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SOX9 transduction increases chondroitin sulphate synthesis in cultured human articular chondrocytes without altering glycosyltransferase and sulfotransferase transcription.

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SYNOPSIS

The transcription factor SOX9 is expressed in all chondrocytes and is essential for the expression of aggrecan, which during biosynthesis is substituted with more than 10 times its weight of chondroitin sulphate (CS) and is secreted by chondrocytes to form the characteristic glycosaminoglycan (GAG)-rich extracellular matrix of cartilage. SOX9 expression rapidly falls during monolayer culture of isolated chondrocytes and this turns off aggrecan and associated CS synthesis. We therefore investigated if SOX9 transduction of cultured human articular chondrocytes had any effect on the gene expression of the glycosyltransferases and sulfotransferases necessary for GAG biosynthesis. Retroviral SOX9 transduction of passaged chondrocytes increased the endogenous rate of GAG synthesis and the total capacity for GAG synthesis assessed in monolayer culture with β -xyloside. Both the endogenous rate and the total capacity of GAG biosynthesis were increased further in chondrogenic cell aggregate cultures. The GAG synthesized was predominantly CS and the hydrodynamic size of the newly synthesized chains was unchanged by SOX9 transduction. Aggrecan gene expression was increased in the SOX9 transduced chondrocytes and increased further in chondrogenic culture, but no comparable effects were found in SOX9 transduced dermal fibroblasts. However, the expression of CS glycosyltransferase and sulfotransferase genes in chondrocytes was unaffected by SOX9 transduction. Therefore SOX9 transduction in chondrocytes increased their CS synthetic capacity, but this was not accompanied by changes in the transcriptional of the CS biosynthetic enzymes and must occur by indirect regulation of enzyme activity through control of enzyme protein translation or enzyme organization.

(a) INTRODUCTION

The transcription factor SOX9 is expressed in all chondrocytes and is essential for the expression of the proteoglycan aggrecan, which is secreted by chondrocytes to form the characteristic glycosaminoglycan (GAG)-rich extracellular matrix of cartilage [1]. In the intracellular biosynthesis in chondrocytes aggrecan is substituted with more than 10 times its mass of chondroitin sulphate chains. In isolated chondrocytes the expression of SOX9 rapidly falls during monolayer culture [2] and this turns off aggrecan gene expression and there is greatly reduced chondroitin sulphate synthesis. SOX9 is expressed early in development transiently in several tissues, but in cartilage its expression is retained throughout life as it regulates the expression of other key cartilage extracellular matrix genes such as COL2A1, COLXI A1 [3-5]. Haploinsufficiency of SOX9 causes the genetic disease campomelic dysplasia, which affects several tissues and results in severe dwarfism caused by inadequate chondrocyte differentiation and poor cartilage formation [6]. It is thus clearly important that the capacity to produce CS in chondrocytes increases in parallel with aggrecan biosynthesis and we therefore investigated whether SOX9 had any direct effect on GAG biosynthesis.

The CS chains synthesized on aggrecan are attached to the protein at serine residues via a tetrasaccharide linkage region composed of glucuronate β 1-3 galactose β 1-3 galactose β 1-4 xylose β 1-serine. The specific enzymes responsible for catalyzing the successive addition of these sugar residues of the linkage oligosaccharide molecules and those responsible for extending the polysaccharide

chain with successive addition of glucuronic acid and N-acetylgalactosamine have been identified, together with the sulfotransferases, which sulphate the glycosaminoglycan chains [for reviews see 7, 8, see table 1 for more detailed references].

We previously showed that passaged human osteoarthritic articular chondrocytes after retroviral transduction of SOX9 regained their chondrogenic response to 3-dimensional cell aggregate culture and to growth factors and greatly increased their production of a GAG-rich cartilage matrix [9]. In this study we investigated whether SOX9 transduction of passaged human articular chondrocytes with SOX9 directly increased GAG production and activated the gene expression of the glycosyltransferases and sulfotransferases required for GAG biosynthesis.

