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Mustn1 is expressed during chondrogenesis and is necessary for chondrocyte proliferation and differentiation *in vitro*

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Abstract

Mustn1 encodes a small nuclear protein expressed specifically in the musculoskeletal system that was originally identified as a strongly up-regulated gene during bone regeneration, especially in fracture callus proliferating chondrocytes. Further experiments were undertaken to investigate its expression and role during chondrogenesis. Initially, whole mount mouse *in situ* hybridization was carried out and revealed *Mustn1* expression in areas of active chondrogenesis that included limb buds, branchial arches and tail bud. To elucidate its function, experiments were carried out to perturb *Mustn1* by overexpression and silencing in the pre-chondrocytic RCJ3.1C5.18 (RCJ) cell line. In these cells, *Mustn1* is normally differentially regulated, with a spike in expression 2 days after induction of differentiation. Further, *Mustn1* was successfully overexpressed in multiple RCJ cell lines by ~2–6 fold, and reduced to ~32–52% in silenced cell lines as compared to parental *Mustn1* levels. Overexpressing, silenced, control, and parental RCJ cell lines were assayed for proliferation and differentiation. No statistically significant changes were observed in either proliferation or proteoglycan production when *Mustn1* overexpressing lines were compared to parental and control. By contrast, both proliferation rate and differentiation were significantly reduced in *Mustn1* silenced cell lines. Specifically, RNAi silenced cell lines showed reductions in populations of ~55–75%, and also ~34–40% less matrix (proteoglycan) production as compared to parental and random control lines. Further, this reduction in matrix production was accompanied by significant downregulation of chondrogenic marker genes, such as Sox9, Collagen type II (Col II), and Collagen type X (Col X). Lastly, reintroduction of *Mustn1* into a silenced cell line rescued this phenotype, returning proliferation rate, matrix production, and chondrogenic marker gene expression back to parental levels. Taken together these data suggest that *Mustn1* is a necessary regulator of chondrocyte function.

Keywords

Chondrogenesis; RNAi; Mustn1; RCJ; Sox9

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Introduction

Previous work in our laboratory has identified *Mustn1* as a small (9.6 kDa) protein that localizes predominantly to the musculoskeletal system [1]. Investigation of *Mustn1*'s sequence revealed that it is highly homologous in vertebrates and contains a nuclear localization sequence but no other significant motifs, only potential phosphorylation and myristoylation sites. GPF-*Mustn1* fusion experiments showed that *Mustn1* localizes to the nucleus but not to the nuclear membrane or nucleoli. Our laboratory has also showed that *Mustn1* is differentially regulated during fracture repair [1,2], specifically, in proliferating chondrocytes, osteoblasts and periosteal osteoprogenitor cells within the fracture callus [1]. In addition, we also found Mustn1 expression in the superficial layer of articular cartilage of a knee joint (unpublished observations), as well as during developing intervertebral discs and limbs [1]. Finally, while proliferating chondrocytes showed *Mustn1* expression in these tissues, terminally differentiated hypertrophic chondrocytes did not. Further, during fracture repair, *Mustn1*'s peak expression correlates to a time point when mesenchymal precursor cells are migrating to the fracture site, differentiating into chondrocytes, and proliferating within the callus prior to becoming terminally hypertrophic. Based on these data, it was speculated that *Mustn1* possibly functions in a transcriptional complex specific to chondrocyte differentiation [1].

In an effort to further characterize this gene, its promoter was identified and analyzed. Within the 1512bp region flanking *Mustn1*'s 5′ end, several transcription factor binding sites were identified that belong to the Activator Protein (AP) family [3]. It was also showed that AP-1 family members (JunD, c-Fos, and Fra-2), known as key transcriptional regulators of musculoskeletal specific genes [3], bind to *Mustn1*'s promoter during both proliferation and differentiation of myogenic C2C12 cells. Knockout studies of these genes showed varying degrees of phenotypic deficiency within the musculoskeletal system including osteoporosis, osteosarcoma, and chondrosarcoma as well as sterility, embryonic lethality, and ocular malformations [4]. However, because AP family members function within multiple signaling pathways, additional analyses are needed to determine within which one(s) *Mustn1* functions.

Based on the aforementioned data, we reasoned that functional perturbation of *Mustn1* during chondrocyte proliferation and differentiation would help to clarify its role during chondrogenesis. To this end, this study sought to modulate *Mustn1* expression in the pre-chondrogenic RCJ cell line. The RCJ line represents a heterogeneous cell population capable of differentiation from proliferating chondrocytes to ultimately terminally differentiated collagen X producing hypertrophic chondrocytes [5]. Also, since *Mustn1* is highly expressed in the early stages of fracture repair and this process is known to recapitulate embryonic development [2,6,7,8], we decided to further investigate *Mustn1* expression during embryogenesis. This combination of *in vivo* and *in vitro* approaches revealed that *Mustn1* is abundantly expressed in developing cartilaginous structures (limb buds, branchial arches and tail bud) and is necessary for both chondrocyte proliferation and differentiation.

Materials and Methods

Materials

The Anti-DIG Antibody, dexamethasone and β-glycerophosphate, bovine calf serum (BCS), Dulbecco's modified Eagle medium (DMEM) and antibiotics (penicillin/streptomycin), and G418 were purchased from Roche (Indianapolis, IN), Sigma-Aldrich (St. Louis, MO), Gibco BRL (Gaithersburg, MD), and Invitrogen (Carlsbad, CA), respectively. Oligonucleotides primers were designed against the coding regions of β-2 Microglobulin, *Mustn1*, Col II, Col X, and Sox9 (Table 1).

Whole mount embryo in situ hybridization

All methods and animal procedures were reviewed and approved by the university's Institutional Animal Care and Use Committee and met or exceeded all federal guidelines for the humane use of animals in research. Riboprobes were created by using T7 and Sp6 primers to amplify sense and antisense RNA probe sequences. These products were then purified and labeled using the DIG RNA labeling Kit (Roche). The original PCR product was degraded using RNase free DNase 1 (Qiagen). This reaction was stopped using 0.2M EDTA (pH 8.0). The final riboprobes were then purified using sephadex G-50 (Qiagen) quick spin columns. The *in situ* analysis was completed following the protocol as described by Hsieh et. al. [9]. Briefly, embryos were dissected out on 9.5, 10.5, and 11.5dpc and fixed with 4% paraformaldehyde for 1hr at room temperature (RT). Larger embryos (10.5 and 11.5dpc) were then permeabilized with proteinase K and all embryos were then bleached with H_2O_2 and stored in 100% methanol at −20°C. Embryos were then rehydrated through a methanol gradient and hybridized overnight at 65°C in a probe concentration of 250ng/ml., followed by a wash with MABT, blocked in MABT+20% Goat serum + 2% Boehringer Blocking Reagent (BM 1096 176) and incubated overnight in 1:2000 dilution anti-DIG 2° antibody. Finally, the embryos were again washed with MABT and expression was visualized with a solution of NTMT/BCIP. Photomicrographs were taken under bright field using a Zeiss Discovery. V8 SteREO microscope with an AxioCam MRc digital camera.

Cell culture

RCJ cells were maintained in DMEM supplemented with 10% BCS, 1% penicillin/streptomycin, and 10^{-7}M dexamethasone. Cells were fed every 3–4 days and passaged prior to confluence.

