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Quantification of type II procollagen splice forms using Alternative Transcript-qPCR (AT-qPCR)

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Abstract

During skeletal development, the onset of chondrogenic differentiation is marked by expression of the $\alpha 1(\text{II})$ procollagen *Col2a1* gene. Exon 2 of *Col2a1* codes for a cysteine-rich von Willebrand factor C-like domain. Chondroprogenitors express the exon 2-containing IIA and IID splice forms by utilizing adjacent 5' splice sites separated by 3 base pairs. There is a shift to expression of the shorter, exon 2-lacking IIB splice form with further differentiation. Alternative splicing analysis of *Col2a1* splice forms has often relied upon semi-quantitative PCR, using a single set of PCR primers to amplify multiple splice forms. We show that this widely used method is inaccurate due to mismatched amplification efficiency of different-sized PCR products. We have developed the TaqMan®-based AT-qPCR (Alternative Transcript-qPCR) assay to more accurately quantify alternatively spliced mRNA, and demonstrate the measurement of *Col2a1* splice form expression in differentiating ATDC5 cells *in vitro* and in wild type mouse embryonic and postnatal cartilage *in vivo*. The AT-qPCR assay is based on the use of a multiple amplicon standard (MAS) plasmid, containing a chemically synthesized cluster of splice site-spanning PCR amplicons, to quantify alternative splice forms by standard curve-based qPCR. The MAS plasmid designed for *Col2a1* also contained an 18S rRNA amplicon for sample normalization, and an amplicon corresponding to a region spanning exon 52-53 to measure total *Col2a1* mRNA. In mouse E12.5 to P70 cartilage, we observed the expected switch between the IIA and IIB splice forms; no IID or IIC splice products were observed. However, in the ATDC5 cultures, predominant expression of the IIA and IID splice forms was found at all times in culture. Additionally, we observed that the sum of the IIA, IIB and IID splice forms comprises only a small fraction of *Col2a1* transcripts containing the constitutive exon 52-53 junction. We conclude from our results that the majority of ATDC5 cells in the assay described in this study remained as chondroprogenitors during culture in standard differentiation conditions, and that additional *Col2a1* transcripts may be present. The validity of this novel AT-qPCR assay was confirmed by demonstrating the expected *Col2a1* isoform expression patterns *in vivo* in developing mouse cartilage. The ability to measure true levels of procollagen type II splice forms will provide better monitoring of chondrocyte differentiation in

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other model systems. In addition, the AT-qPCR assay described here could be applied to any gene of interest to detect and quantify known and predicted alternative splice forms and can be scaled up for high throughput assays.

Keywords

mRNA alternative splicing; quantitative real-time PCR; *Col2a1*; chondrogenesis; differentiation

1. Introduction

Collagens are a major component of most extracellular matrices, and play a pivotal role in many physiological and pathological processes. Expression of the type II procollagen gene (*Col2a1*) (Metsaranta et al., 1991) is essentially restricted to cartilage, the vitreous body of the eye, the inner ear, and the nucleus pulposus of the intervertebral disc in the adult. The gene is more widely expressed during development, and is found in non-chondrogenic as well as chondrogenic tissues (Cheah et al., 1991). *Col2a1*-null mice develop a skeleton with abnormal cartilage and no endochondral bone, and die before or shortly after birth (Li et al., 1995). Additionally, these mice fail to remove the notocord from vertebral bodies, where it is normally dismantled during embryogenesis (Aszodi et al., 1998).

Col2a1 is alternatively spliced during cartilage development, whereby chondroprogenitor cells express predominantly exon 2-containing IIA splice forms while differentiated chondrocytes express mainly the exon 2-lacking IIB splice forms of *Col2a1* mRNA (Ryan and Sandell, 1990) (Fig. 1). We previously reported that two additional alternatively spliced products, which we termed IIC and IID, are expressed during chondrogenic differentiation of mesenchymal stem cells (McAlinden et al., 2008). The murine IIC splice form is generated by the use of an alternative 5' splice site, resulting in an mRNA transcript containing only the first 34 residues of the 204 nucleotide exon 2. This causes a frameshift mutation that subsequently generates three premature termination codons (PTC) within exon 6. In contrast, the IID mRNA transcript contains all of exon 2 plus an additional three nucleotides (GTC in mouse) using an adjacent 5' splice site. The IIA, IIB and IID splice forms contain full-length open reading frames. Recently, expression of the IID splice form was also observed in human articular chondrocytes during expansion in monolayer culture in the presence of BMP-2 (Claus et al., 2010).

The alternatively-spliced exon 2-encoded domain of *Col2a1* encodes a highly conserved cysteine-rich (CR) von Willebrand Factor C-like domain within the amino (NH₂) propeptide (Bornstein, 2002). This exon 2-encoded protein shares some homology with CR domains in other proteins that are known to bind to and regulate growth factors of the TGF- β superfamily (Abreu et al., 2002). *In vitro* studies have shown that recombinant exon 2-encoded proteins can bind to BMP-2 and TGF- β (Zhu et al., 1999) while a BMP-binding function *in vivo* was demonstrated in *Xenopus* by over-expression of full length type IIA procollagen mRNA (Larrain et al., 2000). In IIA null mutant mice, loss of head tissues, holoprosencephaly, and loss of mid-facial structures has been reported (Leung et al., 2010). While this study did not find these effects to be due to dysregulation of BMP activity, the data does suggest that the embryonic IIA isoform of *Col2a1* is important for development of some non-chondrogenic tissues (Cheah et al., 1991; Ng et al., 1993). A functional role for the exon 2-encoded domain in regulating growth factors during cartilage development has not been identified. Mice engineered to express only the embryonic IIA isoform of type II procollagen have no overt phenotype (Lewis et al., 2012) but further analyses shows defects in post-natal cartilage and bone tissue (McAlinden, unpublished observations). This may suggest that the *Col2a1* alternative splicing event is important in production of proper post-

natal skeletal tissue. Also, the $\alpha 1$ chain of type II procollagen and the $\alpha 3$ chain of type XI procollagen are both encoded by *Col2a1* (Eyre and Wu, 1987; Furuto and Miller, 1983). Therefore, alternative splicing may control type XI procollagen isoforms influencing the production and maintenance of proper skeletal tissues. As such, the functional significance of the developmentally-regulated *Col2a1* splicing switch during skeletal differentiation remains to be fully deciphered.

