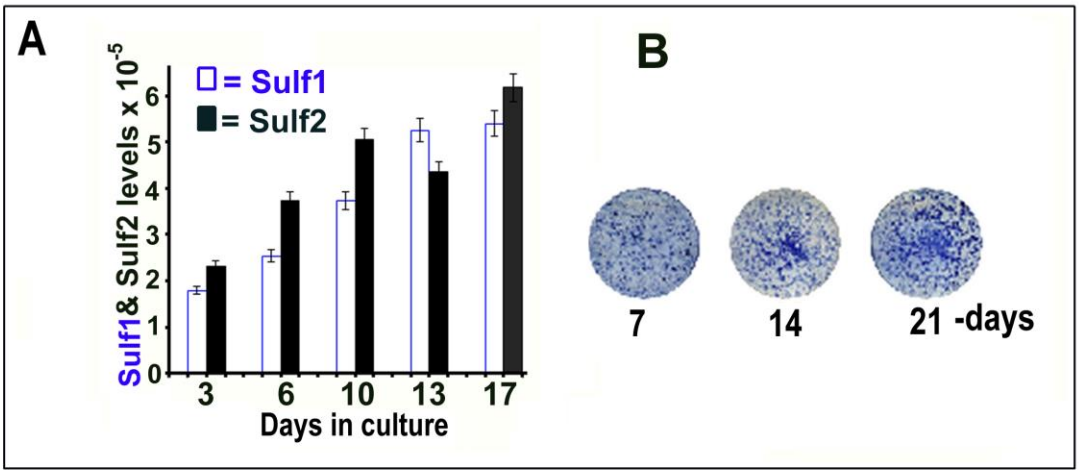

SULF1
ob
ocy
A
SULF1
ob
B
SULF2
ocy
ob
C
PRE-IMMUNE
D
SULF1
E
SULF1
osteoclasts
F
TRAP stain
osteoclasts
G
TRAP stain
Hypertrophic chondrocytes
H
I
SULF2
J

Figure 2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= Sulf2
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2
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4
5
6
3
6
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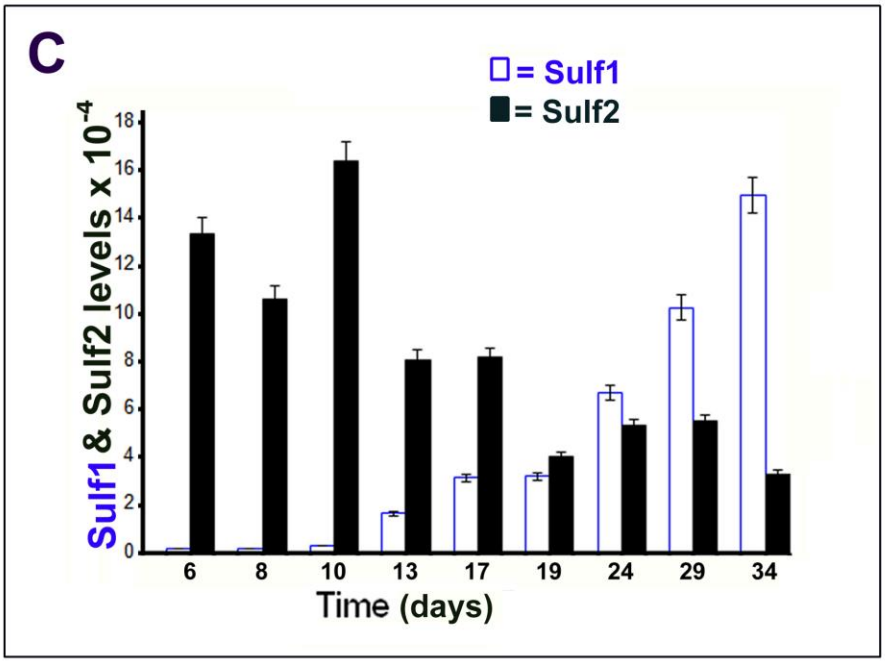
13
17
Days in culture
B
7
14
21 -days