(b) EXPERIMENTAL

Cell culture

Human articular cartilage was obtained, with informed consent and local research and ethical committee approval, following total knee arthroplasties. Chondrocytes were isolated from residual cartilage by trypsin/collagenase digestion and grown as monolayers in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal bovine serum (FBS- from Invitrogen, Paisley UK). At passage 3-5 the chondrocytes were transduced at high efficiency (~80%) with a retrovirus expressing a bicistronic hSOX9-IRES-green fluorescent protein (GFP) cDNA at a titer of 5×10^6 in the presence of the following growth factors which enhance cell proliferation [10]: platelet derived growth factor BB (PDGF-BB, 10ng/ml), transforming growth factor β -1 (TGF β -1, 1ng/ml) and fibroblast growth factor-2 (FGF-2, 5ng/ml) (all from Sigma, Poole UK). Control transduction was with GFP retrovirus (lacking hSOX9) and human dermal fibroblasts (Lonza Biologics, Slough UK) were also similarly transduced with similar efficiency. The transduced chondrocytes were grown as monolayers in DMEM + 10% FBS up to late passage (approximately 8-10 population doublings) for use in these experiments. Chondrogenic cultures were formed of cell aggregates (5×10^5 cells) grown for 14 days in a chondrogenic medium as previously described [9] (DMEM with FBS(10%), dexamethasone (10nM), TGF β -3 (10ng/ml), IGF-1, (100ng/ml), ascorbate (25 μ g/ml 2-phosphate) and ITS1+ (all from Sigma).

Sulphated GAG Analysis

Total GAG content of cell aggregate cultures was determined using the dimethylmethylene blue (DMMB) assay [9]. For analysis of the rate of GAG synthesis cells in monolayer were incubated in DMEM + 10% FBS containing sodium [35 S]-sulphate (20 μ Ci/ml) (MP Biomedicals, London UK) for 24h with or without 1mM *p*-nitrophenyl- β -D-xyloside (Sigma). After culture the combined cell layer and medium were extracted with 4M guanidine hydrochloride, 50mM sodium acetate pH 6.0 for 24 hr at 4°C. Cell aggregate cultures were incubated with sodium [35 S]-sulphate (20 μ Ci/ml) for the final 24h of the 14-day culture. After culture the medium and the cell aggregate were extracted as for the monolayer cultures. Unincorporated radiolabel was separated from macromolecular products in all samples using PD-10 size exclusion columns (Amersham Lifesciences, Amersham UK) eluted in phosphate buffered saline [11]. Radiolabel in the high molecular

weight fraction was measured by scintillation counting. Rates of incorporation were normalized to the DNA content of the cell monolayer or the cell aggregate, measured using a Hoescht 33258 dye binding assay [9]. The CS content of the total GAG was determined by incubating radiolabelled samples with chondroitinase ABC for 90 minutes at 37°C, precipitation of the undigested glycosaminoglycan with cetylpyridinium chloride (Sigma) and scintillation counting. Radiolabelled CS chain length was determined by size exclusion chromatography of CS isolated following alkaline hydrolysis. Samples were fractionated on a Sephacryl S300 column (600mm x 10mm) in 50mM tris HCl, 100mM NaCl, 100mM Na₂SO₄, pH 7.5 and 0.5ml fractions were collected and radioactivity was measured in a scintillation counter. Shark chondroitin sulphate (300µg/ml) (Sigma) was added to the samples as a carrier.

Gene expression analysis

Gene expression analysis was carried out as previously described [9]. Total RNA was prepared from cells using Tri Reagent (Sigma) and cDNA generated with M-MLV reverse transcriptase using random hexamers (both from Promega, Southampton, UK). Expression was analysed with an MJ Research Opticon 2 using a SYBR Green Core Kit (Eurogentec, Seraing Belgium). Primers for real time PCR analysis of GAG synthesis enzymes (Invitrogen) were designed using ABI primer express software and are presented in Table 1. Primers for GAPDH and aggrecan and have been previously described [12].