With respect to investigating the functional significance of *Col2a1* alternative splicing, an assay system is required that can distinguish and quantify expression levels of all four isoforms identified to date. In this study, we report a novel real time PCR approach to quantify the alternative splice forms of murine *Col2a1* mRNA expressed *in vivo* in wild type cartilage tissue at various stages of embryonic and post-natal development, as well as in ATDC5 cultures during *in vitro* chondrogenesis. There are few practical methods for the routine quantitative analysis of alternative mRNA splice forms. Most commonly, PCR-based, co-amplification approaches, generating multiple PCR products have been used, with primers that flank alternative exon-exon junctions (Brown et al., 2004; Connell et al., 2005; Dalski et al., 2000; Davis-Taber et al., 2000; Favy et al., 2000; Kafert et al., 1999; Long et al., 2011; Meidan et al., 2005; Nygard et al., 2010; Sylvestersen et al., 2011; Yoong et al., 2005). Although convenient, the co-amplification approach, is semi-quantitative at best, and may under- or over-estimate relative quantities of alternative splice forms. Quantitative real-time PCR (qPCR) has become the method of choice to measure relative or absolute differences in steady-state mRNA levels. We previously described a standard curve-based qPCR method to quantify splice forms of human $\alpha 1(\text{II})$ procollagen mRNA using separately cloned, splice-site containing cDNA fragments to generate standard curves (McAlinden et al., 2008). In addition to these PCR-based methods for splice form quantification, high-throughput methods to quantify alternatively spliced products on a genomic scale have also been described (Fan et al., 2004; Lin et al., 2010; Wang et al., 2010). Generally, however, differences in splice form expression observed in a genomic-scale assay should still require verification by a smaller-scale gene-specific assay, such as the method described here.

In this report, we demonstrate the use of a novel assay, termed AT-qPCR (Alternative Transcript quantitative real-time PCR), that takes advantage of relatively low-cost gene synthesis technology to produce “engineered” sequence templates to use as multiple amplicon standards (MAS) for quantitative assessment of alternative splice forms. Placing multiple amplicons within a cloned synthetic DNA insert eliminates the need to precisely match the molar concentrations of multiple standard plasmids. This method could be used to measure splice form ratios of individual gene products in cells or tissues when the expense and complex data analysis of current high throughput approaches is not required.

Using the AT-qPCR method, we found that the *Col2a1* mRNA in insulin-induced ATDC5 cultures (Shukunami et al., 1996) comprised predominantly the IIA and the IID splice forms at all times in culture. Although the *Col2a1* IIB mRNA splice form, a marker for mature chondrocytes, increased over time in culture, it was significantly less abundant than IIA and IID isoforms at late stage ATDC5 differentiation. The IIC splice form was not detectable throughout the entire ATDC5 differentiation assay. Also, the apparent copy number of *Col2a1* mRNA transcripts determined using the exon 52-53 assay was greater than the total of the IIA, IIB and IID splice forms, suggesting the interesting possibility that there may be additional transcripts of *Col2a1* mRNA expressed in these cultures. Additionally, we analyzed wild type mouse embryonic and postnatal cartilage by the same method, and observed the expected transition between the IIA splice form in the early embryo to predominantly the IIB splice form in the late embryonic to postnatal cartilage and that *Col2a1* mRNA containing the exon 52-53 junction was more abundant than the total of IIA and IIB. This finding validates the AT-qPCR assay system.

The AT-qPCR assay described here is the first analysis of alternative splicing using a chemically-synthesized multiple amplicon standard, permitting a quantitative determination of splice form abundance in cells or tissues. The AT-qPCR assay may be easily applied to any alternatively spliced gene of interest, is scalable to high-throughput applications, and may be useful diagnostically to assess alternative splicing that may have clinical significance.

2. Results

2.1. Chondrogenesis in ATDC5 cultures

Alcian Blue staining of cultures taken at 2 day intervals revealed the emergence of proteoglycan-rich chondrogenic nodules which increased in number, size and staining intensity over the 30 day culture period (Fig. 2A), although a large proportion of the cell layer retained the appearance of a monolayer. By 30 days of culture, cells and extracellular matrix formed a layer that could be removed intact from the cell culture plate. To demonstrate the presence of cartilage-specific type II collagen, samples of these cell layers were dehydrated, fixed while attached to the plate surface, embedded in paraffin and used for immunofluorescent analysis with an antibody specific for the cysteine-rich exon 2 domain of the IIA and IID splice forms (Fig. 2B, D, and F). Collagen IIA and/or IID were abundant in the Alcian Blue-positive nodules (Fig. 2B and 2D), and at intermittent regions of the monolayer between nodules (Fig. 2F). Adjacent sections were also stained with an antibody to type I collagen, which was present to different extents within the nodules (Fig. 2C and 2D), but also throughout the monolayer between nodules (Fig. 2G) in regions where collagen IIA and/or IID could not be detected..