RNA interference

Three 19-mer RNAi target sequences were designed (5′-AAGGAAGAAGACCTGAAGG -3′, 5′ – CCTGTGAAGGAAGAAGACC – 3′, and 5′ – TATTCAGCCGCAACCGCAC – 3′) which correspond to three different regions of the *Mustn1* coding sequence (first and second sequence overlap). Oligonucleotides of hairpins containing these sequences (M2, M3, and M4 respectively) were generated (Invitrogen) and cloned into the pSuppressorRetro plasmid (Imgenex, San Diego, CA). A random control plasmid was generated by cloning a 19-mer sequence (5′ – GACTCCAGTGGTAATCTAC – 3′), which shows no similarity to any sequence within the mouse genome, into the same expression plasmid. A BLASTN (Basic Local Alignment Search Tool Nucleotide http;//www.ncbi.nlm.nih.gov/BLAST/) search was conducted to ensure that this random control sequence showed no genomic homology. Using a Retroviral GeneSuppressor System kit (Imgenex) these plasmids were co-transfected along with a packaging vector designed to create an ectotropic retrovirus protein coat into 293 cells. Virus containing media was harvested and used to infect proliferating RCJ cells. After a 24hr infection period, RCJ cells were selected using G418 (.5–.75mg/ml) for one week. Selected cells were plated at low density and colony forming units were allowed to grow up over several weeks. Homogeneous colonies were isolated and expanded. Five clones for each RNAi sequence were assayed for *Mustn1* expression and three clones (M2-2, M3-5, and M4-4) showing the most effective reduction in *Mustn1* levels were used in subsequent proliferation and differentiation experiments.

Overexpression

The *Mustn1* coding sequence was cloned into the pCDNA vector downstream of the CMV promoter (Invitrogen). RCJ cells were transfected with this plasmid using Fugene 6 Transfection Reagent (Roche). As a control, an empty pCDNA plasmid was also transfected. After a 24hr transfection period, RCJ cells were selected using G418 (0.5–0.75mg/ml) for one

week. Selected cells were plated at low density and colony forming units were allowed to grow up over several weeks. Homogeneous colonies were isolated and expanded. Eight clones were assayed for *Mustn1* expression and the three clones (OE3, OE4, and OE6) showing the highest *Mustn1* levels were used in subsequent proliferation and differentiation experiments.

Site-directed mutagenesis

Oligonucleotides were designed with alterations to the 3′ nucleotide in each codon within the M2 RNAi sequence (M2 RNAi sense sequence 5′ AAGGAAGAAGACCTGAAGG 3′, Rescue sense sequence 5′ AAAGAGGAGGATCTAAAAG 3′). These oligonucleotides were then used to amplify the original *Mustn1* pCDNA plasmid using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene). The original plasmid was then degraded using Dpn1 restriction digestion and the rescue plasmid was transformed into XL1-Blue Supercompetent Cells. Following Ampicillin selection, the rescue plasmid was isolated, verified and then used to transiently transfect the RCJ M2-2 cell line prior to proliferation and differentiation analyses.

Cell Proliferation

RCJ cells were seeded in 96-well plates (Falcon) at a density of ~2,000 cells/well. The regular RCJ media was removed, cells were washed with PBS, and 2.5%BCS RCJ media was added at the 0hr time point. Triplicate wells for each cell line were assayed during a 72hr time course (at 2hr, 24hr, 48hr, and 72hr) via MTS reagent (Promega). 5μl of MTS reagent was added to each well containing 100μl media. The plates were then incubated at 37°C for 1hr in the dark. The MTS/media solution was then diluted in 400ul dH_2O and analyzed by spectrophotometer (BioRad) at 490nm. Values from control wells (MTS/media solution that did not contain cells) were subtracted from each experimental value. Values were further normalized to the 2hr time point to reflect population doublings. The triplicate values for each cell line at each time point were then averaged and standard deviation (sd) was determined.

Differentiation/Matrix production analysis

RCJ cells from each cell line were seeded in 24-well plates (Falcon) at a density of ~20,000cells/well (for the experiments with the overexpressing cell lines). Since *Mustn1* silencing reduces the proliferative rate of RCJ cells, we conducted preliminary experiments with our silenced cell lines to ensure that all lines reach ~90% confluency (based on manual counts) concurrently. As such, we plated different concentrations, 20,000 – 30,000cells/well at the beginning of our experiments. For all cell lines, at near confluence (~90%) the regular RCJ media was removed and replaced with differentiation media (DMEM with 10% BCS, 1% penicillin/streptomycin, 10^{-7}M dexamethasone, 50μg/ml ascorbic acid, and 10mM β-glycerophosphate) on Day 0 and was replaced every three days. Samples were taken throughout a 14 day time course (on Day 0, 2, 5, 7, 10, and 14). At each time point triplicate wells for each cell line were fixed for 4 min with 10% Buffered Formalin Phosphate (Fisher) and stored in PBS at 4°C. On Day 14, all plates were stained with a 1% Alcian blue solution for 45min at room temperature. Wells were washed with PBS and the Alcian blue was eluted in 4%Guanidine Hydrochloride in PBS for 30min at RT. Samples were then diluted 1:2–1:4 in dH_2O and analyzed at 600nm via spectrophotometer (BioRad) and normalized to the Guanidine Hydrochloride/dH_2O solution. The triplicate values for each cell line at each time point were then averaged and the sd determined. Values were then further normalized to the Day 0 time point to reflect fold change. Also, prior to Alcian blue elution, all wells were examined with a Zeiss Axiovert 200 microscope under bright field light and random fields from each well were captured with a CCD camera (Zeiss).

RNA isolation

RCJ cells were seeded in 24-well plates (Falcon) at a density of ~20,000cells/well. At near confluence the regular RCJ media was removed and replaced with differentiation media (DMEM with 10% BCS, 1% penicillin/streptomycin, 10^{-7}M dexamethasone, 50μg/ml ascorbic acid, and 10mM β-glycerophosphate) on Day 0 and was replaced every three days. Samples were taken throughout a 14 day time course (on Day 0, 2, 5, 7, 10, and 14). At each time point triplicate wells for each cell line were lysed using TriZol reagent (Invitrogen), pooled, and stored at −80°C. Upon completion of the time course RNA was extracted using chloroform and 100% isopropanol using the recommended protocol (Invitrogen). The RNA was then resuspended and diluted in dH_2O in preparation for quantitative real-time RT-PCR (qPCR).

Quantitative real-time RT-PCR (qPCR)

RNA isolated from Day 0, 2, 5, 7, 10, and 14 of the differentiation time course was treated with DNase 1 (Qiagen) and quantified using a ND-1000 Spectrophotometer (Nano Drop). The qPCR experiments were carried out using a QuantiTect SYBR Green RT-PCR Kit (Qiagen) on a LightCycler system (Roche) as previously described by our laboratory [8,10,11,12]. Primer pairs for *Mustn1*, Col II, Col X, and Sox9 were designed (Table 1) to anneal at 58°C. Each run consisted of mRNA (4–20ng/reaction) from Day 0, 2, 5, 7, 10, and 14 (for *Mustn1*) or Day 0, 5, 10, 14 (for Col II, Col X, and Sox9) and assayed together with the housekeeping gene β2-microglobulin, as well as 5 point calibration curves (1.25–20ng/reaction). The calculated concentration value for each gene was normalized to its corresponding β2-microglobulin value. All qPCR products were checked via agarose gel electrophoresis to assess amplification. Each run was replicated three times and results are reported as expression level ± sd.

