Running head: MicroRNAs in chondrogenesis and osteoarthritis

Title: The expression and function of microRNAs in chondrogenesis and osteoarthritis

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Abstract

Objective: To use an *in vitro* model of chondrogenesis to identify microRNAs with a functional role in cartilage homeostasis.

Methods: The expression of microRNAs was measured in the ATDC5 model of chondrogenesis using microarray and verified using quantitative RT-PCR. MicroRNA expression was localised by *in situ* hybridisation. Predicted microRNA target genes were validated using 3'UTR-luciferase reporter plasmids containing either wild-type sequence or mutants of the microRNA target sequence. Signalling through the Smad pathway was measured using a $(CAGA)_{12}$-luciferase reporter.

Results: The expression of a number of microRNAs was regulated across chondrogenesis. This includes 39 microRNAs co-expressed with microRNA-140, known to be involved in cartilage homeostasis and osteoarthritis. Of these, microRNA-455 resides within an intron of *COL27A1* which encodes a cartilage collagen. Comparing human osteoarthritic cartilage with femoral neck fracture controls, both microRNA-140-5p and microRNA-455-3p show increased expression in osteoarthritic cartilage. *In situ* hybridisation shows microRNA-455-3p expression in the developing limbs of chicks and mice and in human osteoarthritic cartilage. The expression of microRNA-455-3p is regulated by TGFβ ligands and the microRNA regulates TGFβ signalling. *ACVR2B*, *SMAD2* and *CHRDL1* are direct targets of miR-455-3p and may mediate its functional impact on TGFβ signalling.

Conclusions: MicroRNA-455 is expressed during chondrogenesis and in adult articular cartilage where it can regulate TGFβ signalling, suppressing the Smad2/3 pathway. Diminished signalling through this pathway in ageing and osteoarthritic chondrocytes is known to contribute to cartilage destruction. We propose that the increase in microRNA-455 in osteoarthritis exacerbates this process and contributes to disease pathology.

Osteoarthritis (OA) is a degenerative joint disease characterised by degradation of articular cartilage, thickening of the subchondral bone and the formation of osteophytes at the joint margin (1). The aetiology of OA is complex with e.g. genetic, developmental, biochemical and biomechanical factors contributing. The chondrocyte is the only cell in cartilage and is responsible for the synthesis and turnover of the extracellular matrix (ECM) which is crucial to tissue function (1).

During development mesenchymal cells aggregate and differentiate into chondrocytes. Chondrocytes undergo a series of differentiation events, including proliferation, hypertrophy, terminal differentiation, mineralization and programmed cell death. Blood vessels penetrate the calcified matrix and bring in osteoblasts which build new bone. The cartilage model grows by rounds of chondrocyte cell division accompanied by secretion of ECM at either end of the bones in the growth plates. Chondrocytes in the articular cartilage are constrained from completing this developmental programme allowing maintenance of a functional cartilage layer, essential for normal joint function (2).

Articular chondrocytes must enact an appropriate pattern of gene expression to achieve tissue homeostasis in response to signals from e.g. locally produced growth factors, mechanical load or changes to the ECM (1). Transcription profiling demonstrates that chondrocytes in normal cartilage express a range of genes and that this expression pattern is significantly altered in OA (3). One facet of this aberrant gene expression in OA is the replay of chondrocyte differentiation with the expression of many genes associated with chondrocyte hypertrophy (e.g. matrix metalloproteinase 13, type X collagen) (2). The transcriptional control of chondrogenesis is known in some detail, however, mechanisms leading to altered gene expression in OA are less well understood (1, 2).

Small non-coding RNAs (19-24 nt long) known as microRNAs (miRNAs) are recognised as important regulators of gene expression (4). miRNAs are first transcribed as primary transcripts (pri-miRNA) with a cap and poly-A tail and processed to short, 70-nucleotide stem-loop structures (pre-miRNA) in the nucleus. The pre-miRNA is then processed by the ribonuclease Dicer, forming two complementary short RNA molecules one of which is integrated into the RNA-induced silencing complex (RISC). After integration into the active RISC complex, miRNAs base pair with their complementary mRNA molecules, usually in the 3'UTR (5). In mammals, RISC functions to suppress translation generally leading to decreased levels of steady-state mRNA.

It has been shown that microRNAs are necessary for normal skeletal development. The conditional knockout of *Dicer* in cartilage leads to decreased chondrocyte proliferation and accelerated hypertrophy with consequent compromised skeletal growth (6). Furthermore, the profile of microRNA expression in mesenchymal stem cells is significantly altered once these cells differentiate in chondrocytes (7, 8). More specifically, BMP signalling regulates the expression of miR-199a, which is reported to target *Smad1* and regulate early chondrogenesis by reducing the expression of key genes such as *Col2a1* and *Sox9* in a BMP-driven model (9). MicroRNA-1 regulates aggrecan expression in a human chondrocyte-like cell line (10) whilst miR-675 indirectly regulates *COL2A1* expression in primary articular chondrocytes (11). The profile of microRNA expression is also altered between differentiated and dedifferentiated adult articular chondrocytes (12, 13). A number of miRNAs have also been identified as regulators of osteoblastogenesis e.g. miR-29, miR-141 and miR-200a, miR-206, miR-210 and miR-2861 (see recent review Kapinas and Delany (14)).

MicroRNA-140 (miR-140) was found to be expressed only in cartilaginous tissues in the developing zebrafish (15). We previously investigated the expression pattern and potential

targets of mouse miR-140 (16). MiR-140 is specifically expressed in cartilage tissues of mouse embryos during both long and flat bone development. We identified and validated histone deacetylase 4 (*Hdac4*), a known co-repressor of Runx2 and MEF2C transcription factors essential for chondrocyte hypertrophy and bone development, as a target of miR-140. We therefore proposed that miR-140 functions in the developing skeleton to promote differentiation by functionally suppressing HDAC4. We also reported that miR-140 also targets *Cxcl12* (stromal cell-derived factor 1) (17) and *Smad3* (18), both of which are also implicated in chondrocyte differentiation. The function of miR-140 *in vivo* has been demonstrated by Miyaki et al. (19) using targeted deletion to create a miR-140 null mouse. This mouse has a mild developmental phenotype in the skeleton, potentially via reduced proliferation of chondrocytes in the growth plate, but displays a premature osteoarthritis phenotype driven at least in part by an increase in *ADAMTS5* expression. *ADAMTS5* is also shown as a direct target of miR-140. A transgenic mouse overexpressing miR-140 in cartilage displays no skeletal phenotype during development, but is protected in an antigen-induced arthritis model. More recently, Nakamura et al (20), also showed skeletal abnormalities in a miR-140 null mouse, with accelerated hypertrophic chondrocyte differentiation. *Dnpep* was identified as a target of miR-140 with the increase in this aspartyl aminopeptidase in the miR-140 null mouse leading to reduced BMP signalling. miR-140 thus plays a key role in cartilage homeostasis and osteoarthritis.