2.2. Quantification of alternative splice forms by AT-qPCR

A multiple-amplicon standard (MAS) plasmid was designed, with a chemically-synthesized insert sequence containing amplicons for each of six custom-designed TaqMan® assays including known alternative splice forms (IIA, IIB, IIC and IID), the exon 52-53 junction of *Col2a1* to directly measure the quantity of total *Col2a1* mRNA, and 18S rRNA as an internal standard (Fig. 3 and Table 1). The AT-qPCR assay is essentially a standard curve-based quantification of each splice form, using the same set of dilutions of the MAS to standardize each assay. Although splice form quantities were determined as “copy number” relative to the copy number of the MAS, the values were not absolute, since the standard was not prepared by cDNA synthesis, and the efficiency of each cDNA synthesis reaction was unknown. Details concerning the assay protocol are found in Methods and Supplementary Materials.

Design constraints on the MAS template required that MAS amplicons, although having the same primer and probe-binding sequences, were different in size from the “native” amplicons of the ATDC5 cDNA. We therefore derived an adjustment calculation (Supplementary Materials) to account for differences in experimentally-determined amplification efficiency of the standard and the cDNAs (Supplementary Fig. 1 and Supplementary Table 1). All qPCR values were adjusted for efficiency (Supplementary Figs. 2–7) before normalization to the mean 18S rRNA value (Supplementary Fig. 8).

To verify the specificity of the PCR reactions, samples were taken directly from the 96-well reaction plates at the end of 40 cycles for agarose gel analysis of PCR products. Amplified DNA products from the *Col2a1* MAS standard, and the ATDC5 cDNA templates were observed to be of the predicted size (Fig. 4, Table 1).

The quantity of each *Col2a1* splice form expressed over 30 days of growth in chondrogenic (+ insulin) medium was determined from *Col2a1* MAS plasmid standard curves (Fig. 5 and

Supplementary Figs. 2–7). The IIC splice form was not expressed at levels above background. The expression levels of the IIA, IIB and IID splice forms, normalized to 18S rRNA, increased throughout the first 14 days of culture and decreased thereafter (Fig. 5A). Since all splice form quantities were calculated from the same dilution series of the MAS plasmid standard, errors due to dilution of standards were largely eliminated, and the quantity of each splice form could be directly compared. The MAS standard was designed to determine the sum of all possible splice forms, by using an amplicon in a region of the *Col2a1* mRNA (exon 52-53 junction) that is not known to be alternatively spliced or absent from any known splice forms. Unexpectedly, *Col2a1* transcripts containing the exon 52-53 junction (Fig. 5B, right Y axis) were 76-fold more abundant at d2 to 145-fold more abundant at d22 than the sum of IIA, IIB and IID transcripts (Fig. 5B, left Y axis). This result suggests that *Col2a1* transcripts other than IIA, IIB and IID may be produced by ATDC5 cells in this culture system.

In Fig. 5C, the quantity of each splice form is plotted as a percentage of the sum of the IIA, IIB, IIC and IID splice forms. The IIA splice form increases initially and decreases steadily over time. The IID splice form exhibits a pattern that is the near inverse of IIA over time. At all stages of differentiation ATDC5 cells express a larger percentage of the IID compared to the IIA splice form. The IIB splice form never exceeds 4% of all splice forms, suggesting that it correlates with the overall number of nodules, and shows a nearly linear increase until late in culture and a decline during late differentiation. Levels of the IIC splice form were never greater than ~1% of total *Col2a1* mRNA, and never exceeded the level expected from the slight cross-reactivity of the IIC assay with the abundant IIA and IID splice forms.

We performed the same *Col2a1* AT-qPCR analysis on RNA purified from wild type murine epiphyseal cartilage samples harvested at E12.5 to P70 (Fig. 6). We found that IIA was the major splice form at E12.5 when the normalized overall *Col2a1* transcript level was lowest, and progressively diminished as a fraction of total *Col2a1* transcripts through to P28. An increase in IIA was observed at P70. The IIB form increased in relative abundance through to P28, and decreased at P70. No IIC or IID splice forms were detected at any time point. *Col2a1* transcripts containing the exon 52-53 junction were more abundant than the total of IIA and IIB transcripts at all time points, varying from approximately 3-fold at P0 to 9-fold at P7, respectively. Similar to our analysis of the ATDC5 cultures, this result suggests that *Col2a1* transcripts other than the IIA and IIB splice forms might also be present in mouse cartilage chondrocytes.

2.3. Comparison of AT-qPCR method with co-amplification method

We determined that the IIB splice form increases in relative abundance during chondrogenic differentiation of ATDC5 cells, but never exceeds 4% of the total of all four splice forms. This result was not in agreement with an earlier published analysis of *Col2a1* splice form ratios in ATDC5 cultures (Hatano et al., 2002). We therefore performed an experiment to investigate the discrepancy. Using the same primers that were used in this previous study, we performed endpoint PCR co-amplification of splice forms in ATDC5 samples that we had previously analyzed by AT-qPCR (Fig. 5). Agarose gel electrophoresis revealed a pair of bands at ~500 bp representing a heteroduplex of the IIA and IID splice forms, previously described by our laboratories (McAlinden et al., 2008), and a 275 bp band amplified from the IIB splice form (Fig. 7A). We subsequently cloned the IIA and IIB PCR products that were generated with these primers, and determined that the amplification efficiency of the isolated IIB template was greater than the isolated IIA template. Following SYBR®-Green-based real-time PCR, we calculated efficiency values of 111.7% for the IIA product (slope = -3.069 ± 0.239) and 135% (slope = -2.695 ± 0.283) for the IIB product. (Fig. 7B). Although we observed no evidence in the melt curve analysis for primer-dimer formation or mispriming in either set of reactions, efficiencies exceeding 100%, could be accounted for

by a small amount of primer dimer formation in both dilution series. The r^2 values were 0.98 for the IIA product, and 0.97 for the IIB product.