Statistical analysis

All quantitative data are reported as the average results of three or more independent experiments with sd indicated by error bars. Significance was determined in all analyses by one way ANOVA followed by a Holm-Sidac Post-hoc when comparing to initial (Day 0 or 2hr) time points and Mann-Whitney tests when values from a single time point were compared.

Results

Mustn1 expression during development

In order to further elucidate *Mustn1* expression pattern during mouse development, we performed whole mount *in situ* hybridization. Mouse embryos incubated with the antisense *Mustn1* riboprobes showed staining in several developing tissues at different time points. In a 9.5dpc embryo, staining was present throughout the embryo, possibly in musculoskeletal tissues such as the paraxial mesoderm on either side of the unstained neural tube as well as craniofacial structures (Fig. 1A). At 10.5dpc, hybridization was localized to several areas of cartilage and bone formation. For example, the craniofacial region showed intense staining, especially the branchial arches (Fig. 1B, green arrows), as do the fore and hind limb buds (Fig. 1B, black arrows), and somites in the typical array pattern perpendicular to the spinal column (Fig. 1B). As development proceeds to 11.5dpc, staining was again observed along the entirety of both the fore and hind limb buds (Fig. 1C and E, white arrows). *Mustn1* expression was also observed in the craniofacial region, particularly the developing first branchial arch that is beginning to divide into the maxillary and mandibular components (Fig. 1C and D, green arrows). In addition, hybridization is also detected in the frontonasal process (Fig. 1C and D, yellow arrows), the lens of the eye (Fig. 1D, red arrow) and posterior tip of the tail (Fig. 1E,

blue arrow). Mouse embryos incubated with a *Mustn1* sense control riboprobe showed no staining at any time point (data not shown).

Mustn1 and chondrogenic marker gene expression in differentiating RCJ cells

In order to get a baseline expression pattern for *Mustn1* and three cartilage marker genes, Sox9, Col II, and Col X during RCJ differentiation, we assayed mRNA levels via qPCR. *Mustn1* expression in RCJ cells showed a peak of ~5.2 fold two days after the induction of differentiation as compared to Day 0, followed by a decline to Day 0 levels for the remainder of the time course (Fig. 2A). In contrast, Sox9 showed a steady increase throughout the time course assayed and culminated in a peak of ~4.7 fold over its Day 0 expression value on Day 14 (Fig. 2B). Similarly and as expected, both Col II and Col X showed a similar expression pattern, steadily increasing to a large peak of ~51.4 and ~21.1 fold over Day 0 values on Day 14, respectively (Fig. 2B). Lastly, to compliment the molecular data and verify histologically that these cells do indeed undergo differentiation and produce extracellular matrix, we show the Alcian blue stained cultures from the same time points (Fig. 2C).

Modulation of Mustn1 mRNA levels

Next we sought to alter *Mustn1* expression in RCJ cells via functional perturbation, overexpression and silencing. Overexpression of *Mustn1* by stable transfection resulted in multiple homogeneous clones. Three clones were selected and showed significant increased *Mustn1* expression al levels of ~6.1, 3.1, and 2.1 fold as compared to those of the parental cell line (OE3, OE4, and OE6, respectively, Fig. 3A). Transfection of the empty expression vector (control) into the parental RCJ cell line caused no significant alteration in *Mustn1* expression levels as expected (Fig. 3A). Based on these data, OE3 and OE6 (highest and lowest overexpression levels) were chosen as representative cell lines for investigating the effects of *Mustn1* overexpression on cell differentiation.

Silencing of *Mustn1* by RNAi infection also resulted in multiple homogeneous cell clones. Three clones, M2-2, M3-5, and M4-4, one resulting from each of the three different RNAi sequences used, were selected and their *Mustn1* expression was also analyzed via qPCR. *Mustn1* expression was significantly reduced to ~0.4, 0.3, and 0.6 fold of the parental cell line expression levels in M2-2, M3-5, and M4-4, respectively (Fig. 3B). The introduction of the random control sequence showed no significant change in *Mustn1* expression (Fig. 3B).

Based on these data, M2-2 was chosen as a representative silenced cell line for investigating the effects of *Mustn1* silencing on differentiation, and as such we also targeted it for the rescue experiments. To verify that the rescue worked in terms of restoring *Mustn1* levels back to those of parental cells we measured mRNA levels and results from this experiment showed that our strategy work and the rescue sequence did indeed restore *Mustn1* levels back to parental levels (Fig. 3B).

Functional perturbation of Mustn1

To identify any functional effects caused by overexpressing or silencing *Mustn1* on chondrocyte proliferation, we assayed RCJ population growth in low density cell cultures. When the proliferation rate of the overexpressing cell lines, OE3, OE4, and OE6 were compared to both the parental cell line and the empty vector control over 72 hrs, there were no statistically significant differences found at any time point (Fig. 4A). In contrast, when the proliferation rate of the silenced cell lines, M2-2, M3-5, and M4-4 were compared to both the parental and the random control cell lines, significant differences were observed at several time points (Fig. 4B). Specifically, at the 24hr time point, random control, M2-2, M3-5, and rescue cell lines with averages of ~2.2, 1.5, 1.7, and 2.3 population doublings, respectively, showed no significant differences from parental (avg. ~2.2). However, the M4-4 cell line showed

significant reduction in population level (~1.1) when compared to parental ($p<.01$). At 48hrs, the random control, M3-5, and rescue cell lines with population averages of ~4.0, 1.7, and 2.5, respectively, showed no significant differences from parental (~3.9). However, the M2-2 and M4-4 cell lines with averages of ~1.8 and 1.7, respectively, showed significant reduction in population levels when compared to parental ($p<.05$, Fig. 4B). Interestingly, at 72hrs, only the random control and rescue cell lines with averages of ~5.7, and 4.3, respectively, showed no significant differences from parental (~5.6). However, all three silenced lines, M2-2, M3-5, and M4-4 with averages of ~2.4, 2.5, 1.4, respectively, showed significant reduction in populations when compared to parental ($p<.001$ for M2-2 and M4-4 and $p<0.05$ for M3-5, Fig 4B). Furthermore, there was a significant difference in population size between the M2-2 and rescue cell lines at 24hr ($p<.05$) and 72hr ($p<.01$, Fig. 4B).