(c) RESULTS

Glycosaminoglycan synthesis is regulated by SOX9 in chondrocytes

Transduction of human articular chondrocytes at passage 3-5 with SOX9-GFP retrovirus resulted in ~80% GFP positive cells and a 10-fold increase in SOX9 mRNA, which persisted during subsequent expansion in monolayer culture. We have previously shown that after SOX9 transduction, these human articular chondrocytes regained a strong chondrogenic response to 3-dimensional cell aggregate cultures and over 14 days established a more GAG-rich ECM than control cultures [9]. We therefore investigated the rate of GAG biosynthesis in SOX9 transduced chondrocyte aggregate cultures in comparison with GFP transduced controls. The results showed that over the 14-day culture, the SOX9 transduced chondrocytes showed a greater increase in wet weight and had a significantly higher GAG content (Figure 1A) and from [³⁵S]-sulphate incorporation they synthesized 3.2 times more GAG than controls during the final 24h of the 14-day culture (Figure 1B). We next examined whether SOX9 similarly affected GAG biosynthesis in monolayer culture and there was a 7.6-fold increase in sulphate incorporation following SOX9 transduction (Figure 2A). These results showed the effects on endogenous GAG biosynthesis, which in chondrocytes is likely to reflect increased expression of CS on proteoglycans such as aggrecan. In order to assess the effects of SOX9 on the total capacity of the cells for CS biosynthesis we determined sulphate incorporation in the presence of β-D-xyloside, which acts as an artificial primer for CS biosynthesis [13]. This therefore provides a measure of the total capacity for CS synthesis, which is independent of the rate of synthesis of endogenous acceptors for CS chains. In SOX9 transduced chondrocytes there was a 2.6-fold increase in [³⁵S]-sulphate incorporation in the

presence of β -D-xyloside (Figure 2A). This showed that the total capacity for CS biosynthesis was increased following SOX9 transduction. The effect of SOX9 on GAG biosynthesis appeared to be cell specific. Transduction of human dermal fibroblasts, which have lower endogenous rates of CS biosynthesis, with SOX9 did not cause a significant increase in endogenous, or in total CS biosynthesis in monolayer culture (Figure 2B). In SOX9 transduced chondrocytes and controls, CS was the predominant GAG (83-85% of total) produced as shown by enzymatic digestion of radiolabelled GAGS with chondroitinase ABC (Figure 3). Furthermore, size exclusion chromatography showed that GAGs in both control and SOX9 transduced chondrocytes were of similar hydrodynamic size (Sephacryl S300 K_{av} = 0.45) indicating no change in average chain length.

Aggrecan gene expression

Due to the increased rate of CS synthesis in the SOX9-transduced chondrocytes, without β -xyloside, we examined the expression of the mRNA encoding the aggrecan core protein. Previous reports have demonstrated that SOX9 regulated aggrecan expression and we found that following transduction with the SOX9, aggrecan mRNA expression was significantly increased 2.1-fold compared with GFP-transduced controls (means and standard errors of $2^{\Delta Ct}$ values obtained from 3 experiments using cells from different donors: GFP transduced = 0.08 ± 0.02 ; SOX9 transduced = 0.17 ± 0.02 , $p = 0.038$ unpaired students t-test) and we previously showed that aggrecan expression was increased in transduced chondrocytes following transfer to chondrogenic cell aggregate cultures [9]. Therefore the increased rate of endogenous CS biosynthesis in the SOX9-transduced chondrocytes was correlated with increased aggrecan gene expression.

Gene expression of CS biosynthetic enzymes

To determine whether the increase in the total capacity for CS biosynthesis caused by SOX9 transduction was correlated with an increase in transcription of the biosynthetic enzymes, we determined the expression by qRT-PCR of the genes encoding CS synthesis enzymes (see Table 1). There was significant expression of all the enzymes necessary for CS biosynthesis, which gave levels of mRNAs in the range 0.8% to 6.0% of GAPDH for glycosyltransferases and 0.2% to 2.0% for the sulfotransferases. Analysis of the monolayer cultured chondrocytes showed that there was no significant effect of SOX9 transduction on the gene expression of either the tetrasaccharide linkage region glycosyltransferases, (figure 4A) or the CS glycosyltransferases (figure 4B) or the CS sulfotransferases (Figure 4C). It was also found that the expression levels of the genes for these enzymes were also at similar levels in the dermal fibroblasts (results not shown). The increased endogenous expression of CS and the total capacity in the presence of xyloside were not correlated with any detectable change in gene expression of the enzymes involved in CS chain synthesis.