Using the AT-qPCR values as the starting template quantity, the amplification efficiencies of the IIA and IIB splice forms were used to predict the quantity of each splice form following 30 and 35 cycles of amplification by rtPCR (details are described in Supplementary Materials). At 30 and 35 cycles the lower band (IIB) appears to become more abundant than the upper bands (IIA/IID heteroduplex containing ~40% IIA) with increasing time in culture. We observed that the predicted IIB/IIA ratios appeared to match the ratios of the stained bands on the agarose gel, and differed significantly from the ratios determined by AT-qPCR (Fig. 7A). Due to the IIA/D heterodimer, it was not possible to precisely measure the ratio of the band intensities. It was apparent from these data, however, that co-amplification resulted in a significant overestimation of the IIB/IIA ratio.

3. Discussion

The mouse embryonal carcinoma-derived cell line ATDC5 (Atsumi et al., 1990; Shukunami et al., 1996; Wahl et al., 2004) has been frequently used as a model system for chondrogenic differentiation. Upon stimulation with insulin, condensation occurs to form cartilaginous nodules surrounded by cells having a fibroblastic morphology, analogous to events occurring in condensing limb mesenchyme during skeletal development (Hall and Miyake, 1995). As has been shown to occur during chondrogenesis *in vivo* (Fukada et al., 1999; Kosher et al., 1986; von der Mark et al., 1976; von der Mark, 1980), ATDC5 cells initially synthesize type I collagen, and following condensation switch to synthesis of type II collagen. Our immunohistochemical analysis of the ATDC5 cell layer shows an overall increase in Alcian Blue-positive nodules with time in culture, and demonstrates the heterogeneity of the culture with flat regions containing only type I collagen, and nodular regions containing both type II and type I collagen. In the present study, we have used an improved version of the AT-qPCR assay to analyze the differentiation-related transitions known to occur between different splice forms of *Col2a1* mRNA in ATDC5 cell culture. In addition, the same assay system was used to determine expression of *Col2a1* alternatively spliced transcripts *in vivo* from mouse cartilage tissue at different stages of embryonic and postnatal development.

Using AT-qPCR, we observed that insulin-treated ATDC5 cells expressed mainly the IIA and IID splice forms. This study provides the first demonstration that IID procollagen mRNA is produced by ATDC5 cells, which if translated, may be functionally distinct from IIA procollagen. Our results suggest that most ATDC5 cells in these cultures maintained a chondroprogenitor phenotype, and that a small fraction of the cells are further differentiated, likely residing within the chondrogenic nodules. In many published reports of ATDC5 cultures, the number of nodules per unit area following differentiation is somewhat variable (Hashimoto et al., 2005; Kirimoto et al., 2005; Miura et al., 2002), and it is possible that the IIB splice form may be more abundant in cultures having more numerous, or more differentiated nodules. We have observed that the onset of chondrogenesis in ATDC5 cultures may be delayed in cells with a higher passage number (unpublished observation), which may explain this variability. Confluent cultures of ATDC5 cells treated with BMP-2 have been shown to undergo chondrogenic differentiation to form a uniform monolayer of chondrocyte-like cells expressing markedly upregulated *Col2a1* mRNA, without forming nodules (Shukunami et al., 1998). The *Col2a1* alternative splice forms expressed under these conditions are not yet known.

In our previous work we have shown that IIC expression in MSC cultures could be detected only by Southern blot analysis of PCR products prior to expression of IIA and IID. The IIC

splice form is a candidate for nonsense-mediated decay (NMD) due to the presence of three translation termination codons in exon 6. We considered that the apparent low level of expression could be due to NMD-related turnover. We found that the IIC splice form was undetectable above background in ATDC5 cultures, even in the presence of cycloheximide (not shown) which can cause accumulation of mRNAs normally turned over rapidly by the NMD mechanism (Maquat et al., 2010).

To further validate the AT-qPCR method, we analyzed murine epiphyseal cartilage samples harvested at E12.5 to P70. AT-qPCR analysis showed that the *Col2a1* IIA splice form was predominant at E12.5, and the IIB splice form was more abundant in late embryonic and postnatal murine epiphyseal cartilage as expected. The AT-qPCR values for IIA and IIB splice forms in the E12.5, E16.5 and P0 samples were similar to values obtained for whole mouse embryos from E9.5 to E17.5 using a ribonuclease protection assay (Ng et al., 1993). We observed an apparent increase in both the IIA and IIB forms at P70, consistent with earlier data from our lab (Lewis et al., 2012). The maximum abundance of *Col2a1* transcripts, normalized to 18S rRNA occurred at P7.

We have also constructed an MAS plasmid that is specific for human *COL2A1* splice forms. Using this construct, we have recently published an analysis of *COL2A1* splice forms in day 28 human MSC aggregate cultures (Ravindran et al., 2011). The IIB splice form was the predominant splice form in differentiating MSC aggregates, as we have previously published from our laboratories (McAlinden et al., 2008) using an earlier version of this assay based on separately cloned standards.