To determine if altering *Mustn1* expression affected chondrogenic differentiation, we monitored proteoglycan production (as a function of cartilage matrix) via Alcian blue staining. Using this assay, the two overexpressing cell lines, OE3 and OE6, showing the highest and lowest up-regulation of *Mustn1*, respectively, demonstrated no significant difference at any time point as compared to both the parental cell line and empty vector control (Fig. 5A). In contrast, when Alcian blue binding was quantified in the silenced cell lines, M2-2, M3-5, and M4-4, and compared to both the parental and the random control cell lines significant differences were found at all time points (Fig. 5B). Specifically, on Day 5, the random control cell line with a value of ~5.7 showed no significant difference from the parental cell line (~5.3). However, the M2-2, M3-5, M4-4 and rescue cell lines, with values of ~3.5, 3.6, 3.7, and 4.9 fold, respectively, over Day 0 levels, showed significant differences in matrix production ($p<.001$ for M2-2, M3-5, and M4-4 and $p<.03$ for the rescue cell line when compared to parental). On Day 7, the random control cell line (~7.3 fold change) showed no significant difference from the parental cell line (~6.8). However, the M2-2, M3-5, M4-4, and rescue cell lines with averages of 4.0, 4.6, 4.0, and 5.8 fold, respectively, showed significant differences in matrix production ($p<.001$ for M2-2, M3-5, and M4-4 and $p<.02$ for the rescue line when compared to parental). On Day 10, the random control and rescue cell lines with averages of ~9.3 and 8.2 fold change, showed no significant difference from the parental cell line (~9.5). However, the M2-2, M3-5, and M4-4 cell lines with averages of 5.4, 5.9, and 6.4 fold change, respectively, showed significant reduction in matrix production ($p<.001$ for all three cell lines when compared to parental). Finally, on Day 14, the random control and rescue cell lines with averages of ~9.3 and 9.7 fold change, showed no significant difference from the parental cell line (~8.7). And again, the M2-2, M3-5, and M4-4 cell lines with averages of ~5.4, 5.8, and 5.3 fold change, respectively, showed significant reduction in Alcian blue staining ($p<.001$ for M3-5 and M4-4 and $p<.03$ for M2-2 when compared to parental. A significant difference was also found when M2-2 was compared to the rescue cell line on Day 7 ($p<.05$) as well as Days 10 and 14 ($p<.01$, Fig 5B). Lastly, Figure 5C shows representative Alcian blue stained cultures at Day 14 to visualize these decreases in matrix production, particularly in the lack of nodule formation and size (degree of differentiation) in the representative silenced M2-2 cell line as compared to Random control and Rescue.

Since we observed inhibitory effects on the differentiation of the cells with reduced *Mustn1* levels and since we speculate that *Mustn1* functions as a part of a transcriptional complex, we wanted to investigate if this reduction in matrix production is accompanied by molecular changes, especially in chondrogenic gene marker expression (Col II, Col X and SOX9). Thus, *Mustn1* and marker gene mRNA levels were determined via qPCR and compared to Random control. When *Mustn1* mRNA levels were analyzed in the silenced cell line, M2-2, they were reduced to ~47% as compared to random control at Day 0 (Fig. 6A). In contrast, the rescue cells showed 89% expression when compared to random levels at the same time point (Fig. 6A). No significant differences were found between the Random and Rescue cell lines at any time point and each peaked on Day 2 with increases of ~4.5 and 5.3 fold over Day 0,

respectively (consistent with the data shown in Fig. 2A). However, in the silenced cell line, *Mustn1* mRNA expression remained suppressed. This was the case at all time points except at Day 10, where M2-2 *Mustn1* levels reached those of Random and Rescued cell lines. While the only significant difference between the Random control and M2-2 cell lines was on Day 2, it was indeed very dramatic ($p<.001$, Fig. 6A).

When Sox9 mRNA levels were measured, the Random and Rescue cell lines showed steady increases culminating in peaks on Day 14 with ~5.6 and 9.4 fold increases over Day 0, respectively. The M2-2 cell line did not show this increase in expression and instead Sox9 levels remained suppressed at Days 5 (~1.0), 10 (~1.1) and especially 14 (~0.5, Fig. 6B). The Sox9 values for M2-2 were significantly different on Day 10 ($p=0.012$) and Day 14 ($p<.001$) as compared to Random values on those days. The results with Col II were even more dramatic. While both Random and Rescue cell lines showed steady increases in expression culminating in peaks on Day 14 of ~15 and ~7 over Day 0 values respectively, the Col II levels in the M2-2 cell line were completely suppressed (Fig. 6C). The Col II values for M2-2 were significantly different on Day 10 ($p<.001$) and Day 14 ($p<.001$) as compared to Random values on those days. Finally, Col X mRNA levels also showed steady increases in the Random and Rescue cell lines culminating in peaks on Day 14 of ~5.7 and ~6.1, respectively, while in the M2-2 cell line they remained severely reduced (Figure 6D). In contrast, Col X expression for M2-2 was remained significantly suppressed on both Day 10 ($p<.05$) and Day 14 ($p=.002$) as compared to Random values on those days. There were no significant differences between random and rescue values at any time point in any analysis.

Discussion

The expression pattern of *Mustn1* during embryogenesis suggests that this gene is active in areas of early cartilage, bone and muscle formation, especially in limb buds, branchial arches, vertebrae, and somites, all tissues containing differentiating mesenchymal cells, as observed in this and our previous study [1]. Although this correlation does not necessarily reveal its function, it does suggest that *Mustn1* is involved in these processes.

Mustn1's involvement in the early phases of chondrogenesis is further supported by *in vitro* analyses on cell proliferation and differentiation presented herein. The fact that overexpressing *Mustn1* did not cause any significant alteration in either proliferation or matrix (proteoglycan) production can be explained by the notion that the modest increases in *Mustn1* expression in RCJ cells (~2 – 6 fold) that were achieved in this study were not enough to alter these processes (when compared to the parental or control cell lines). Unfortunately, we were unable to achieve higher levels of experimental overexpression and thus the possibility remains that higher levels of *Mustn1* may be required for inducing changes in cell behavior, although this is highly unlikely given our hypothesis that *Mustn1* functions as a co-activator or co-regulator of transcription and as such it is necessary but not sufficient for transcriptional activation. This suggests that increasing the amount of *Mustn1* within each cell would not cause any effect(s) as the other molecules in the transcriptional complex would also be required. Lastly, *in vivo* overexpression on *Mustn1* in a model of *Xenopus laevis* development has no effect (unpublished observations). In contrast, the silencing experiments indicate that *Mustn1* is necessary for both proliferation and differentiation. In the silenced cell lines where *Mustn1* expression was downregulated to ~33 – 58% expression compared to parental cells, both proliferation and matrix production were clearly suppressed. This decrease was also accompanied by a dramatic reduction in the expression of the three chondrogenic marker genes, Sox9, Col II and Col X, assayed.

While the proliferation and matrix production analyses do not completely elucidate the role of *Mustn1*, the suppression of Sox9, Col II and Col X mRNA expression does provide a greater

understanding of *Mustn1*'s function during chondrocyte differentiation. Further, the observation that matrix production is reduced in *Mustn1* silenced cell lines, could simply be a result of reduced proliferation rate. However, matrix production was tested on visually normalized confluent cells, ensuring consistent cell number across all cell lines tested at initiation of differentiation. Further all cell lines were fully confluent by Day 2 of the time course suggesting that the cells had ceased proliferation and begun the differentiation process concurrently. In addition, the suppression of Col X, a well known marker of hypertrophy [13,14,15] observed in the silenced cells suggests that the lack of matrix production is caused by the inability of these cells to differentiate into terminal hypertrophic chondrocytes and not reduced proliferation rate. Moreover, the parallel and complete suppression seen in Col II, as well as the dramatic reduction in Sox9 expression indicate that even the earlier stages of differentiation [14,16,17] are affected in these silenced cells as a result of *Mustn1* downregulation.