MicroRNA profiling in human cartilage has also been performed, leading to the identification of miR-9 impacting upon IL1-stimulated MMP expression (21) and miR-22 as a regulator of PPARα and BMP7 signalling (22). Studies have shown that miR-27a (23) and miR-27b (24) regulate MMP13 expression in human osteoarthritic chondrocytes. MicroRNA-34a has also been reported to modulate chondrocyte apoptosis (25).

ATDC5 cells are a murine embryonic carcinoma line that can differentiate *in vitro* through chondrogenesis. In this model, the regulation of a number of known markers of chondrocyte proliferation or hypertrophy (e.g. type II and type X collagen) has been shown to mirror the *in vivo* process e.g. (26).

In this study, we aimed to explore whether key microRNAs which were regulated in chondrogenesis were also regulated in osteoarthritic cartilage. Hence we profiled the expression of microRNAs in the ATDC5 model and measured expression of key microRNAs in human cartilage. This has identified groups of microRNAs which may function cooperatively and we demonstrate that in common with miR-140, miR-455 regulates and is regulated by Smad signalling. We hypothesise that these miRNAs regulate the switch from Smad2/3 signalling to Smad1/5/8 signalling in endochondral ossification and contribute to the alteration of TGFβ signalling in OA cartilage.

Materials and Methods

Cell culture and RNA purification: SW1353, C3H10T1/2 and 3T3 cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Invitrogen) supplemented with 10% (v/v) FBS (fetal bovine serum; Sigma), 2mM glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. ATDC5 cells were maintained at 37°C, 5% CO_2 in DMEM/HAM's F12 medium containing 5% (v/v) FCS, 2mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 5ng/ml sodium selenite, 10µg/ml human transferrin. For assay, cells were seeded at $6x10^4$ cells per well of a 6-well plate in the above medium containing 10 µg/ml bovine pancreatic insulin and the medium changed every second day. After 21 days, the medium was changed to alphaMEM with the same supplements and the atmosphere changed to 3% CO_2. Cells were grown to day 42 and GAG stained using alcian blue. Cells were fixed in methanol for 2 minutes, 0.1% (w/v) alcian blue in

0.1M HCl was added and incubated at room temperature overnight. At selected time points, cells were scraped into Trizol reagent (Invitrogen) for RNA purification according to the manufacturer's instructions.

Collection of human cartilage and RNA purification: Human articular cartilage was obtained from femoral heads of patients undergoing total-hip-replacement surgery at the Norfolk and Norwich University Hospital. Samples from patients with OA (n = 10, 5 female, 5 male; age range, 37 - 86 years) were compared with cartilage from patients undergoing hip replacement following fracture to the neck of femur (NOF; n = 10, 5 female, 5 male; age range, 68 - 94 years). OA was diagnosed using the clinical history and an examination, coupled with X-ray findings; confirmation of gross pathology was made at time of joint removal. The fracture patients had no known history of joint disease and their cartilage was free of lesions; 80% of these patients underwent surgery within 36 hours of fracture. This study was performed with Ethical Committee approval and all patients provided informed consent. Cartilage samples were chopped into 2–5-mm pieces and snap frozen in liquid nitrogen within 15–30 minutes of surgery. Cartilage was ground under liquid nitrogen using the Type 6750 Freezer Mill (Spex Certiprep). RNA was purified using mIRVana (Ambion), reverse transcribed using sequence specific primers and microRNA expression measured using Taqman low density array (Life Technologies) or for miR-455-3p, individual assay as below.

Profiling of microRNA and mRNA expression:

For the differentiating ATDC5 cells, RNA samples were analysed on an Agilent Bioanalyser 2100 and a Nanodrop spectrophotometer (Thermo Scientific). For each time point, RNA from six culture replicates was pooled for array. Samples were labelled using the

miRCURY Hy3/Hy5 power labelling kit and hybridized on the miRCURY v.10.0 LNA array by Exiqon A/S (Denmark). Signal was corrected for background, and normalized using the global Lowess regression algorithm. For transcriptomic analysis, samples were hybridized on the Illumina Mouse WG6 whole genome array by Cambridge Genomic Services (UK). Signal was corrected for background and normalized by quantile normalisation using the R package lumi.

Quantitative RT-PCR:

cDNA was synthesised from RNA using Superscript II reverse transcriptase (Invitrogen) and either random hexamers or miRNA specific primers according to the manufacturer's instructions. cDNA was stored at -20°C. The relative quantitation of gene expression was performed on the ABI Prism 7700 sequence detection system (Applied Biosystems) following the manufacturer's protocol.

In situ hybridization (ISH):

Whole mount ISH of mouse embryos and isolated tissues was performed as described in (16). Embryos were treated with proteinase K and endogenous alkaline phosphatase activity was blocked by pre-treatment of tissues with 2 mM levamisole. Hybridisations were performed at 50 °C overnight in hybridisation mix containing 100 pmol of double labelled LNA oligonucleotides (Exiqon). The NBT/BCIP staining reaction was carried out at room temperature after which the embryos were fixed in 4% PFA and stored in PBS at 4 °C. Embryos were then blocked in 3% agar and serially sectioned (100µm) using a Lancer 1000 series Vibratome. Long bones from E18 embryos were stained in an identical manner, paraffin embedded and sectioned (10µm). Sections were then counter stained in haematoxylin and eosin.

Whole mount in situ hybridization for chick embryos was performed as described in (27). Briefly, embryos were fixed in 4% PFA, dehydrated into methanol, rehydrated and treated with proteinase K. Hybridization with double labelled LNA probes (Exiqon) was at 50 °C overnight. After NBT/BCIP colour development embryos were embedded in OCT and sectioned on a cryostat.

Transient transfection

The 3' UTR of potential target mRNAs was amplified by PCR and subcloned into the Hind III / Spe I sites of pMIR-Report (Ambion). Mutation of the microRNA seed sequence was achieved using QuikChange (Agilent). The positive control construct contains a concatamer of three copies of the reverse complement of the mature microRNA sequence downstream of the luciferase gene in pMIR-Report. SW1353 or 3T3 cells were plated at 2×10^4 cells per well in a 24 well plate and grown overnight to approx. 80% confluency. Cells were transiently transfected with 200ng luciferase reporter plasmid, 50ng β-galactosidase expression plasmid (Promega) and 30 - 50nM microRNA mimic or control (AllStars, Qiagen) using Lipofectamine 2000 according to manufacturer's instructions (Invitrogen), and incubated for 48h. For growth factor induction, $pCAGAC_{12}$-luc was as previously described (18). Cells were serum starved for 24 hours post-transfection and then treated with TGFβ1 (4ng/ml) / TGFβ3 (4ng/ml) or Activin A 20ng/ml (R & D systems) for 3 hours. For luciferase assay, cells were washed with ice-cold PBS and lysed in 1×Reporter Lysis Buffer and assayed according to the manufacturer's instructions (Promega). β-galatosidase assays were performed using Beta-Glo assay kit according to manufacturer's instructions (Promega). The data are presented as relative light units normalised to β – galactosidase.