(d) DISCUSSION

Synthesis of CS by articular chondrocytes plays a critical role in establishing and maintaining the cartilage extracellular matrix. SOX9 is essential for the chondrocyte phenotype and necessary for gene expression of the major cartilage matrix proteoglycan, aggrecan. In this study we have now shown that SOX9

transduced into human articular chondrocytes increased both the endogenous CS synthesis and the total capacity for CS synthesis in monolayer culture and there was a further increase in the capacity under chondrogenic conditions. As the rate of CS biosynthesis in chondrocytes is entirely dependent on the supply of proteoglycan protein for chain attachment, which in the chondrocyte is predominantly aggrecan, it is the rate of translation of aggrecan which largely determines the endogenous rate of CS synthesis. Increased aggrecan core protein translation is therefore likely to account for the higher rate of endogenous GAG synthesis detected in SOX9 transduced chondrocytes. However, as in SOX9 transduced chondrocytes there was also a large increase in the total synthetic capacity of GAG, which was much above that required for aggrecan synthesis, it was clear that the capacity for CS biosynthesis was being co-ordinately upregulated in parallel with aggrecan gene expression. In human dermal fibroblasts there was no comparable increase in the GAG synthesis in response to SOX9, which showed that the SOX9 effect was specific to the chondrocytes.

GAG synthesis requires a range of glycosyl- and sulfotransferase enzymes. It is therefore interesting that there were no significant changes in the expression of a large panel of enzymes involved in CS synthesis after SOX9 transduction, despite the increase in CS biosynthesis in these cells. It was also notable that the levels of expression of these transferase genes were similar in fibroblast and chondrocytes and they were expressed in the range similar to many cellular proteins. Their expression in chondrocytes and fibroblasts was therefore not correlated with the rate of endogenous CS synthesis, which was much lower in fibroblasts than in chondrocytes (Figure 1A and B). The regulation of CS synthesis in chondrocytes by SOX9 does not therefore appear to occur through a change in gene transcription of the enzymes responsible for their biosynthesis, but must be regulated by other mechanisms. Aggrecan expression is important to chondrocyte function and sets the major requirement for their CS synthesis capacity. It is possible that the effect of SOX9 on CS synthesis may be through its control of aggrecan transcription and translation and this would also account for the lack of any significant effect in fibroblasts in which we have shown that SOX9 transduction does not increase aggrecan expression [9]. As dermal fibroblasts show negligible expression of aggrecan it raises the possibility that, in chondrocytes, aggrecan core protein itself triggers the increased activity of the enzymes required for CS chain synthesis.

CS biosynthesis occurs in a Golgi compartment of the cell and is a fast, efficient process, rapidly adding CS chains to proteoglycans in the secretory pathway [14, 15]. As regulation of the capacity for CS biosynthesis did not appear to be by direct changes in transcription of the enzymes involved, it must involve other mechanisms. One possibility is that regulation is by the control of translation of the enzymes, or alternatively it may be by the post-transcriptional control of the enzyme activities. This may, therefore involve controlling the translation and turnover of functional enzymes, or by activating mechanisms that guide their organization into more active units with greater efficiency for making CS chains within the Golgi apparatus, where they are localized. Some evidence for the latter type of regulatory mechanisms has been reported. The Golgi contains an assembly line for protein glycosylation and modifications. The order in which the enzymes