C
= Sulf1
= Sulf2
Sulf1 & Sulf2 levels x 10-4
0
2
4
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17
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24
29
34
Time (days)

Figure 3

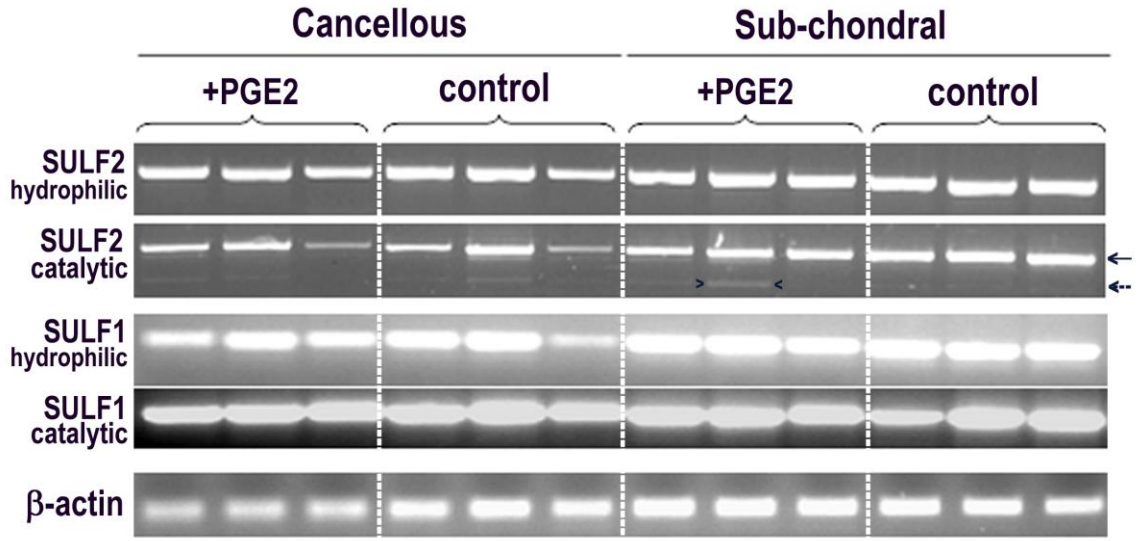
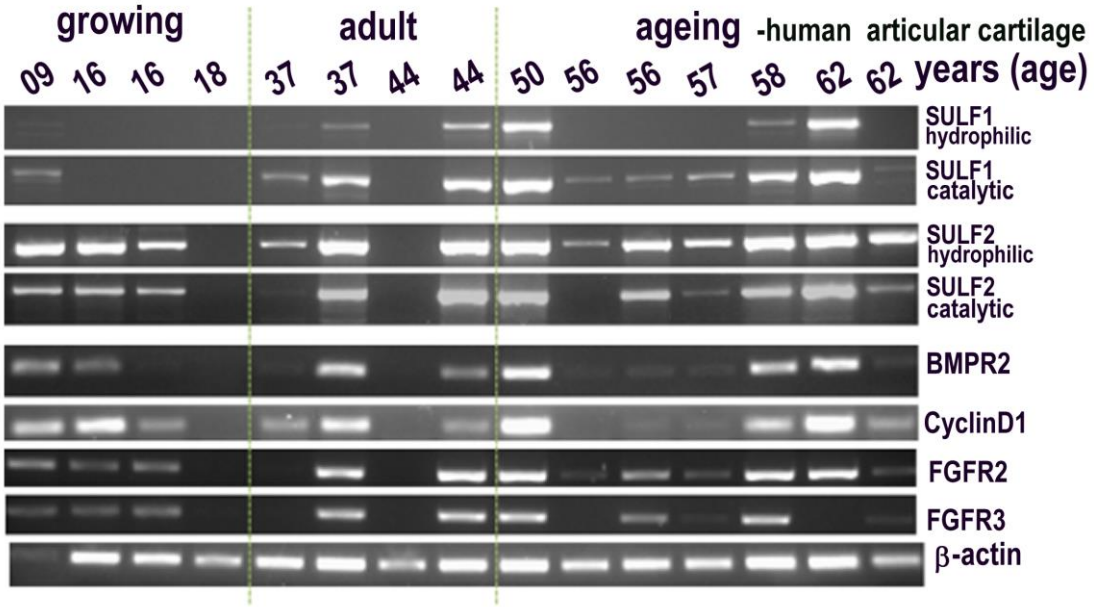