Cluster analysis

Hierarchical cluster analysis and visualisation was performed using Cluster and TreeView (28).

Results

ATDC5 cell differentiation

ATDC5 cells were differentiated over a 42 day time course. Chondrogenesis was followed by staining for glycosaminoglycan accumulation using alcian blue as shown in Figure 1A. We profiled the expression of all mRNAs by Illumina microarray in pooled RNA samples from six replicate wells at each time point. Two markers of chondrocyte differentiation (*col2a1* and *col10a1*) are shown (Figure 1B) with an early increase in *col2a1* expression and a later and more modest increase in *col10a1* expression.

We have recently demonstrated that miR-140-5p, a microRNA selectively expressed in cartilage, targets at least at least HDAC4, CXCL12 and Smad3, all of which are implicated in chondrocyte differentiation (16-18). We thus profiled the expression of all miRNAs in the same pooled RNA samples as above. Figure 2 shows graphs of the average expression for microRNAs in seven individual groups from a two-way unsupervised hierarchical clustering of the data, which demonstrates regulation of miRNAs across chondrocyte differentiation. Groups 1a and b are sub-clades but clearly have different expression patterns; groups 3a and b similarly. The heat maps are shown in Supplemental Figure 1.

Thirty-nine miRNAs group with a pattern of expression similar to that of miR-140, though several of these are found as genomic clusters and are therefore potentially co-regulated, collapsing this group to 23 miRNA loci (Figure 2, Group 3b). The expression of these microRNAs increases across the time course of differentiation. The expression of miR-140-5p

and miR-455-3p (miR-455*, but shown to be the guide strand on www.miRBase.org) was validated using qRT-PCR in triplicate samples from each time point (Figure 3A and B).

For the miR-140-containing group, 7 microRNAs are located within the introns of protein-coding genes (Table 1) with the remainder in intergenic regions. Of these genes, the expression of *Col27a1* (containing miR-455), *Wwp2* (containing miR-140) and *Gpc1* (containing miR-149) are clearly regulated across the ATDC5 cell model in the parallel mRNA microarray experiment whilst *Aatk* is expressed by not regulated (data not shown). *Sfmtb2* and *Eda* are not detected by the probes on the array and ENSMUST00000114231 is not on the array. The expression of *Wwp2* and *Col27a1* were validated by qRT-PCR (Figure 3C and D) showing the general co-regulation of the gene and microRNA. The earlier decrease in mRNA expression compared to miRNA may reflect RNA stability.

Localisation of miR-455-3p in chick and mouse development

We have previously shown that the expression of miR-140 is restricted to the developing mouse skeleton (16). Since miR-455 resides in an intron of the collagen XXVII gene, a collagen expressed in cartilage, we examined the expression of miR-455-3p in development in both chick and mouse embryos (Figure 4A and B). In the developing chick embryo miR-455 was expressed in the skeleton of the developing limbs. Expression was first detected at day 6.5 (Hamburger-Hamilton (HH) stage 30, approximately equivalent to E12.5 in the mouse) with strong expression in the developing long bones and in the developing digits (Figure 4A, iii-vi). Later in development (day 7.5-8, approximately equivalent to E14.5-16.5 in the mouse), expression became more restricted to the developing joints (Figure 4A, vii-xii) with staining in cartilage and perichondrium (Figure 4A, ix, xii). In the developing mouse embryo, expression in the developing long bones was less apparent, potentially due to issues of probe penetration. In the

E18 embryo, whole mount staining and sectioning of the isolated joint shows expression in the growth plate and perichondrium (Figure 4B, ix, x). Expression is also seen in the interdigital region (Figure 4B, iii-vi) and in the sutures of the developing skull (Figure 4B, vii, viii).

Expression of microRNAs in human articular cartilage

The microRNA fraction was purified from human articular cartilage obtained from total hip replacement for either osteoarthritis or fracture to the neck of femur. Similar samples have previously been used and validated as controls in profiling studies (29, 30). Measurement of miR-140-5p was taken from a Taqman® low density array used to profile the expression of 365 microRNAs in these samples (D.A Young, unpublished), with miR-455-3p measured using a separate Taqman® assay in the same samples. The data were normalised using a recently described method based on mean expression value of all expressed microRNAs in a given sample (31), and results for miR-140-5p and miR-455-3p shown in Figure 4C. Both of these microRNAs were expressed at higher levels in the OA samples compared to the fracture controls.

We have also localised expression of mIR-455-3p in adult articular cartilage from osteoarthritic knees using *in situ* hybridisation. Figure 4D shows expression predominantly in the intermediate zone.

Regulation and function of microRNA-455-3p

We previously showed that miR-140 regulates *Smad3* expression and could regulate TGFβ-induced signalling (18). The expression of miR-455-3p is induced by TGFβ1, TGFβ3 and activin A in human SW1353 chondrosarcoma cells (Figure 5A-C) and murine C3H10T1/2 cells (data not shown).

MiR-455 mimic diminishes Smad-dependent signalling (driven by either TGFβ1 or activin A) to a $(CAGA)_{12}$-luciferase construct in a similar manner to miR-140 (Figure 5D and E). The impact of miR-455 is generally greater on activin-induced luciferase compared to TGFβ-induced signalling.

Using MiRNA Body Map (http://www.mirnabodymap.org) *SMAD2*, *activin* receptor 2B (*ACVR2B*), and chordin-like 1 (*CHRDL1*) are predicted as targets for miR-455-3p with potential impact upon TGFβ signalling. We have validated these as direct targets of miR-455-3p by cloning the 3'UTR downstream of the luciferase gene in the pMiR-Report vector and showing that a miR-455 mimic reduces luciferase activity whilst mutation of the seed sequence for miR-455 in the 3' UTR abolishes these effects (Figure 6A-C). For *CHRDL1* and *ACVR2B* the scrambled siRNA shows some non-specific effects on the wild-type construct compared to the mutant.

Discussion

We and others have shown that miR-140 is expressed in a cartilage selective fashion in the developing skeleton (15, 16), in chondrocyte differentiation and in human articular cartilage (22). Here, we aimed to identify additional microRNAs with functions in cartilage development and osteoarthritis.