are arranged from the cis- to the trans-Golgi thus has significant effects on their access to appropriate substrates [16] and indirectly controls their functional activity. Furthermore, heparan sulphate biosynthesis, which assembles GAG chain closely related to CS, provides two examples of the ways in which GAG chain synthesis may be controlled independently of gene expression by requiring enzyme complexes of EXT1/EXT2, [17] and the interaction between glucuronosyl 5-epimerase and 2-O-sulfotransferase [18] This shows the potential importance of further levels of enzyme organization in determining the cellular capacity for GAG biosynthesis. The possibility that enzyme complex formation is a determinant of CS synthesis capacity is entirely compatible with many observations on the high efficiency of CS chain biosynthesis in chondrocytes. In radiolabelling experiments of CS chain biosynthesis on aggrecan, shortened partially finished chains are not detected, which implies that each chain once started is finished very rapidly and aggrecan with fewer chains attached is also not detected, which suggests that the 100 chains on each aggrecan are started within a narrow window of time as it passes through the Golgi [15] and are finished with great efficiency [14]. Kinetic experiments also suggest that the synthesis of all the CS chains on an aggrecan molecule is complete in less than a few minutes [11]. So this is highly compatible with some level of organization of the CS biosynthetic enzymes that makes chain assembly coordinated, efficient and fast. As regulation appeared to be linked to aggrecan expression it is possible that aggrecan core protein is the trigger that increases enzyme translation, or increased organization and activity. This might occur because of the increased availability of CS acceptor sites on newly synthesized aggrecan, or possible following the addition of xylose, which is known to occur in the late ER and in an earlier compartment in the in the secretory pathway than CS elongation. So arrival of xylosylated protein in the Golgi could provide the signal to upregulate CS biosynthesis. The effect of SOX9 on CS synthesis in chondrocytes is thus suggested not to be by direct transcriptional control of gene expression of the biosynthetic enzymes, but by indirect control (possibly through aggrecan) of the enzymes' translation and organization, which increases the total capacity to synthesise CS chains within the chondrocyte.

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FIGURE LEGENDS

Figure 1: Effect of SOX9 Transduction on Glycosaminoglycan Synthesis in Articular Chondrocytes cell aggregate cultures: (A) Glycosaminoglycan content of 14 day chondrocyte cell aggregate cultures using DMMB assay normalized to DNA content. (B) [^{35}S] – sulphate incorporation in the final 24 h of 14 day chondrocyte cell aggregate cultures, normalized to DNA content. Data presented as mean and standard error in each case (n = 3).

Figure 2: Effect of SOX9 Transduction on Glycosaminoglycan Synthetic Capacity in Articular Chondrocytes or Dermal Fibroblast monolayer cultures: (A) [^{35}S] – sulphate incorporation over 24 h in chondrocyte monolayer culture combined cell and medium fractions. Cultures were incubated with *p*-nitrophenyl- β -D-xyloside (1mM) as indicated and results normalized to DNA content in the cell layer. (B) [^{35}S] – sulphate incorporation over 24 hours in dermal fibroblast monolayer cultures normalized to DNA content. Cultures were incubated with *p*-nitrophenyl- β -D-xyloside (1mM) as indicated. * = significant increase in SOX9 transduced culture compared to control (p < 0.05, unpaired students t-test). Data presented as mean and standard error of normalized [^{35}S] – sulphate incorporation (n = 3).

Figure 3: Effect of SOX9 Transduction on Chondroitin Sulphate Synthesis in Articular Chondrocytes in Monolayer Culture: Glycosaminoglycans labeled with [^{35}S] – sulphate for 24 h were digested with chondroitinase ABC (see methods). Undigested radioactive glycosaminoglycans were counted and normalized to DNA content. Data presented as the mean and standard error from 3 experiments.

Figure 4: Gene Expression of Glycosyltransferases and Sulfotransferases of Chondroitin Sulphate Biosynthesis: Real time PCR analysis of cDNA samples from GFP expressing control (black bars) or SOX9 transduced (grey bars) human articular chondrocytes grown in monolayer culture. Results are presented as (A) Linkage region glycosyltransferases, (B) Chondroitin sulphate synthesis enzymes (C) Sulfotransferases. Refer to Table 1 for the full name of each enzyme. Bars and error bars represent mean expression levels and the standard error of three independent cultures.

Table 1**Real time PCR primers sequences and references for glycosyltransferases and sulfotransferases examined in this study**