Figure 4



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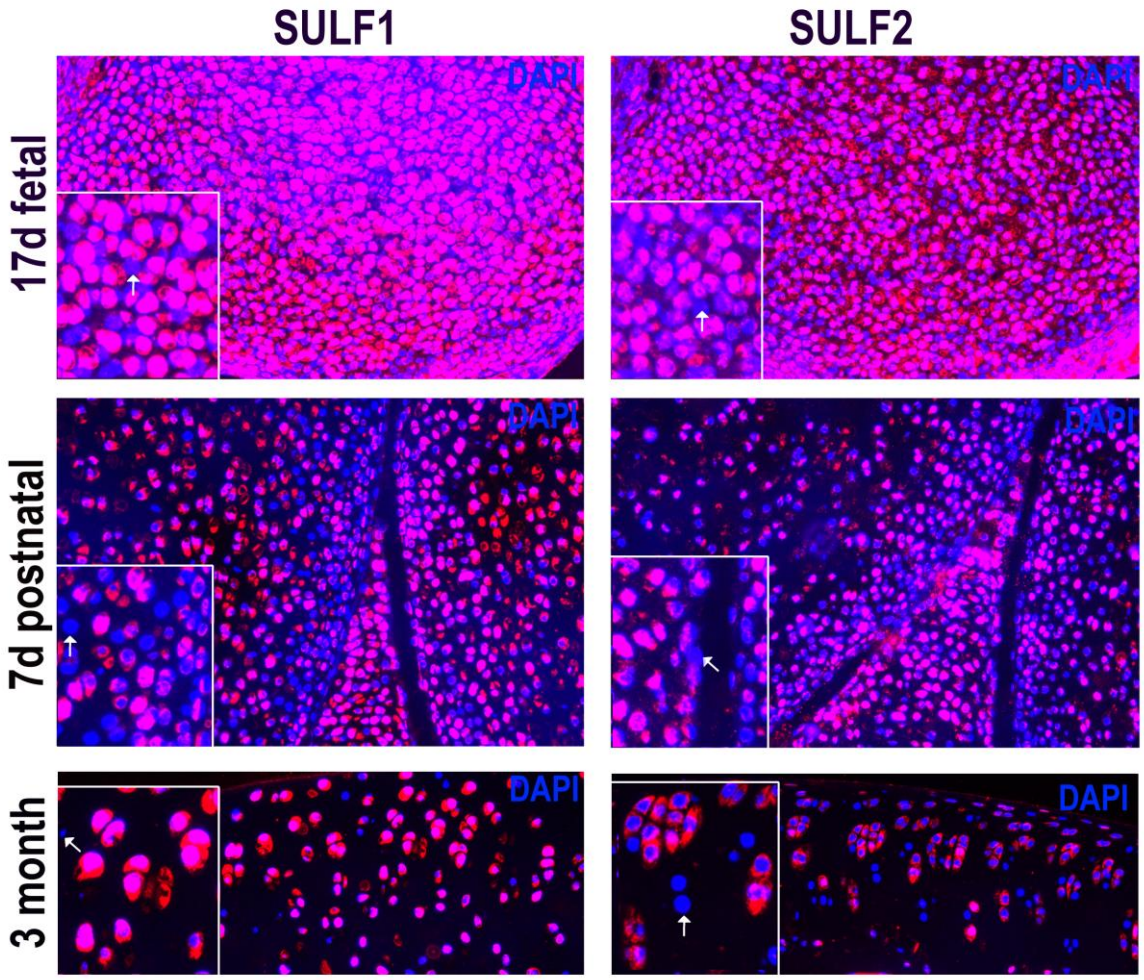
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SULF2