MicroRNA microarrays across ATDC5 cell differentiation identified seven clusters of co-expressed microRNAs. Groups 1 and 2 show decreased expression of microRNAs in the induced cultures compared to control, with Group 1b showing this only across days 5 to 26. Groups 3, 4 and 5 show increased expression of microRNAs in the induced cultures compared to control, with Group 3a showing this increase from day 15 onwards, Group 3b from day 10

onwards and Group 4 from day 5 to 26. Group 5 shows an alternating pattern of expression across the time course.

A comparison of microRNAs expressed in human mesenchymal stromal cells (MSCs) under chondrogenic differentiation (7) or with mouse MSCs differentiated via culture on polyhydroxyalkanoates (8) shows some overlap with our data, but many differences. This is difficult to interpret, since no time course of differentiation is presented and each model likely measures different facets of chondrocyte differentiation.

A number of microRNAs regulated across the ATDC5 model have also been described with a role in osteoblast differentiation (7, 9, 14). Whilst no studies have compared microRNA expression during osteogenic, adipogenic and chondrogenic differentiation from the same starting population of precursor cells, one study has examined the former two together (32). In this study, approximately half of the microRNAs regulated during differentiation were common to both adipogenesis and osteogenesis and therefore may have a role in the process of differentiation per se rather than differentiation to a specific lineage.

Group 1a contains miR-146a, miR-155 and miR-125b all of which are regulated by inflammatory mediators (e.g IL-1, TNFα, LPS) and have a role in regulating inflammation / innate immunity (33-35). miR-125b has also been shown to inhibit osteoblastic differentiation from mouse ST2 cells (36) and to be negatively regulated by BMP-2 treatment in C2C12 cells (9). Group 1b contains miR-29 which has been shown to promote osteogenesis, regulating several collagen genes and a number of inhibitors of osteoblast differentiation and chondrogenesis (14). Group 2 contains miR-199a, a BMP-2 responsive microRNA which regulates chondrogenesis via suppression of Smad1 (9).

In Group 3a and b miR-466, miR467, miR-669 and miR-297 are all part of a genomic cluster and therefore potentially co-regulated. Mmu-miR-99a and let-7c-1 also form a cluster.

Group 5 contains miR-675, processed from a longer non-coding RNA called H19 and shown to be regulated by Sox9 and during chondrocyte de-differentiation and re-differentiation in vitro (11).

The expression of miRNAs in Group 3b increased with differentiation and hypertrophy. This included miR-140 (miR-140-5p), but also miR-140* (miR-140-3p), the passenger strand. Whilst the passenger strand is generally thought of as non-functional, a recent publication demonstrated that miR-140* was induced by nicotine and targeted the 3'UTR of the dynamin 1 gene in the nervous system (37). MiR-455-3p and 5p were also expressed in this group. MiR-140 and miR-455 are both located within introns of protein-coding genes (*Wwp2* and *Col27a1* respectively) and both these latter genes were regulated across the ATDC5 model with similar kinetics to the microRNAs. Of particular interest is that collagen XXVII, the product of the *Col27a1* gene is a cartilage collagen (38).

Whole mount *in situ* hybridisation showed expression of miR-455 in developing long bones in a chick model. With time, expression becomes more restricted to the developing joints, with expression in cartilage and perichondrium. There is also evidence of expression in muscle, in line with a previous report showing that miR-455 was expressed in myotubes treated with the proinflammatory cytokine TWEAK (39). *In situ* hybridisation in the developing mouse embryo confirmed expression in the long bones and joints. Interestingly, we also observed expression in the sutures of the developing skull and in the interdigital region of the developing mouse paw. Both of these processes involve apoptosis and it is possible therefore that miR-455 regulates apoptosis in these tissues in development. *Col27a1* is expressed in the cartilage anlagen of the developing skeleton, most prominently in the hypertrophic region of the growth plate. In adult mice staining is also seen in articular cartilage (38, 40, 41). Expression of miR-455 has also been reported in the differentiation of brown adipocytes (42) and miR-455 may also have role in

innate immunity since both heat-killed *Candida albicans* and lipopolysaccharide induced its expression in macrophages (43) in an NFκB-dependent manner. We could not detect the induction of miR-455 following stimulation of human articular chondrocytes with a variety of Toll-like receptor ligands which can activate gene expression in these cells (data not shown).

We have previously shown that miR-140 directly targets Smad3 expression and regulates Smad-dependent TGFβ signalling (18). Here we demonstrated that miR-455 also abrogates Smad-dependent signalling and validated three direct targets of miR-455-3p, the predicted guide strand: Smad2, activin receptor 2B and chordin-like 1. The expression of miR-455 is induced by TGFβ1, TGFβ3 and activin. The Smad signalling pathway has been shown to regulate the maturation of some microRNAs by the Drosha complex (44), but pri-miR-455 is also induced by TGFβ1 (data not shown) and both *COL27A1* and *WWP2* are also induced by TGFβ1 (data not shown), suggesting transcriptional induction is the most likely mechanism here.

In the growth plate, TGFβ signalling through Smad 2/3 is known to block chondrocyte terminal differentiation, conversely, BMP signalling through Smad 1/5/8 promotes this process. The common mediator Smad4 is required for both of these pathways and where this is limiting, signalling can be regulated through competition for Smad4 (45). TGFβ itself can signal through Smad 1/5/8 in chondrocytes by engaging the ALK1 receptor rather than the ALK5 receptor. This has led to an elegant hypothesis where a change in the ratio of ALK5 and ALK1 with age, shifts signalling towards the ALK1-mediated Smad1/5/8 pathway with differentiation to a catabolic phenotype contributing to cartilage destruction (45).

We therefore suggest that terminal differentiation is regulated by microRNAs, with miR-140 and miR-455 decreasing Smad2/3 and consequently both decreasing TGFβ signalling and promoting Smad1/5/8 dependent BMP signalling via increasing availability of Smad 4. Recently, miR-199a* has been shown to regulate early chondrogenesis by directly targeting

Smad1 (9). Our data shows miR-199a* decreased in expression during late chondrogenesis in the ATDC5 model which fits with our hypothesis. Similarly, miR-21 has been shown to target BMPRII (46), and this microRNA is also repressed particularly during late chondrogenesis in our data. We have shown that activin receptor IIB (ACVR2B) is a direct target of miR-455-3p, and recently, miR-210 was shown to target ACVR1B to promote osteoblastic differentiation (14). Activin signalling uses the Smad2/3 pathway, so the downregulation of these targets would again decrease Smad2/3 signalling and potentially enhance Smad1/5/8 signalling. Chordin-like 1 is a BMP antagonist which, whilst it has not been implicated in osteoarthritis, has been shown to impact upon mesenchymal stem cell proliferation (47).