Enzyme	Real time PCR primer sequence (5'-3')	Reference
Xylosyltransferase-1 (XT1)	F- GTGGATCCCGTCAATGTCATC R- GTGTGTGAATTCGGCAGTGG	[19]
Xylosyltransferase-2 (XT2)	F- CGAATCGCCTACATCATGCTGG R- TAAACGGCCTTGAGGAGACG	[19]
Galactosyltransferase-1 (GalT1)	F- TCACCCCACTTTTGTTCCTT R- CATGGCCCCTACACTGTGTCT	[20, 21]
Galactosyltransferase-2 (GalT2)	F- GCGCACGTCTCGGTTTTTT R- TTGACGGTACAGGCACCAAGT	[22]
Glucuronyltransferase (GlcAT)	F- GGCGCGCCATGAAG R- ATGGCTGGCCGAGCTCTA	[23]
Chondroitin Synthase-1 (CHSY1)	F- CCCGCCCCAGAAGAAGTC R- TCTCATAAACCATTCATACTTGCCAA	[24]
N-acetyl galactosaminyltransferase-1 (GalNAcT1)	F- CCCCAATCACCGTCCTTACA R- TGTTTGTGGTCCCCTTTGAAG	[25]
N-acetyl galactosaminyltransferase-1 (GalNAcT2)	F- GGGCTAATGGCATAGGCTATCA R- CTTTGTCAATTTGGGAATGAAGAA	[26]
Chondroitin sulphate glucuronyltransferase (CSGlcUAT)	F- TTGAGACGAACGGCGAAGAC R- CGGCGATCAGACACCAAGTC	[27]
Chondroitin polymerizing factor (ChF)	F- AGATCCAGGAGTTACAGTGGGAGAT R- CCGGGCGGGATGGT	[28]
Chondroitin-6-O-sulfotransferase-1 (C6ST1)	F- CCCGGACGGTTTTCTACTTGATG R- GCGTCGCCCCGGATACAG	[29]
Chondroitin-6-O-sulfotransferase-2 (C6ST2)	F- TGCTCGATCTGGGCGTG R- AAAGCTGCACCACCTTAGG	[30]
Chondroitin-4-O-sulfotransferase-1 (C4ST1)	F- GGCCCTGCGCAAAG R- GGGTGTGTGGGTCGATGAG	[31]
Chondroitin-4-O-sulfotransferase-2 (C4ST2)	F- AACGAGGAGTTCTACCGCAAGT R- GCTTGTGTGGTTGGCGTACA	[31]
N-acetylgalactosamine-4-O-sulfotransferase-2 (GalNAc4ST2)	F- AGAAGATGCCAATTACTTTTACAGATG R- TGCTACCTTAAAGTTGGGAAAT	[32]
N-acetylgalactosamine-4-sulphate-O-6-sulfotransferase (GalNAc4S-6ST)	F- CGAAAGCTAGAAAGGGCTATTGC R- CGAGGGCCATCCATTGTATG	[33]
Uronosyl-2-O-sulfotransferase (UA2OST)	F- AGATCCAGGAGTTACAGTGGGAGAT R- CCGGGCGGGATGGT	[34]