Despite these results, low level Alcian blue staining is still present in the *Mustn1* silenced cells indicating the presence of glycosaminoglycans (GAGs) within cartilage [18]. One reason for this maybe that even pre-chondrocytes produce minimal levels of proteoglycans, but more probable is the fact that we do not completely eliminate *Mustn1* expression in the silenced cells. The residual lower levels of *Mustn1* (~30–50%) as detected by qPCR in these cells supports this idea. Even though GAGs are still being produced, the main component of the cartilage extracellular matrix is collagen. Collagen makes up two-thirds of cartilage dry weight with over 75% of that comprised of collagen II [19,20]. The dramatic suppression of Col II mRNA suggests that the matrix produced by the silenced cell lines is probably very different and to some extent structurally deficient when compared to that produced by the parental or random cell lines. although this remains to be experimentally verified.

The most interesting result of these molecular analyses is the suppression of Sox9 observed in the silenced cell lines, which is subsequently rescued after restoring *Mustn1* levels into the cells (rescue experiment). These data suggest that *Mustn1* functions at an earlier phase during chondrogenic differentiation, as Sox9 is responsible for the transcriptional activation of Col II in multiple chondrogenic and osteogenic tissues [21,22,23]. Thus, it is this reduction in Sox9 expression that likely causes the subsequent suppression of Col II levels. Sox9 activation itself has been shown to be both required and sufficient for chondrogenesis alone or with Sox5 and Sox 6 [22,24,25]. The vital role of Sox9 during chondrocyte differentiation makes *Mustn1*'s affect on this gene's expression an interesting avenue to explore. Although these data implicate *Mustn1* in the activation of Sox9, they do not indicate which signaling pathway in involved with *Mustn1* function as several pathways affect Sox9 expression. Lastly, *in vivo* downregulation of *Mustn1* in a model of *Xenopus laevis* development using an antisense morpholino oligonucleotide approach resulted in gross morphological defects that included small or lack of eyes, shortened body axis, and tail/body kinks and this was accompanied by an ~40% downregulation of Sox9 mRNA in the developing tadpoles (unpublished observations).

When these data are taken in conjunction with what is previously known about *Mustn1*, i.e. its expression restricted to the musculoskeletal system, its promoter, and the fact that it is a nuclear protein [1,2], a clearer picture of *Mustn1*'s function begins to emerge. These data suggest that *Mustn1* works within the nucleus, possibly as part of a transcription initiation complex. The lack of a DNA binding motif within its sequence precludes *Mustn1* from being a transcription factor *per se* and thus supports the notion that *Mustn1* possibly functions as a musculoskeletal co-activator or co-regulator, although more conclusive experiments have yet to validate this hypothesis. Further, results from the experiments reported herein support the notion that *Mustn1* is a co-activator of a transcriptional complex involved in both the proliferation and differentiation of chondrocytes as both processes are negatively affected when *Mustn1* is

silenced *in vitro*. However, that *Mustn1* helps to inhibit a repressor involved in the transcriptional regulation of these processes cannot be ruled out. Further, if *Mustn1* is indeed a transcriptional co-activator, the identification of its target genes holds much promise in the elucidation of chondrogenesis. Because *Mustn1* is active very early in chondrocyte differentiation, cartilage and bone development, as well as bone regeneration, finding the direct targets of this gene will aid in identifying the early initiators of each of these vital processes. While *Mustn1* itself is not sufficient for chondrogenic differentiation, it is possible that its target gene(s) may be. And although these results are promising and further provide evidence of *Mustn1*'s critical regulatory role in chondrogenic differentiation, additional research is required to conclusively elucidate *Mustn1*'s role in the overall process of chondrogenesis.

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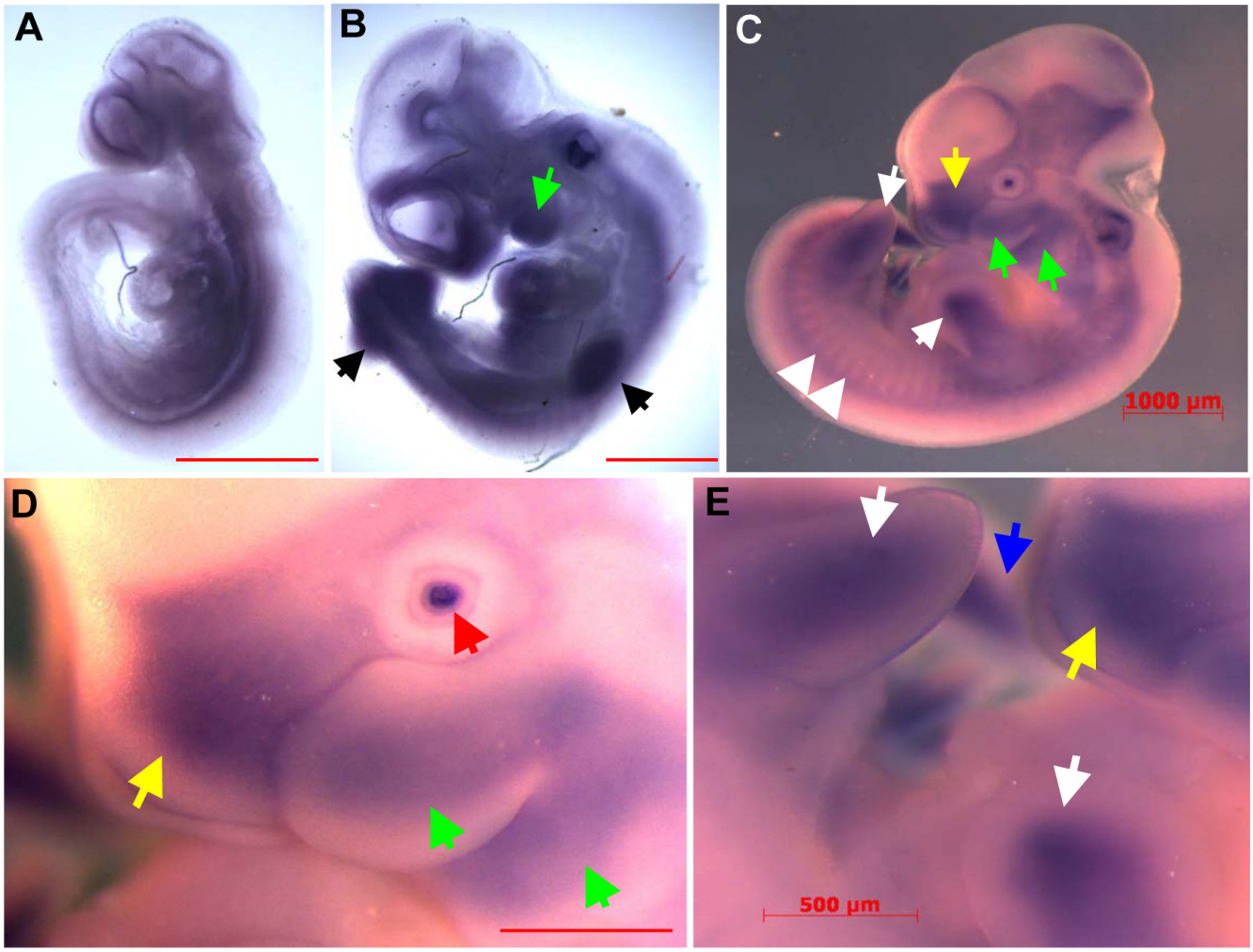

Figure 1. *Mustn1* expression localizes to areas of chondrogenesis and myogenesis during mouse embryogenesis

Whole mount embryos at 9.5dpc (**A**), 10.5dpc (**B**) and 11.5dpc (**CDE**) were hybridized with *Mustn1* antisense riboprobes. 9.5dpc embryos stain broadly for *Mustn1* throughout mesodermal tissues, while those at 10.5dpc and 11.5dpc embryos show distinct staining in fore and hind limbs (black and white arrows, respectively), branchial arches (green arrows), frontonasal process (yellow arrows), somites (white arrowheads), lens (red arrow) and posterior tail bud (blue arrow). Scale bars = 1000 µm (**ABC**) and 500µm (**DE**).