Both miR-140 and miR-455 are increased in expression in OA cartilage compared to fracture controls. This goes against the data from miR-140 null mice, demonstrating that loss of miR-140 leads to premature osteoarthritis and data showing miR-140 decreased in human OA cartilage (19, 48). This is likely explained by differences in the human cartilage samples used in each study since in our comparison of osteoarthritic cartilage from the hip compared with fracture controls, ADAMTS5 expression (a demonstrated target of miR-140) is always decreased in osteoarthritis (29). Average expression of DNPEP (the other miR-140 target coming from the null mouse studies) is also decreased in our OA samples compared to fracture, though this doesn't reach significance (49). The kinetics of microRNA expression across disease initiation and progression will be important and we are currently addressing this in murine models of OA.

Pursuing our hypothesis above, a change in miR-140/miR-455 expression would lead to altered TGFβ/Activin signalling through the Smad2/3 pathway (Figure 7). It is known that TGFβ signalling is important for the maintenance of articular cartilage and that decreased TGFβ signalling through the Smad 2/3 pathway leads to OA-like changes in the joint (45). This

provides a potential mechanistic link between microRNAs which regulate the Smad pathway and the pathology of OA.

In conclusion, microRNA-455 is expressed across chondrogenesis, in adult articular cartilage and is differentially expressed in osteoarthritis. It has the potential to modulate TGFβ signalling in cartilage, modulating cartilage homeostasis. We are currently pursuing this through generation of the miR-455 null mouse.

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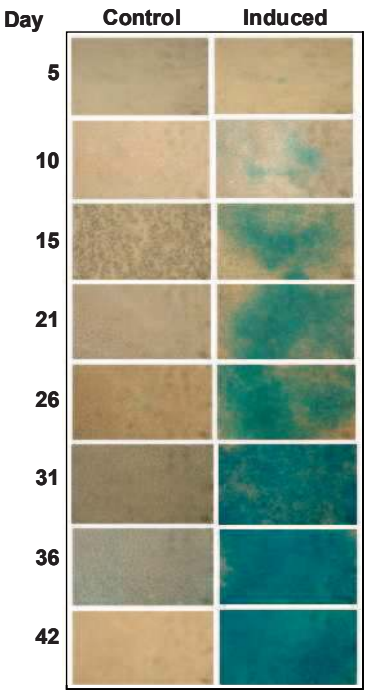
Table 1: Intronic location of microRNAs clustering with miR-140 (see www.mirbase.org)

MicroRNA	Gene	Intron number
miR-99a	ENSMUST00000114231	1
miR-140	Wwp2	7, 15 or 16*
miR-149	Gpc1	1
miR-338	Aatk	3, 4 or 7*
miR-455	Col27a1	7 or 10*
miR-466 and miR-467	Sfmbt2	9, 10 or 11
miR-676	Eda	2

* depending on transcript

A.



B.

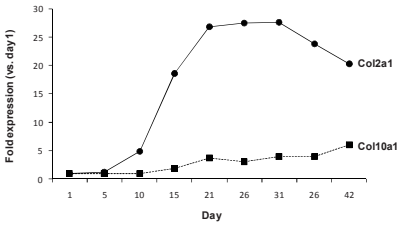


Figure 1: ATDC5 model of chondrogenesis
A. ATDC5 murine embryonic carcinoma cells were differentiated over a 42 day time course; chondrogenesis was followed by staining for glycosaminoglycan using alcian blue as shown. B. The expression of type II and type X collagen (plotted as fold compared to day 1) is regulated across chondrogenesis with an early increase in *Col2a1* and a later more modest increase in *Col10a1* mRNA.

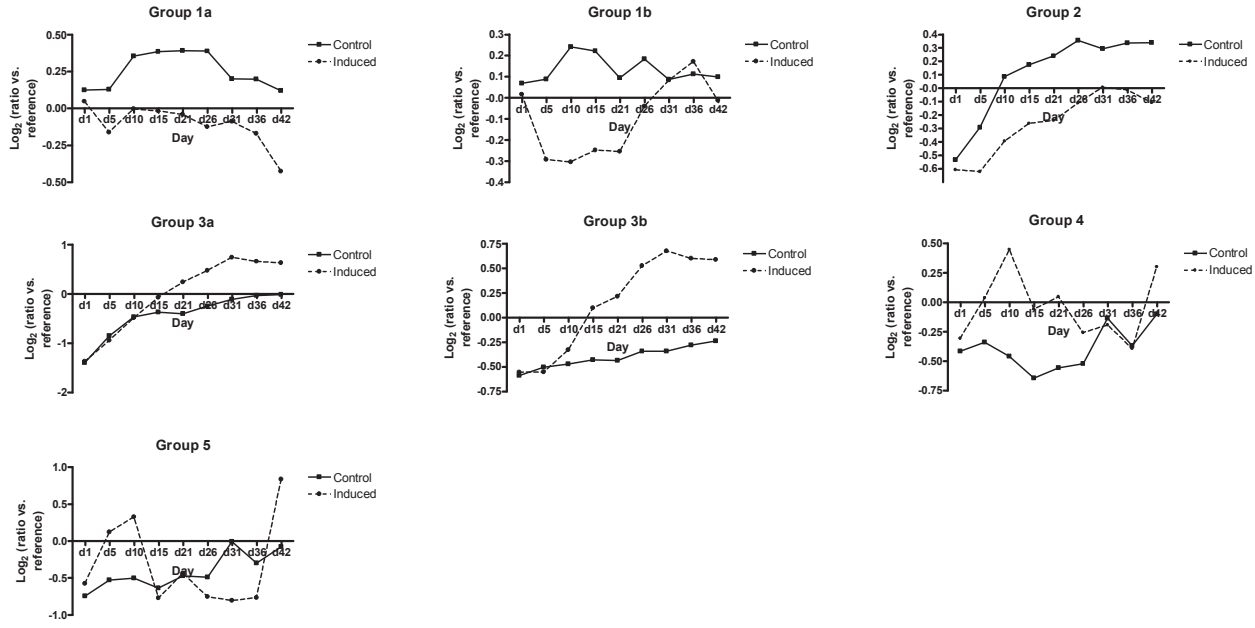


Figure 2: Expression of microRNAs in the ATDC5 model

For each time point, RNA from six culture replicates was pooled, labelled using the miRCURY Hy3/Hy5 power labelling kit and hybridized on the miRCURY v.10.0 LNA array (Exiqon, Denmark). The experiment utilized a dual label approach comparing each test sample to a common reference sample. Signal was corrected for background, and normalized using the global Lowess regression algorithm. Hierarchical cluster analysis and visualisation was performed using Cluster and TreeView (28) to generate heat maps. The average expression of microRNAs in each cluster is plotted.