17d fetal
7d postnatal
3 month
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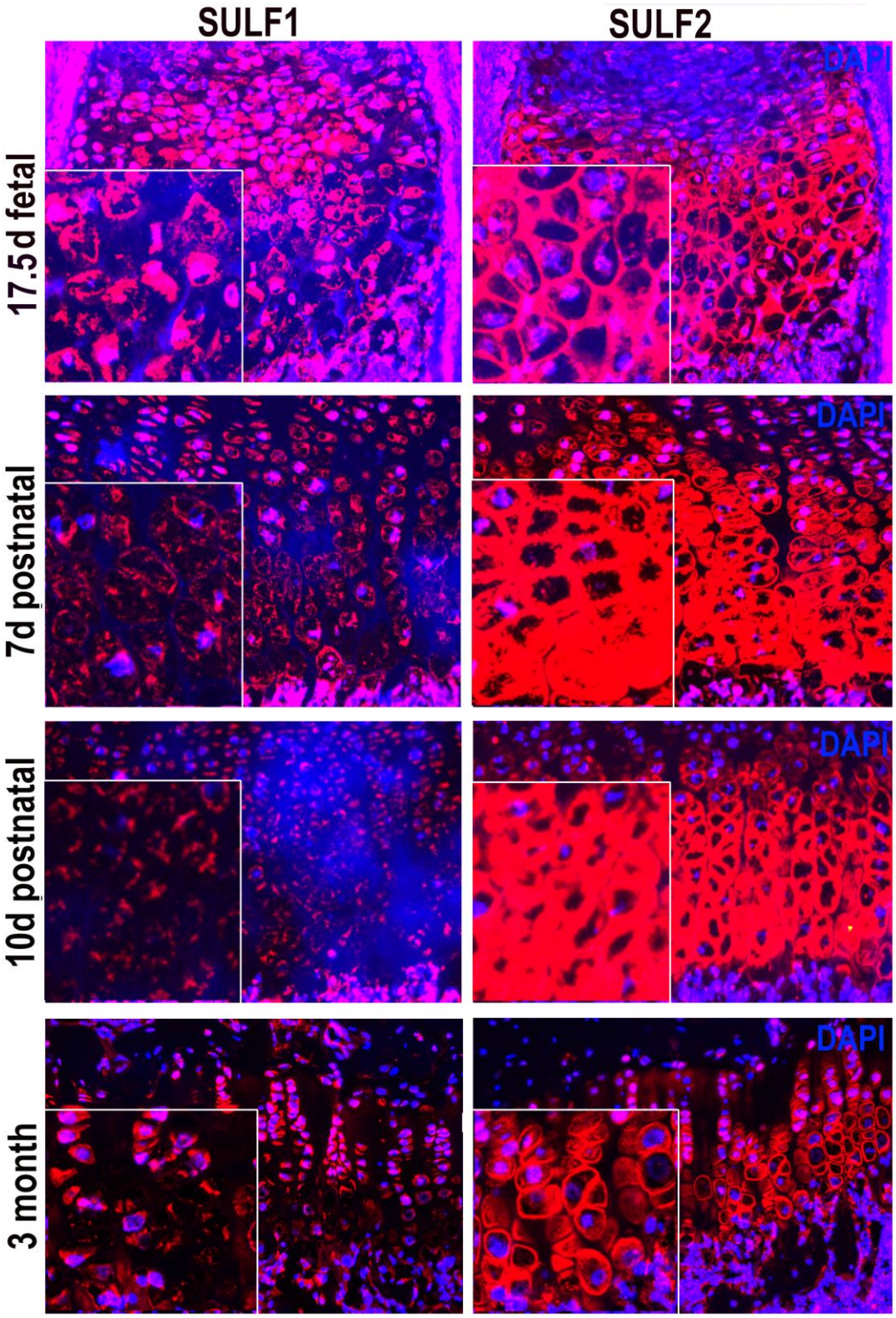
Figure 6