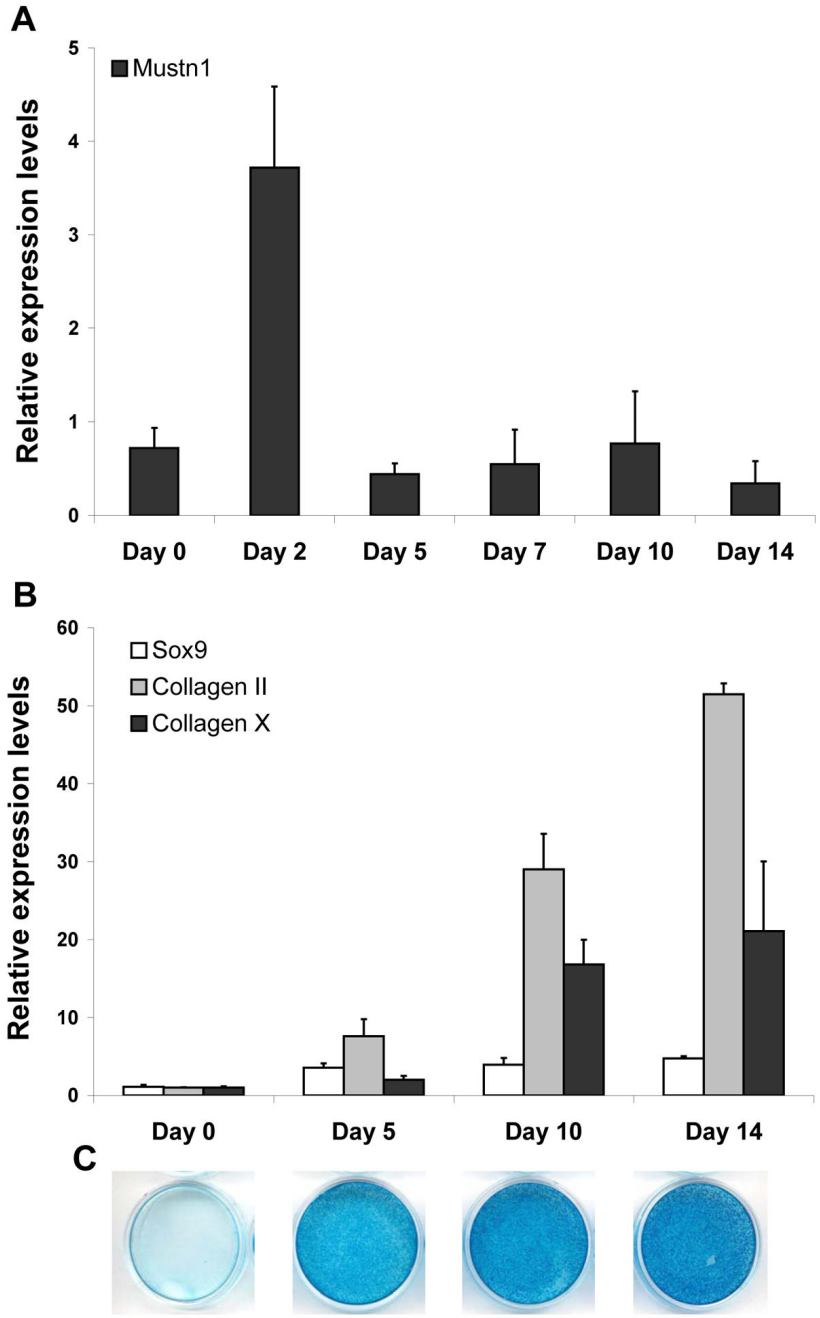

Figure 2. *Mustn1* and chondrogenic marker gene expression is differentially regulated during RCJ cell differentiation

Expression of *Mustn1* (**A**), and Sox9, a pre-chondrocyte cell marker, Collagen II, a proliferating chondrocyte cell marker, and Collagen X, a hypertrophic chondrocyte cell marker (**B**) as assayed via qPCR during RCJ differentiation. Confluent RCJ cells were stimulated to differentiate at Day 0 and mRNA was isolated and assayed at the days indicated. All bars represent average values of raw gene expression normalized to β2-microglobulin in pooled mRNA (n=3). Error bars indicate standard deviation (st. dev.) between qPCR runs (n=3). **C.** Photomicrographs of tissue culture dishes showing respective Alcian stained cell lines to indicate degree of differentiation on Day 0, 5, 10, and 14.