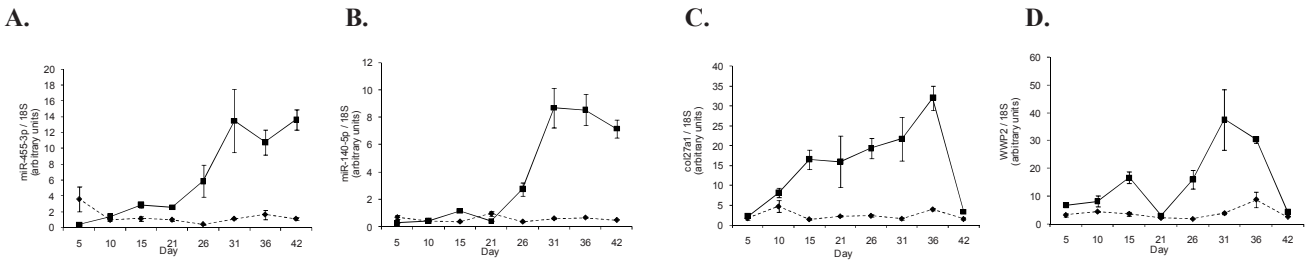


Figure 3: Expression of miR-455-3p, miR-140-5p, Col27a1 and Wwp2 in the ATDC5 model
Microarray data was validated using qRT-PCR in the individual replicate samples (n=6). Both microRNAs (A. miR-455-3p; B. miR-140-5p) were increased in expression across chondrogenesis. Expression of the genes in which the microRNAs are encoded (C. Col27a1 for miR-455; D. Wwp2 for miR-140) shows earlier induction of expression and a return to non-induced levels at day 42.

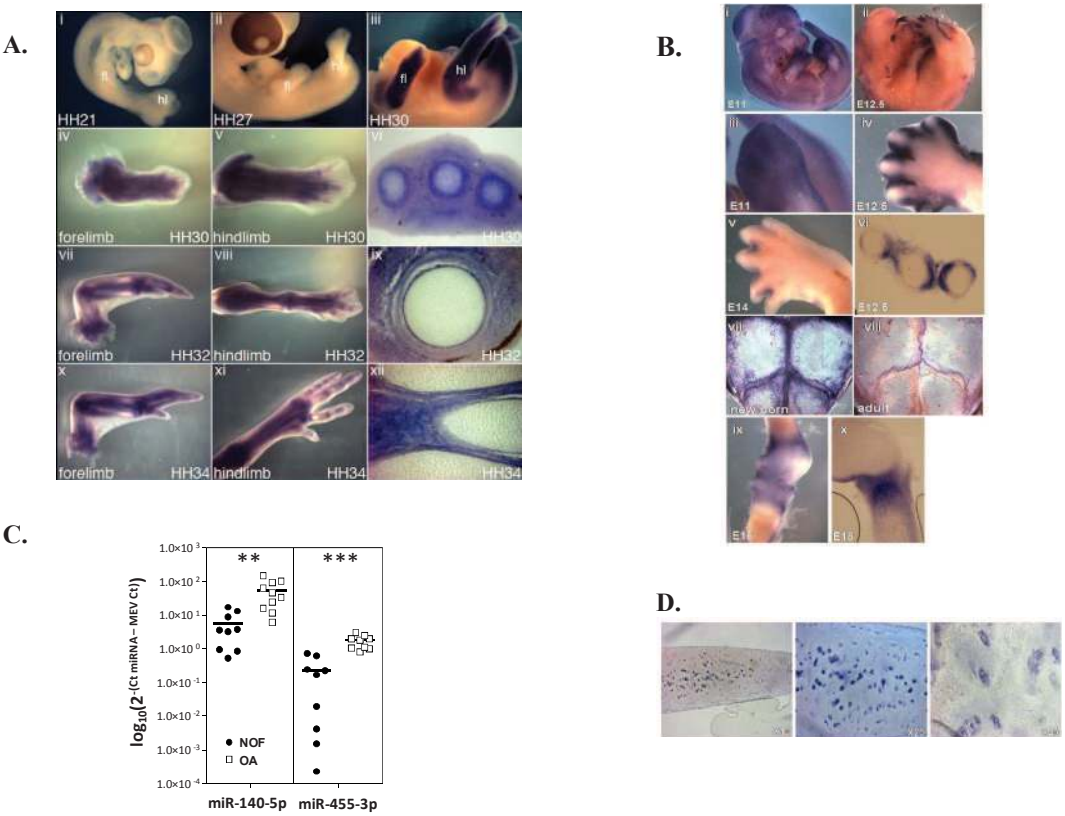


Figure 4: Expression of miR-455 during chick and mouse development and in adult human articular cartilage
In situ hybridization was performed on A: whole mount chick embryos (i-iii), dissected limbs (iv, v, vii, viii, x, xi) and sections through these at different stages of development (vi, ix, xii). Expression was not detected at Hamburger-Hamilton stage 21 or 27 (i, ii) but was seen from HH30, approximately day 6.5, onwards (iii - xii). Sections through limb buds show staining in the perichondrium (vi, ix, xii). In B: the mouse embryo (i, ii), expression was seen in the interdigital regions (iii - vi) and the developing joint in the growth plate and perichondrium (ix). Sectioning shows staining in and around the cartilage (x). Staining of the newborn calvaria shows strong staining in the developing sutures (vii, viii). C: Human articular cartilage samples obtained from femoral heads of patients with OA (n=10) were compared with those from patients undergoing hip replacement following fracture to the neck of femur (NOF; n = 10). RNA was purified, reverse transcribed and assayed by qRT-PCR for miR-455-3p and mIR-140-5p. Statistical differences were calculated using Mann-Whitney U test where ** = $p<0.01$, *** = $p<0.001$. D. *In situ* hybridisation of human articular cartilage from an osteoarthritic knee shows staining predominantly in the intermediate zone.