In ATDC5 cultures we found that the apparent copy number of *Col2a1* mRNA transcripts determined using the exon 52-53 assay was greater (~76 to 145-fold) than the total of the IIA, IIB and IID splice forms. This suggests the interesting possibility that there may be additional transcripts of *Col2a1* mRNA expressed in these cultures that are not detected by the IIA, IIB, or IID assay. Mouse cartilage mRNA also contained exon 52-53 junction-containing mRNA that was in excess of the sum of *Col2a1* splice forms, but was significantly less abundant (~3 to 9-fold) than in ATDC5 cultures. This difference may be related to the embryonal carcinoma origin of the ATDC5 cell line. Further study to determine the nature of the additional *Col2a1* transcripts may help to resolve this issue. Analysis of the *Col2a1* gene for alternative promoter sequences with FPRM (<http://linux1.softberry.com/berry.phtml>) revealed 12 potential alternative promoter sites, including a TATA box (TATAAAGA) within exon 2, which could also contribute to transcript diversity.

The PCR co-amplification approach has been frequently used to estimate ratios of alternative splice forms. Using this technique, PCR is performed using primers that span an alternative exon, followed by analysis of the relative amounts of different-sized PCR products containing or lacking the exon of interest. While this technique may be useful for initial identification of other alternatively spliced transcripts, a major drawback to this approach is that the PCR products are unlikely to be amplified at the same efficiency, with the result that the ratio of the products inaccurately reflects the ratios of starting amplicons. Furthermore, the more efficiently amplified product can compete more effectively for primers, polymerase and dNTPs to further alter ratios of products. Co-amplification of alternative splice forms using the same primer set is similar in effect to competitive PCR (Becker-Andre and Hahlbrock, 1989; Gilliland et al., 1990) in which competing templates having the same amplification efficiency mutually inhibit amplification of one another until an equivalency point is reached.

We performed an experiment to directly compare the AT-qPCR method with end point co-amplification PCR. Using primers from a previous study of ATDC5 differentiation (Hatano et al., 2002) which amplify fragments containing or lacking exon 2, we found that the IIA and IIB PCR products were not amplified with the same efficiency. To see if the efficiency difference could result in over-estimation of the IIB splice form, we calculated the expected quantity of each product following amplification using our AT-qPCR values for starting quantities of IIA and IIB, and the amplification efficiency of the co-amplification products. Our calculation indicated that after co-amplification the ratios of IIB/IIA PCR products would be higher than ratios obtained by AT-qPCR. This was, in fact, the observed result. It is possible that any previous analysis of splice form ratios determined using co-amplification may be subject to a similar error. In addition to the efficiency mismatch problem, it was previously reported (Mathieu-Daude et al., 1996) that the rate of amplification of abundant PCR products declines more rapidly than that of less abundant products amplified in the same reaction, due to rehybridization of PCR products that interfere with primer annealing or extension. This phenomenon, termed the “C₀T” effect results in a progressive increase in the apparent abundance of the minor product. Either, or both of these mechanisms may explain the difference between the AT-qPCR result and the co-amplification result.

As we have shown here, the AT-qPCR assay can be a valuable tool in studies of gene expression during cellular differentiation. The assay method we have developed could be useful when the interrogation of an interesting genomic subset may be more informative and practical than large-scale microarray screening. Using recently developed microfluidics-based PCR platforms, the AT-qPCR assay could be scaled up to a high throughput analysis. It has been estimated that over 60% of disease-causing mutations may affect splicing (Lopez-Bigas et al., 2005). Although the clinical significance of splicing is only beginning to be realized, there are numerous potential diagnostic applications of AT-qPCR. To name a few, splice variants of many cancer-associated proteins have been recognized as potential biomarkers (Brinkman, 2004). A rapid AT-qPCR assay for informative alternative splice forms could expedite diagnosis and influence treatment strategy. Aberrant alternative splicing may underlie certain neurodegenerative disorders (Dalski et al., 2000) including Alzheimer’s disease (Connell et al., 2005). Splice site mutations of the CFTR gene may relate to the phenotypic expression of cystic fibrosis (Noone and Knowles, 2001).

Annotated and cDNA supported sequence data for alternative splice variants is available through the NCBI AceView database (Thierry-Mieg and Thierry-Mieg, 2006) (www.ncbi.nlm.nih.gov/IEB/Research/Acembly). Martelli et al. (Martelli et al., 2010) have recently described a database resource called ASPicDB (Alternative Splicing Prediction Data Base; <http://t.caspar.it/ASPicDB/>). These and other database resources could enable the pre-design of multiple amplicon standards for human genes, providing a new tool to study the diversity of the human transcriptome, and the interrogation of tissue-specific mRNA populations for patterns of splicing having diagnostic or prognostic significance.

4. Experimental Procedures

4.1. Cell culture

Early passage ATDC5 cells were provided by Dr. Brian Johnstone (currently at Oregon Health & Science University, Portland, OR), and were cultured as previously described (Shukunami et al., 1996). ATDC5 cells were plated at 1.2×10^5 cells/35mm dish (6 well plates) in DMEM: Ham’s F-12 (1:1) containing 5% FBS, 10 μ g/ml human transferrin (Sigma Aldrich) and 3×10^{-8} M sodium selenite (Sigma Aldrich). After 7 days of culture, the medium was supplemented with 10 μ g/ml bovine insulin (Sigma Aldrich) to induce chondrogenesis for 30 days. Cells were maintained at 37° C in a humidified atmosphere of 5% CO₂ for the entire culture period, and medium was changed every two days. Cells were

harvested at 2 day intervals, lysed in Trizol reagent and frozen at -80°C . Two wells in each plate were fixed with methanol for 2 min. and stained overnight with 0.1% Alcian Blue in 0.1N HCl, rinsed with PBS, and imaged on a color flatbed scanner. RNA was prepared from cultures using a combination of the Trizol and Qiagen RNeasy kit method, and cDNA was synthesized using random primers and Superscript II reverse transcriptase (Invitrogen) from a 2 μg aliquot from each time point.