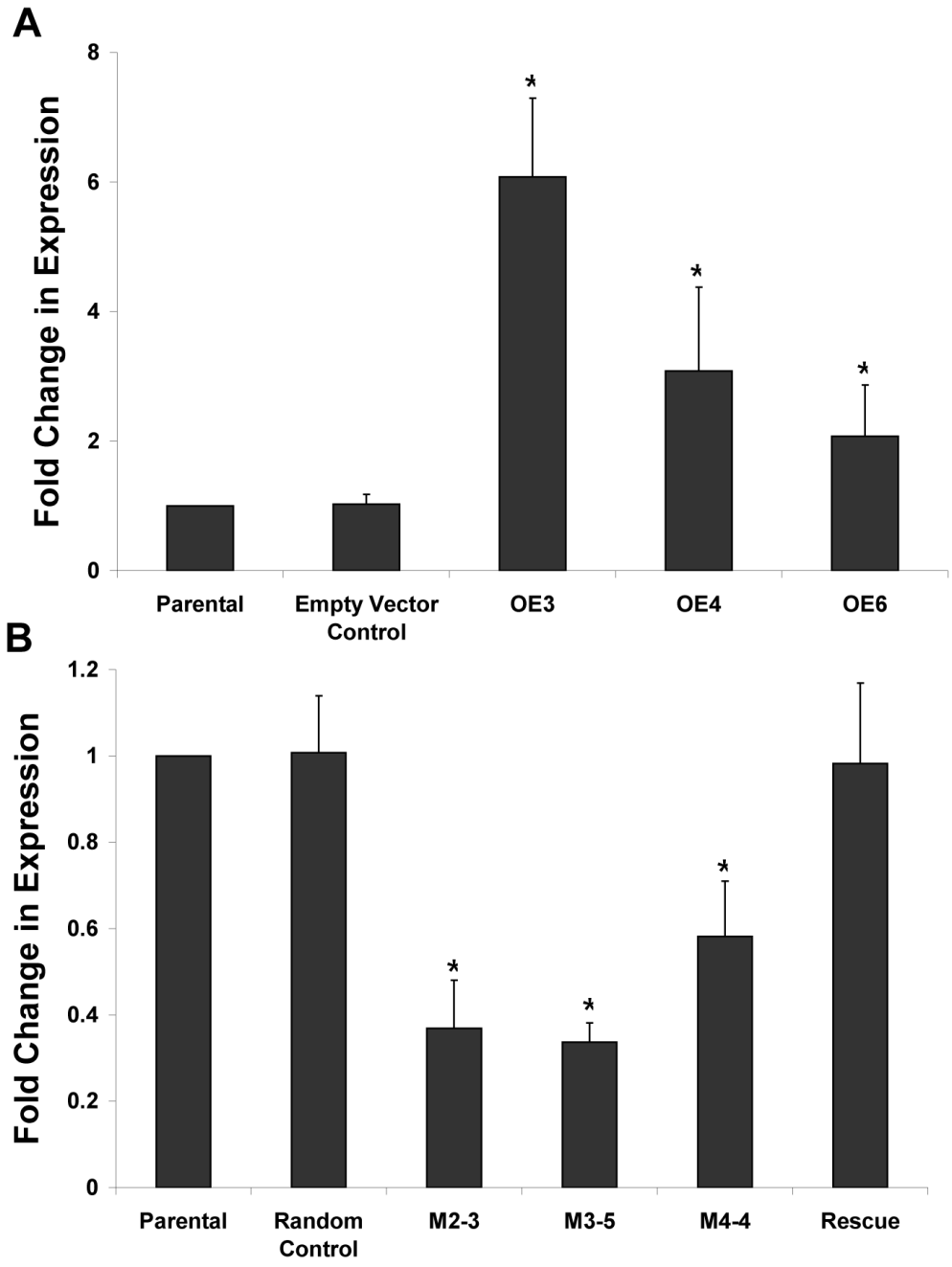

Figure 3. Modulation of *Mustn1* expression via overexpression and silencing

A.*Mustn1* was overexpressed in RCJ cell lines, OE3, OE4 and OE6 at ~6, 3, and 2 fold level of that of the parental cells, respectively. **B.** *Mustn1* was silenced in RCJ cell lines, M2-2, M3-5 and M4-4 at ~0.4, 0.3, and 0.6 fold level of that of the parental cells, respectively. A random sequence with no homology to the rat genome was used as a control (Random Control). The Rescue cell line was created by transiently transfecting the M2-2 cell line with a *Mustn1* containing plasmid. *Mustn1* expression was assayed by qPCR in proliferating RCJ cells and normalized to β2-microglobulin in pooled mRNA (n=3). Fold change was then determined by normalizing values to those of parental cells. Error bars indicate st. dev. (n=3). *p<.01 determined by Mann-Whitney test vs. Parental.

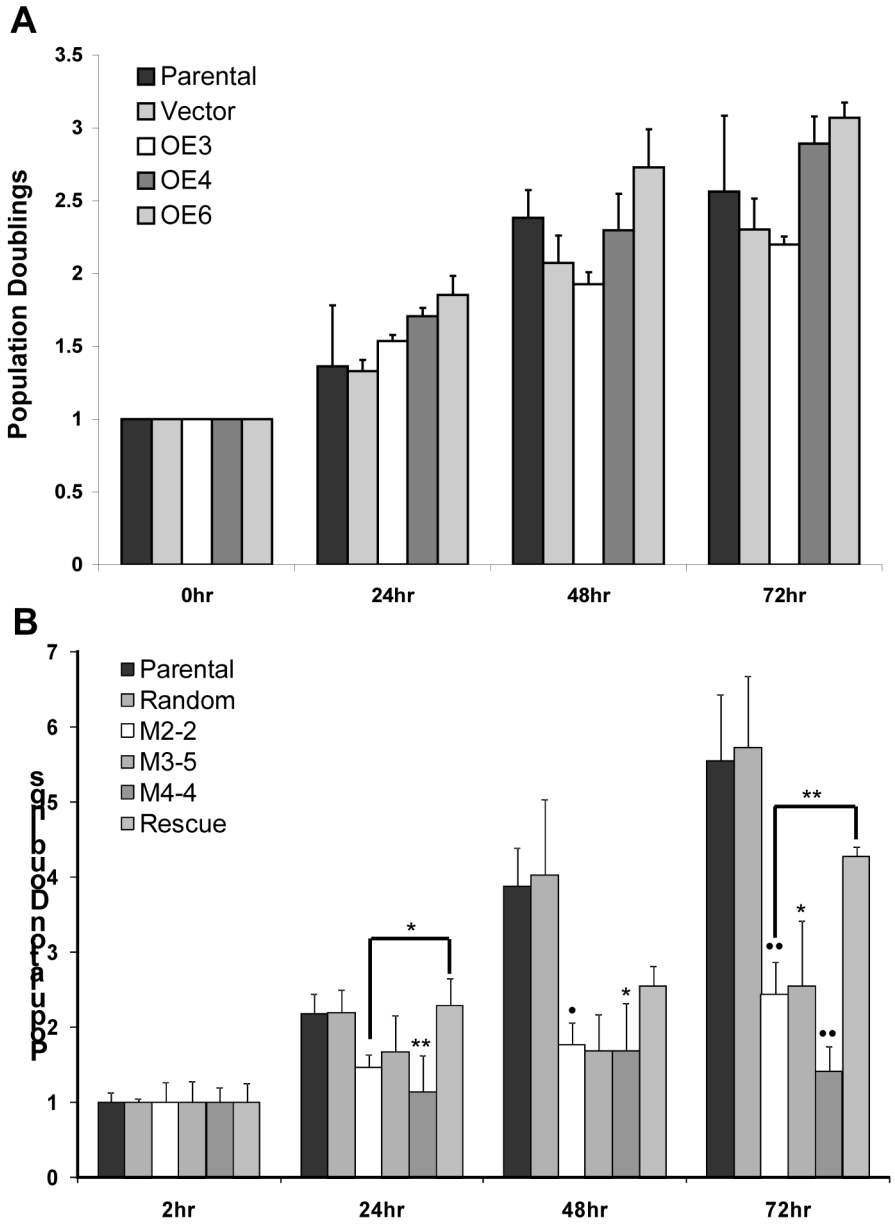

Figure 4. Cell proliferation is unchanged when *Mustn1* is overexpressed but is reduced in silenced cells

A.Cell proliferation measurements in Parental, Vector (control), OE3, OE4 and OE6 cell lines via MTS over a 3 day time course. There was no statistical significance between any cell line at any time point. **B.** Similar analysis of Parental, Random (control), silenced cell lines, M2-2, M3-5, M4-4 and Rescue. There was no statistical difference between Vector, Random, or Rescue and Parental cell lines at any time point. Each data point represents the average of triplicate MTS experiments (n=3). The value of each cell line was normalized to the 0hr time point to show population doublings. Error bars represent st. dev. All statistical significance was determined by ANOVA on ranks with Tukey post-hoc. Significant difference was only observed in between Parental and silenced cell lines, as well as M2-2 and Rescue. *p<.05, **p<.01, •p<.006, ••p<.001.