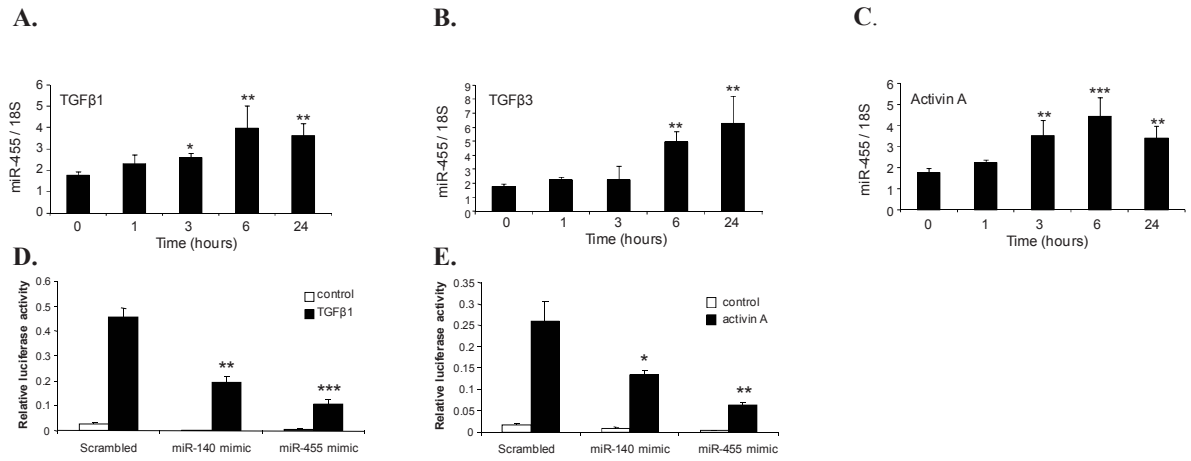


Figure 5: MiR-455 regulates and is regulated by Smad2/3 signalling.
Human SW1353 chondrosarcoma cells were serum starved for 24 hours before the addition of A. TGFβ1 (5ng/ml), B. TGFβ3 (5ng/ml) or C. activin A (20ng/ml) and miR-455-3p was measured by qRT-PCR (n=3). Cells were transfected with the Smad2/3-responsive reporter $(CAGA)_{12}$-luciferase in the presence of miR-140 mimic, miR-455 mimic or scrambled control at 50nM. Cells were serum starved for 24 hours before the addition of D. TGFβ1 (5ng/ml), or E. activin A (20ng/ml) for 6 hours (n=3). Relative light units were measured on a luminometer and normalised to β-galactosidase activity from a co-transfected expression construct. Statistical differences were calculated using t-test where * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

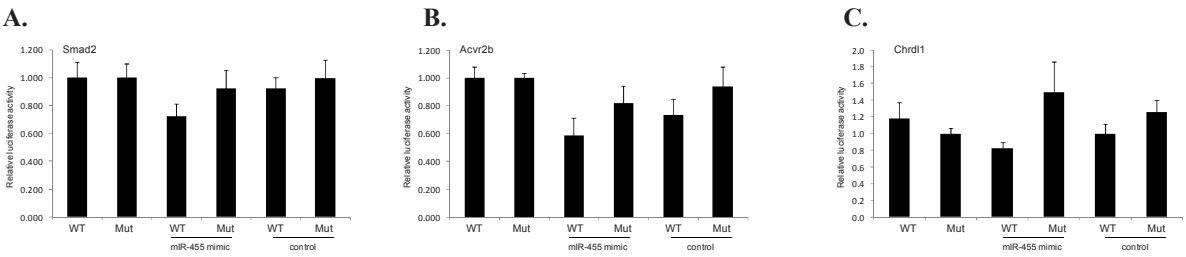


Figure 5: MiR-455-3p targets components of the TGFβ signalling pathway.
Cells (3T3) were transfected with the 3' UTR of A. SMAD2 (n=18), B. ACVR2B (n=12) or C. CHRDL1 (n=18) cloned into pMiR-Report in the absence or presence of control siRNA or an miR-455 mimic, incubated for 24 hours. Relative light units were measured on a luminometer and normalised to β-galactosidase activity from a co-transfected expression construct. Statistical differences were calculated using t-test where * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

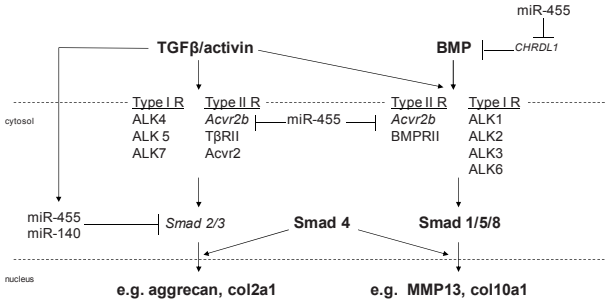
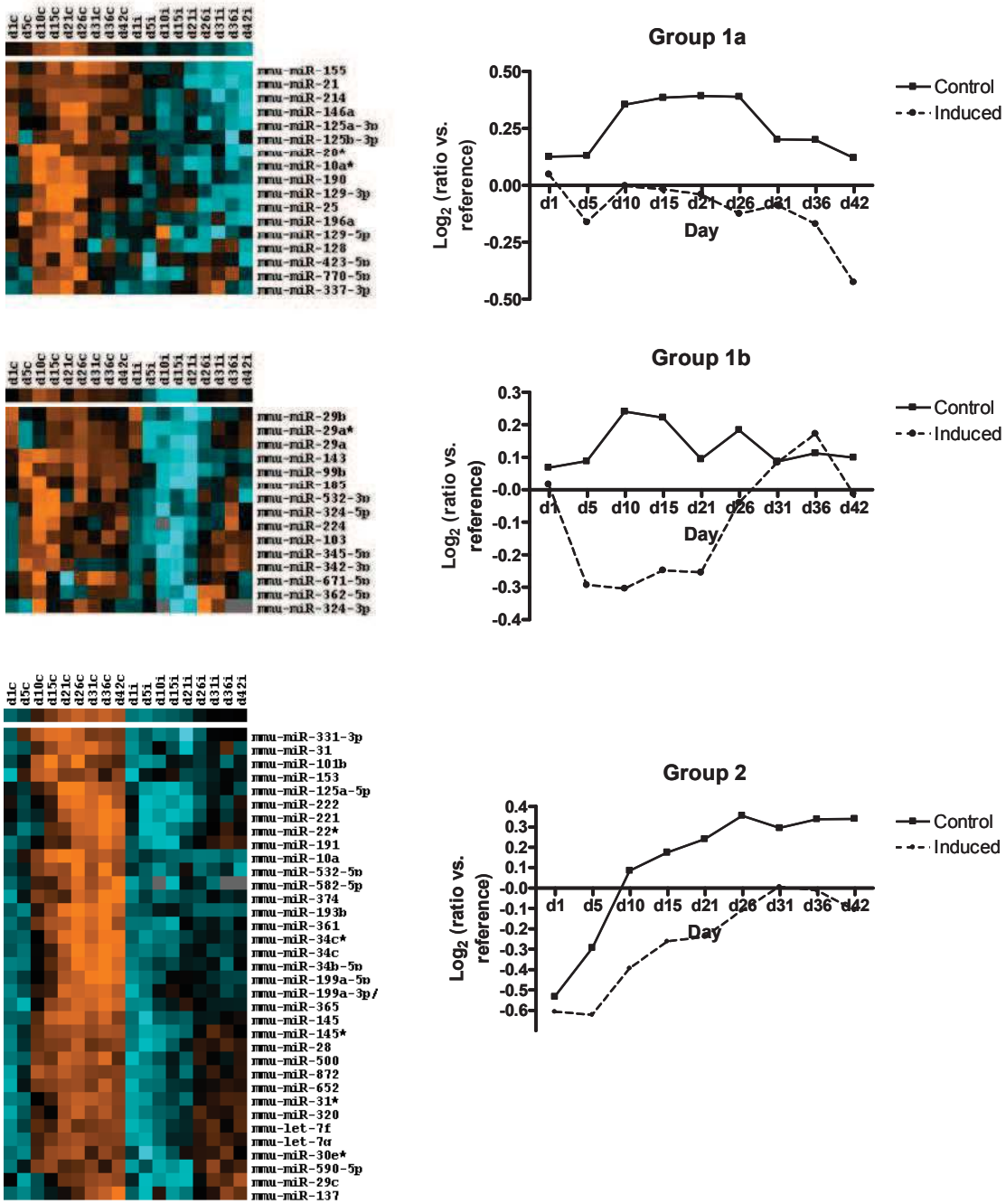
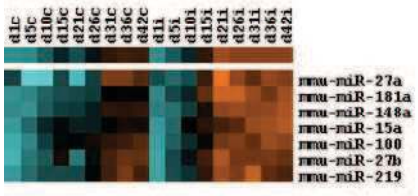