4.2 RNA preparation from murine cartilage

Hind limbs were harvested from wild type C57BL/6 litters collected at various time points of development (E16.5, P0, P7, P14, P28, P70) and epiphyseal cartilage was dissected. At E12.5, the whole embryo was harvested since hind limbs were not developed enough to permit accurate dissection. RNA was extracted from all tissues using Trizol reagent (Invitrogen) following the manufacturer's instructions and finally dissolved in 30–40 μl of RNase free water and quantified by A_{260} measurement with a Nanodrop spectrophotometer.

4.3. Immunofluorescent staining of ATDC5 cell layer

An ATDC5 culture following 30 days of insulin induction was fixed with 4% paraformaldehyde while attached to the culture dish, detached from the surface, embedded in paraffin and sectioned (10 μm) onto glass slides. Sections were then de-paraffinized in xylene, rehydrated through decreasing grades of ethanol and treated with 1% hyaluronidase (Sigma Aldrich) for 30 min at 37°C . Sections were rinsed with PBS, blocked with 10% goat serum for 1 hr at room temperature and then incubated overnight at 4°C with rabbit polyclonal primary antibody (1/300 dilution in 2% goat serum) that recognizes the exon 2-encoded domain of type IIA/IID procollagen (gift from Dr. Linda Sandell) (Oganesian et al., 1997) or an antibody recognizing collagen type I. Following PBS washes, sections were then incubated with goat anti-rabbit antibodies conjugated to Alexa-488 dye (1/250 diluted in 2% goat serum) (Invitrogen) for 45 min at room temperature. Following PBS washes, slides were mounted with DAPI-containing media. Negative controls without addition of primary antibody were also carried out. Images were viewed on a Nikon Eclipse E800 fluorescence microscope. The FITC band pass filter set was used to view Alexa 488-stained tissue and the DAPI filter set was used for viewing cell nuclei.

4.4. Alternative Transcript-Quantitative PCR (AT-qPCR)

A method to quantify absolute levels of each alternatively-spliced mRNA isoform of the human *COL2A1* gene (IIA, IIB, IIC and IID) has been previously described (McAlinden et al., 2008). A modified and improved method for analysis of mouse *Col2a1* splice forms was used in the present study. Custom TaqMan® assays (Applied Biosystems™ by Life Technologies Corp., Carlsbad, CA) were designed using Primer Express® software (Applied Biosystems) to specifically anneal on either side of each mRNA isoform splice site. FAM™/MGB probe oligonucleotides that specifically anneal to exon-exon junctions corresponding to each *Col2a1* mRNA splice form, were also designed (Table 1). A chemically synthesized DNA insert containing all of the relevant primer and probe binding sites for each splice form (referred to as a multi-amplicon standard, MAS) was cloned into pUC57 (GenScript USA Inc., Piscataway, NJ). The mouse $\alpha 1(\text{II})$ MAS plasmid also contained an 18S rRNA amplicon for normalization of each sample, and an amplicon spanning the presumed constitutive exon 52-53 junction for determination of all possible *Col2a1* transcripts. The MAS was used to generate standard curves for absolute quantification of each collagen splice form, 18S rRNA, and exon 52-53. A ten-fold dilution series of the MAS plasmid, spanning from 3×10^8 to 3×10^4 copies per 2.5 μL sample was prepared and the same dilution series was used in each of the six AT-qPCR assays (IIA, IIB, IIC, IID, 18S rRNA and exon 52-53). These serially diluted standards and samples were used to calculate the amplification efficiency of each reaction. Aliquots of cDNA were

diluted to provide sufficient volume for analysis of each of the six assays in triplicate. Samples for 18S rRNA quantification were diluted 1/5000 to stay within the linear range of the MAS standard dilution series. Real time quantitative PCR was performed using a StepOne Real-Time PCR System (Applied Biosystems). Values were adjusted for differences in amplification efficiency between the MAS amplicons and cDNA amplicons, and normalized based upon the 18S rRNA value. The AT-qPCR protocol and amplification efficiency adjustment are described in detail in Supplementary Materials. Essential aspects of the assay are also briefly described in Section 2.2.

4.5. Analysis of PCR product length

Representative samples of MAS and ATDC5 PCR products were taken from 96-well PCR plates following 40 cycles of amplification, and electrophoresed in a 5% NuSieve 3-1 agarose horizontal gel in 1X TBE buffer containing 5 ml GelStar® nucleic acid gel stain (Lonza Rockland Inc.) per 50 ml agarose. Gels were visualized under UV illumination on a KODAK Gel Logic 100 Imaging System.