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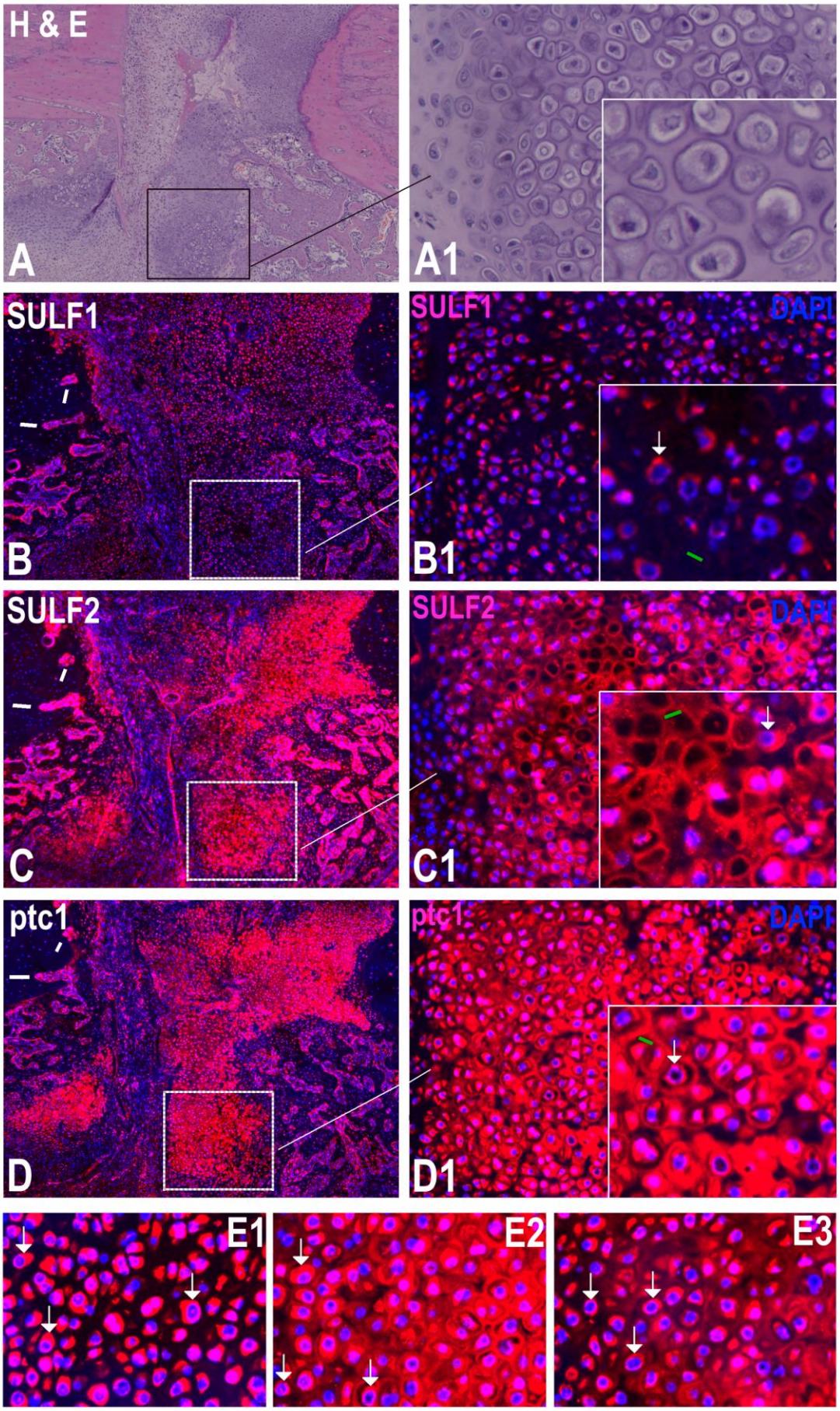
H & E
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D
ptc1
DAPI
D1
E1
E2
E3

Figure 8