Figure 7: Overview of miR-455 and miR-140 impact on TGFβ signalling in cartilage.

Supplemental Figure 1: Expression of microRNAs in the ATDC5 model

For each time point, RNA from six culture replicates was pooled, labelled using the miRCURY Hy3/Hy5 power labelling kit and hybridized on the miRCURY v.10.0 LNA array (Exiqon, Denmark). The experiment utilized a dual label approach comparing each test sample to a common reference sample. Signal was corrected for background, and normalized using the global Lowess regression algorithm. Hierarchical cluster analysis and visualisation was performed using Cluster and TreeView (28) to generate heat maps. The average expression of microRNAs in each cluster is plotted.

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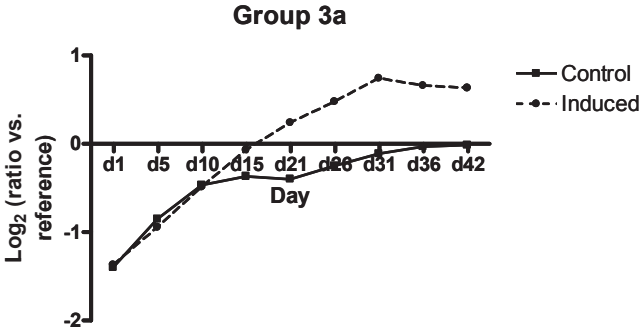
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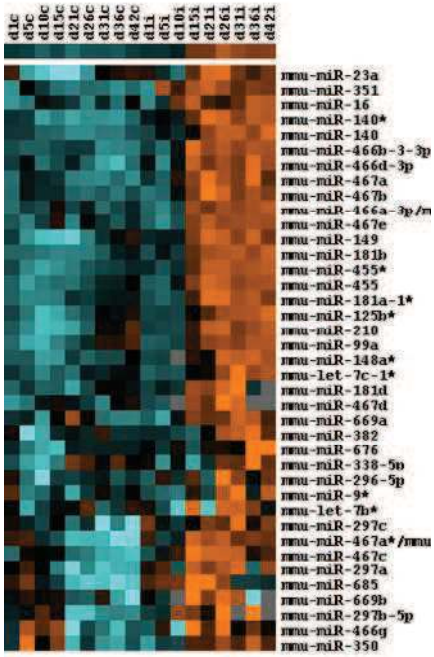
d10
d15
d21
d26
d31
d36
d42
Day
Control
Induced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d5i
d10i
d15i
d21i
d26i
d31i
d36i
d42i
mmu-miR-23a
mmu-miR-351
mmu-miR-16
mmu-miR-140*
mmu-miR-140
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mmu-miR-467b
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mmu-miR-149
mmu-miR-181b
mmu-miR-455*
mmu-miR-455
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mmu-miR-99a
mmu-miR-148a*
mmu-let-7c-1*
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mmu-let-7b*
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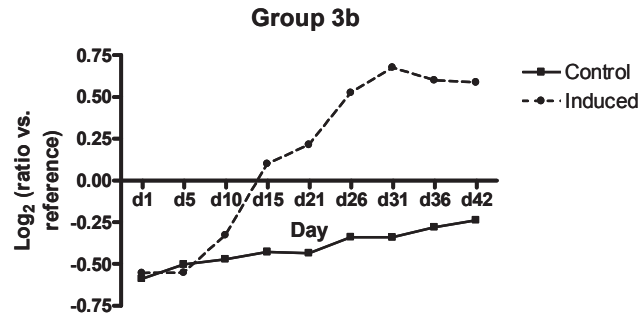
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d10
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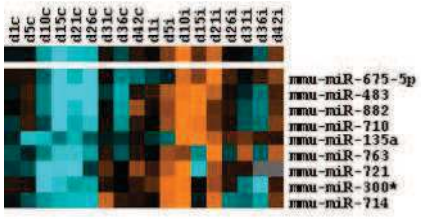
d21
d26
d31
d36
d42
Day
Control
Induced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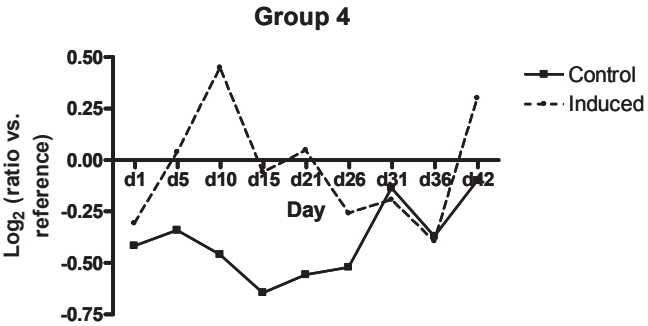
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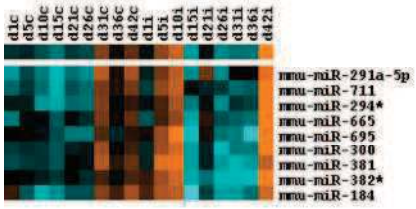
d31
d36
d42
Day
Control
Induced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mmu-miR-711
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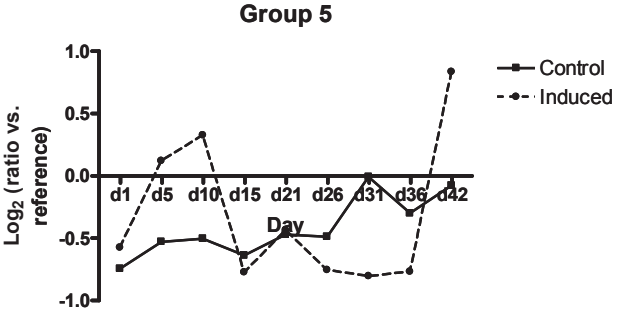
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d10
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d36
d42
Day
Control
Induced