4.6. Comparison of the rt-PCR co-amplification method and the AT-qPCR method

Co-amplification based, semi-quantitative *Col2a1* splice form analysis was carried out on the same samples used for AT-qPCR analysis using a previously published (Hatano et al., 2002) exon 1-7 primer set. PCR products were ligated into the pCRII-Topo vector (Invitrogen), and clones were isolated containing the IIA and IIB splice forms. The identity of these clones was verified by DNA sequencing. Serial tenfold dilutions of the IIA and IIB cloned cDNAs were used to determine PCR efficiency of each amplicon by SYBR®-Green qPCR analysis using the same primers that generated the original PCR products. Assuming that the AT-qPCR values for the IIA and IIB form calculated in our assays were correct, we used the calculated PCR efficiency of the IIA and IIB exon 1-7 amplicons to predict the relative amounts of the IIA and IIB PCR products after 30 and 35 cycles. The detailed methods for this analysis are described in Supplementary Materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AT-qPCR	alternative transcript-quantitative real-time polymerase chain reaction
MAS	multiple amplicon standard
MSC	mesenchymal stem cells
PTC	premature termination codon
qPCR	quantitative real-time PCR
rtPCR	reverse transcriptase polymerase chain reaction

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Highlights

- We have developed a novel 5' nuclease (TaqMan®) assay (AT-qPCR) to quantify mRNA alternative transcripts.
- AT-qPCR was used to quantify *Col2a1* splice forms during chondrogenesis in ATDC5 cells and mouse cartilage development.
- The IIA and IID splice forms persisted in ATDC5 cultures, while mouse cartilage showed progression from IIA to IIB.
- Total *Col2a1* mRNA exceeds the sum of the IIA, IIB and IID splice forms in ATDC5 cells and developing mouse cartilage.
- AT-qPCR can precisely determine splice form ratios for any gene of interest, and may have clinical diagnostic applications.

Fig. 1.

Alternative splice forms of *Col2a1* mRNA. The IIA splice form contains the 204 nucleotide exon 2A that is absent from the IIB splice form. The IIC splice form contains only the first 34 nucleotides of exon 2, which disrupts the reading frame and introduces three premature termination codons (PTC) within exon 6. The IID splice form contains an additional 3 nucleotides (GTC in the mouse) at the 3' end of exon 2, resulting in the insertion of an additional codon (arginine) at the end of translated exon 2. Bars indicate location of amplicons for AT-qPCR.

Fig. 2. ATDC5 chondrogenic differentiation. (A) Alcian Blue-stained chondrogenic nodules imaged at 4 to 28 days of culture in differentiation medium. Scale bar, 10 mm. (B,D,F) Sections through ATDC5 nodules (B, D) and flat portion of cell layer (F) at 30 days; immunofluorescent staining with an antibody recognizing the exon 2A and 2D domains of procollagen $\alpha 1(\text{II})$. Scale bars, 100 μm . (C,E,G) Adjacent sections were stained with an antibody to collagen type I.

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18S rRNA (F)      18S rRNA (P)      18S rRNA (R)
TTCGTATTGCGCCGCTAGATGAAATTCCTTGACCGGC GAAGACGGACCAGAGCGAAAGG

Total Col2a1 (F)   Total Col2a1 (P)   Total Col2a1 (R)
GTGGCTTCCACTTCAGCTATGGCACCGCTAACGTCCAGATTTCTCTCCGTCTACTGTCCACTG
G

IIC(F)
CGCGGTCTCTACGGTGTCA
IIA (F)           IIB (F)           IID (F)           IIA (P)
GAGAGTGCTGTCCCATCTGCGCGTCTACGGTGTCAAGCTGACCTCGCCACTGACTGCCAGT
G

IIA(R)
CCAAAGGGGCAGAAAGGAGAA
IIB (R)
CCAAAGGGGCAGAAAGGAGAA
IIB (P)           IID (P)           IID (R)
GGAAAAATGCCCGAAAATTAGCAGTGGTCGAAAATTACCAAAGGGGCAGAAAGGAGAA

IIC (P)           IIC (R)
CAGGAGGCTGGCAGCAATGGGCAGAGGAAAATTAGGG

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Fig. 3.

Sequence and organization of the *Mus musculus Col2a1* MAS plasmid insert sequence. The DNA plasmid (pUC57) contains a chemically synthesized multi-amplicon standard (MAS) cDNA insert representing the different splice forms of interest. TaqMan® forward (F) and reverse (R) PCR primer sites (smaller font), and a fluorescent dye-labeled oligonucleotide probe site (P) (larger font) to amplify each splice form are shown. The insert contains amplicons for the IIA (green), IIB (black), IIC (blue) and IID (purple) splice forms, respectively. The insert also contains an 18S rRNA amplicon (gray) for normalization, and an amplicon spanning exon 52-53 of *Col2a1* (red) to measure total *Col2a1* levels.

Fig. 4. Agarose gel analysis of murine *Col2a1* MAS PCR products. PCR products amplified from the MAS insert and ATDC5 cDNA are shown. Left lane contains a 20 bp DNA ladder. Products were of the expected size (Table 1).

Fig. 5. AT-qPCR analysis of *Col2a1* alternative splice forms during ATDC5 differentiation. (A) Comparison of quantification of IIA, IIB, and IID splice forms, normalized to 18S rRNA. The Y axes are scaled differently in each panel. (B) Evidence for additional *Col2a1* alternative transcripts; stacked bar graph shows the relative contributions of the IIA, IIB, and IID splice forms to the sum of all four splice forms (left Y axis) compared to exon 52-53-containing transcripts (right Y axis). (C) Changes in the abundance of IIA, IIB, IIC and IID alternative splice forms of *Col2a1* mRNA plotted as percentage of the total of IIA, IIB, IIC and IID splice forms; the Y axis was split, since the AT-qPCR technique permits measurement of alternative splice forms over multiple orders of magnitude.

Fig. 6. AT-qPCR analysis of *Col2a1* alternative splice forms in wild type mouse epiphyseal cartilage at different time points of development. Stacked bar graph shows contribution of IIA and IIB splice forms of *Col2a1* to their sum (left Y axis), compared to exon 52-53-containing transcripts (right Y axis) at the indicated stages of embryonic (E) development or postnatal (P) development. Note differences in scale of left and right Y axes.