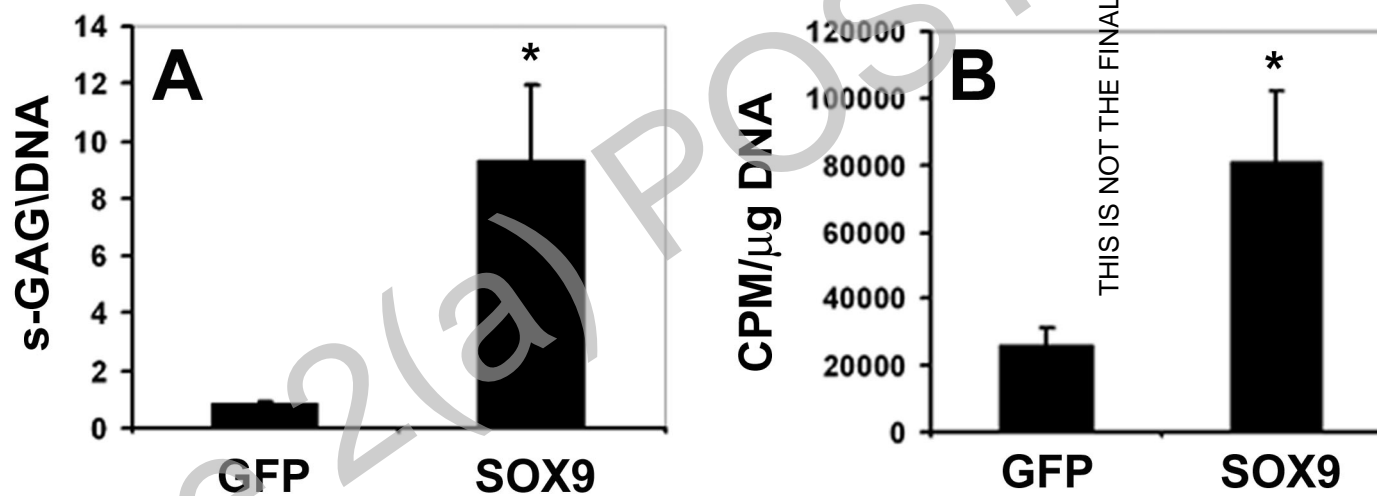
Figure 1

Figure 2

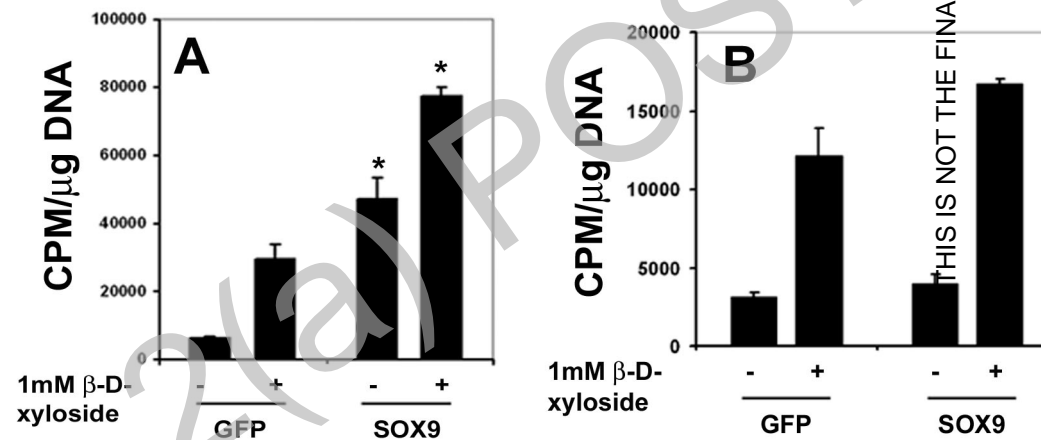
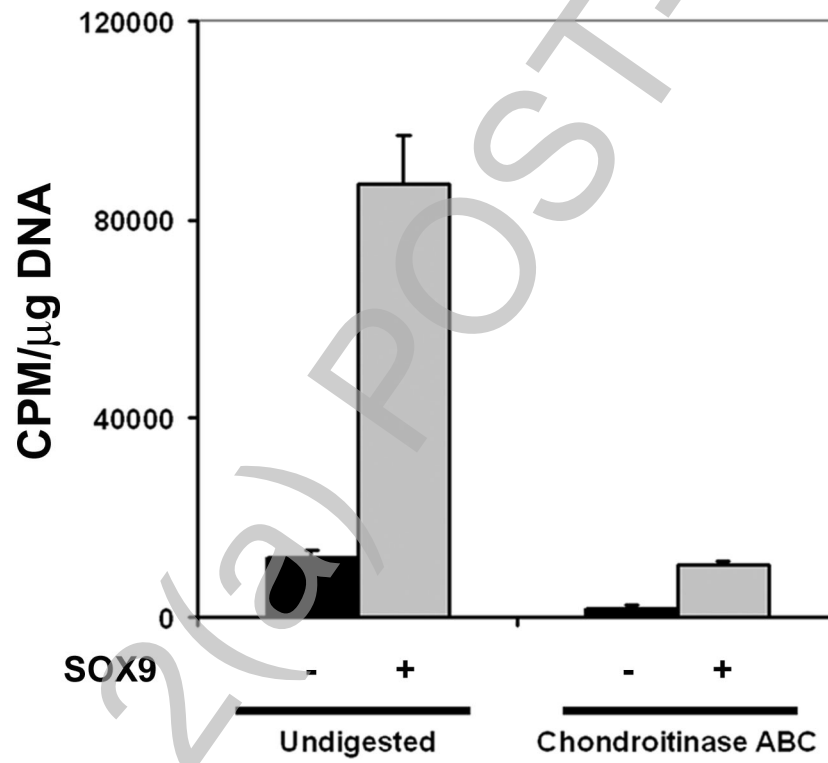


Figure 3



THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080262

Figure 4