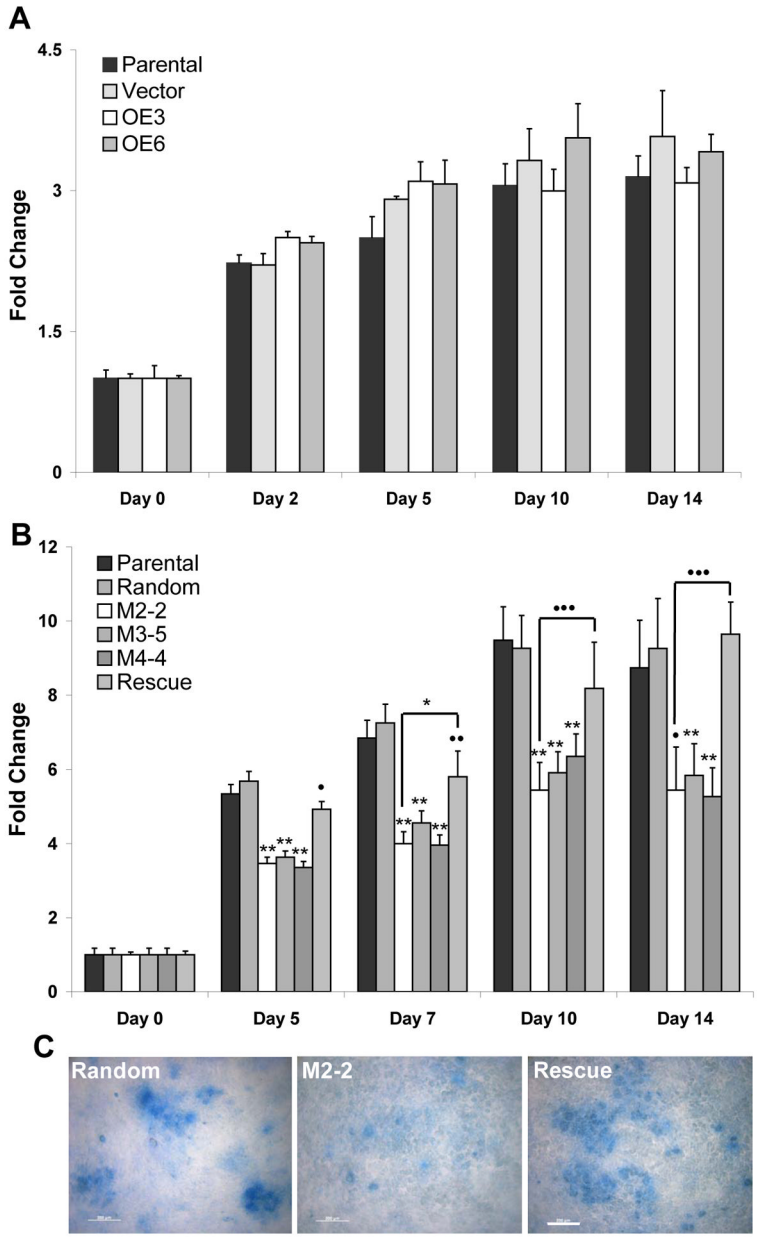

Figure 5. Matrix production is unaffected when *Mustn1* is overexpressed, but is reduced in silenced cells

Confluent cells from each line were stimulated to differentiate on Day 0. At specific times during differentiation all cell lines were fixed and stained with Alcian blue. The dye was then eluted and measured via spectrophotometry to quantitatively determine the amount of matrix produced at each time point. **A.** Parental, OE 3, OE6 and empty vector cell lines were assayed at Day 0, 2, 5, 10, and 14. **B.** Parental, Random, M2-2, M3-5, M4-4, and Rescue cell lines on Day 0, 5, 7, 10 and 14. **C.** Photomicrographs showing a random region from the respective Alcian stained cell line to indicate nodule formation and size (degree of differentiation). All bars indicate average of triplicate experiments ± st. dev. Significance was determined by ANOVA on ranks with a Tukey Post-hoc between the silenced cell lines vs. Parental levels or Mann-Whitney to compare M2-2 to Rescue. *p<.05, **p<.001, •p<.03, ••p<.02, •••p<.01.

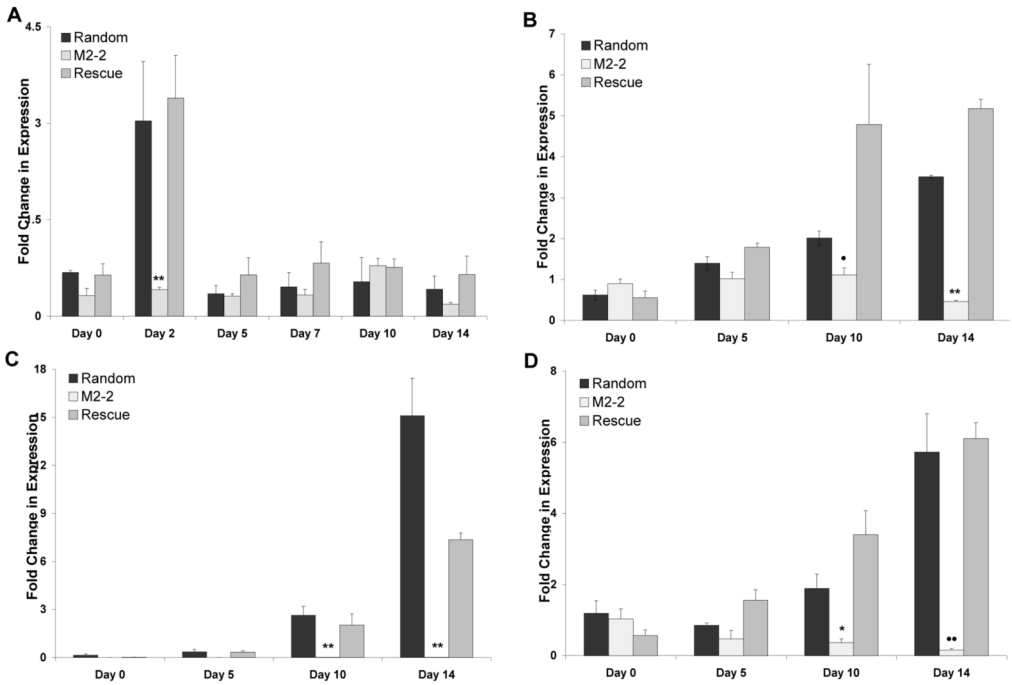

Figure 6. *Mustn1* and chondrogenic marker gene expression is reduced in Mustn1 silenced cells

Confluent RCJ cells were stimulated to differentiate at Day 0 and mRNA was isolated and assayed via qPCR (normalized to β2-Microglobulin) at Day 0, 2, 5, 7, 10 and 14 for *Mustn1* (**A**) and Day 0, 5, 10 and 14 for Sox9 (**B**), Collagen type II (**C**), and Collagen type X (**D**). All bars represent average raw gene expression values of pooled mRNA (n=3). Error bars indicate st. dev. between qPCR runs (n=3). Significance was determined by Mann-Whitney test vs. Random Expression levels. *p<.05, **p<.001, •p<.01, ••p=.002.

Table 1

List of primers and conditions used for qPCR.

Target Gene	Accession Number	Primer Sequence	Amplicon Size	Tm(oC)
β-2microglobulin	NM 012512	5′ - TGGTGTGCTCATTGCTATTC 3′ - CTCTGAAGGAGCCCAAAAC	152	58
Mustn1	NM 181390	5′ - GCTTTTCCTCTGCCACCTC 3′ - ATTCCCCGACCCACCTC	129	58
Collagen II	NM 012929	5′ - TGTGCTTCTTCTCCTTGCTC 3′ - GACCTGAAACTCTGCCACC	187	58
Collagen X	AJ131848	5′ - ACCTGGGGCAACTTAGAAAA 3′-CAGTGGAATAGAAGGCACACA	179	58
Sox9	AB073720	5′ - CCGACACGGAGAACACAC 3′ – CAGTCATAGCCCTTCAGCAC	98	58