Fig. 7.

Co-amplification error due to mismatch in amplification efficiency of IIA and IIB splice forms using exon 1 and exon 7 PCR primers. (A) Co-amplification of cDNA from ATDC5 cultures at day 2, 10, 18 and 26 (lanes 1–4). (B) Cloned murine IIA and IIB PCR products were used to calculate amplification efficiency by performing separate real-time PCR reactions with SYBR®-Green detection on dilutions of the cloned templates. Log10 dilutions of the templates were plotted against the C_T . Using the actual initial quantities of the IIA and IIB splice forms determined by AT-qPCR, and the amplification efficiency calculated from the standard curve slopes, the IIB/IIA ratios at 30 and 35 cycles using the co-amplification method were predicted. The predicted IIB/IIA ratios are similar to the ratios of IIB/IIA on the gel, and differ significantly from the actual ratios calculated from the AT-qPCR data.

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Comparison of Col2a1 amplicons in MmCol2a1_MAS and murine cDNA

Table 1

Amplicon	Template	Length	Sequence
Ex52-53	MmCol2a1_MAS	63	<i>GTGGCTTCAC</i> <i>TTTC</i> <u><i>TC</i></u> <i>TC</i> <i>CGTCTACTGT</i> <i>TC</i> <i>CACTG</i>
	murine cDNA	87	<i>GTGGCTTC</i> <i>CACTTT</i> <i>CAGCTATGGCGATGGCAAC</i> <u><i>CTGGCTCCCAAC</i></u> <i>ACCGCTAACGTCTCCAGATGAC</i> <i>TTTCC</i> <i>TCCGTC</i> <i>TACTGT</i> <i>CCACTG</i>
18S	MmCol2a1_MAS	59	<i>TTCCGTA</i> <i>TTGGCGCCGCTAGATGAAAT</i> <i>CTTGGACCGGCGAAGACGGAC</i> <i>CCAGAGCGGAAAGG</i>
	murine cDNA	61	<i>TTCCGTA</i> <i>TTGGCGCCGCTAGAGGTGAAAAT</i> <i>CTTTGGACCCGGCGCAAGAC</i> <i>CGGAC</i> <i>CAGAGCGGAAAG</i>
IIA	MmCol2a1_MAS	122	<i>GGAGAGT</i> <i>GCTGTC</i> <i>CCATCTG</i> <i>CGCGGTCT</i> <i>ACGGTGTCAA</i> <u><i>GCTGACCTCGCCACTG</i></u> <i>ACTGCCAGTGA</i> <i>AAATG</i> <i>CCCGAAAAT</i> <u><i>TAGCCAGTGGAAAAAT</i></u> <i>TAGCACTGTGTCGAAAAATT</i> <i>ACCAAGGGGC</i> <i>AGAAAGGAGAA</i>
	murine cDNA	78	<i>GGAGAGT</i> <i>GCTGTC</i> <i>CCATCTG</i> <i>CCCCAGCTGACCTCGCCACTGCCAGTG/GAAAAA</i> <i>TTAGGGCCAAAGGGGGCAGAAAAAGGAGAA</i>
IIB	MmCol2a1_MAS	101	<i>GCGGTCTAC</i> <i>GGTGTCAA</i> <u><i>AGCTGACCTCGCCACTGACTGCACTGCCAGTGGAAAAAT</i></u> <i>TAGCCGAAAAAT</i> <u><i>TAGCCGAAAAAT</i></u> <i>TAGCACTGTGTCGAAAAATT</i> <i>ACCAAGGGGC</i> <i>AGAAAGGAGAA</i>
	murine cDNA	63	<i>GCGGTCTAC</i> <i>GGTGTCAA</i> <u><i>GGGCCAGGATGCCCG/GAAAAA</i></u> <i>TTAGGGCCAAAGGGGCAGAAAAAGGAGAA</i>
IIC	MmCol2a1_MAS	139	<i>CGCGGTCTAC</i> <i>GGTGTCAA</i> <u><i>AGCTGACCTCGCCACTGACTGCCAGTGGAAAAAT</i></u> <i>TGCCGAAAAAT</i> <u><i>TAGCCAGTGGTCTGAAAAATT</i></u> <i>TAGCACTGTGTCGAAAAATT</i> <i>ACCAAGGGGCAGAAAGGAGAA</i> <i>TGGGCAGAGGAAAAATTAGGG</i>
	murine cDNA	77	<i>CGCGGTCTAC</i> <i>GGTGTCAA</i> <u><i>GGGCCAGGATGCC/CAGGAGGCTGGC/AGCTGTCTGCAGAA</i></u> <i>TGGGCAGAG/GAAAAA</i> <i>TTAGGG</i>
IID	MmCol2a1_MAS	84	<i>AGCTGACCTCGCCACTGACT</i> <i>GGCCAGTGGAAAAAT</i> <u><i>TGCCGAAAAATTAC</i></u> <i>CAGCTGGTCTGAAAAATT</i> <i>ACCAAGGGGCAGAAAGGAGAA</i>
	murine cDNA	58	<i>AGCTGACCTCGCCACTG</i> <i>CCAGTGGTC/GAAAAA</i> <i>TTAGGGCCAAAGGGGCAGAAAGGAGAA</i>

Boldface italic indicates forward and reverse PCR primer binding sites. Underlined text indicates probe binding site. Slash indicates exon/exon junction in TaqMan® probe site or PCR primer site. Amplicon length is indicated in bp.