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ABSTRACT

Background: Chordoma are malignant tumors of the axial skeleton, which arise from remnants of the notochord. The Notochord (chorda dorsalis) is an essential embryonic structure involved in the development of the nervous system and axial skeleton. Therefore, the notochord seems to be the most biologically relevant control tissue to study chordoma in molecular biology research. Nevertheless, up to now mainly different tissues but not the notochord have been used as control for chordoma, due to difficulty of isolating notochordal tissue. Here, we describe a fast and precise method of isolating notochordal cells.

Methods: Examination of human fetuses, with a gestation of 9, 11 and 13 weeks, using (immuno) histochemical methods was performed. To isolate pure notochord cells for further molecular biology investigation five flash frozen fetuses between 9 and 10 weeks of gestation were dissected by microtome slicing. Thereafter pure notochord cells for further molecular biology investigation where harvested by using laser capture microdissection (LCM). RNA was extracted from these samples and used in quantitative PCR.

Results: This study illustrates notochord of embryonic spines in three different stages of gestation (9–11–13 weeks). Immunohistochemical staining with brachyury showed strong staining of the notochord, but also weak staining of the intervertebral disc and vertebral body. LCM of notochord slices and subsequent total RNA extraction resulted in a good yield of total RNA. qPCR analysis of two housekeeping genes confirmed the quality of the RNA.

Conclusion: LCM is a fast and precise method to isolate notochord and the quality and yield RNA extracted from this tissue is sufficient for qPCR analysis. Therefore early embryo notochord isolated by LCM is suggested to be the gold standard for future research in chordoma development, classification and diagnosis.

1. Introduction

Chordoma are malignant tumors of the axial skeleton, which occur most frequently at the skull base and the sacrum (McMaster et al., 2001). Complete removal of the tumor is often a challenging and not always possible mainly at the skull base. A 5 year recurrence is reported in up to 49.7% of all cases (Colli and Al-Mefty, 2001). Chordoma are thought to arise from remnants of the embryonic notochord (Yamaguchi et al., 2008; Vujovic et al., 2006). These remnants are destined to remain in the spine throughout life and can become malignant at any age, with a median between 53 and 58.5 years (McMaster et al., 2001; Noel et al., 2005; Lee et al., 2012). Little is known about the underlying mechanisms that orchestrate the switch from benign notochord cell to malignant chordoma. To study these mechanisms in detail, molecular and cellular analysis of chordoma tissue is needed. Notochordal tissue seems to be a reliable control for this purpose. However, up to now most studies in chordoma do not use the notochord as a control tissue for various reasons. Either it was not available (Rinner et al., 2013), or other types of tissue were used such as nucleus pulposus (Schwab et al., 2009; Bayrak et al., 2013; Ji et al., 2010; Aydemir et al., 2012; Zou et al., 2014), which might contain notochordal cells. If nucleus pulposus is used, it is important to use only the tissue of young patients as throughout age there is a transition

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from a notochordal cell rich tissue to that of more fibrotic and more populated by nucleus pulposus cells (also defined as chondrocyte-like cells, or nucleus pulpocytes) (McCann and Seguin, 2016). Throughout age, it is shown that the intervertebral disc cells do not show notochord-like cells after the age of 8 years, and notochordal cell markers disappear almost completely after 25 years (Weiler et al., 2010).

Other studies use muscle tissue (Duan et al., 2010), osteoblast cells (Duan et al., 2014), blood (Rinner et al., 2013) or other tumors like chondrosarcoma (Vujovic et al., 2006). Therefore it is essential to develop a feasible method how to isolate highly purified notochord cells for further basic investigations.

The human notochord, also called chorda dorsalis, is an embryologic structure that arises from the bilaminar disc in the third week of the gestation. The most important functions of the notochord are the patterning of the surrounding ectoderm, mesoderm and endoderm (Cleaver and Krieg, 2001), as well as the induction of formation of different tissues (neuroectoderm, dermatotome, myotome, sclerotome) (Stemple, 2005). In the early stages, the notochord is a rod-shaped structure, positioned where the axial skeleton will form later on. During embryogenesis, most notochordal cells are replaced by bone in the vertebral body (Hunter et al., 2004). Notochordal cells in the intervertebral discs are considered to be the precursor of nucleus pulposus cells (McCann and Seguin, 2016). After 8 weeks of gestation, the notochord involutes into clusters in the intervertebral disc, which are connected by an acellular sheath (Pazzaglia et al., 1989). After 26 weeks of gestation, the notochord becomes less distinct from the intervertebral disc (Pazzaglia et al., 1989). Only few studies have been conducted to visualize the notochord in 8–13 weeks of gestation foetuses (Pazzaglia et al., 1989; Babic, 1991). To better understand the anatomy of the human notochord in the tissue that is provided to us, and to establish which age of gestation is best to be used for dissection, the first part of this study focuses on the notochord from human foetuses of 9–13 weeks of gestation.

Since the notochord clusters are microscopically small and completely surrounded by mesodermal tissue, it is impossible to dissect this macroscopically without significant contamination with the surrounding tissue. So far, no study described an adequate way to dissect pure human notochordal tissue. The primary aim of this study was to establish an easy-to-use protocol to isolate notochord, which can be used as a gold standard for collecting control tissue in chordoma research. Our secondary aim was to investigate whether the quality of the extracted RNA from this tissue is sufficient for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis. QRT-PCR is a powerful tool to provide quantitative measurements of gene transcription, and is commonly used in tumor biology. Here, we used laser capture microdissection to isolate the notochord from five foetuses between 9 and 10 weeks of gestation. RNA was extracted from this tissue and used in a qRT-PCR.

2. Materials and methods

2.1. Ethical considerations

For this study, spines from aborted fetuses were used. Prior to the study, an approval from the Medical Ethics committee of Maastricht University Medical Center (MUMC, Maastricht, The Netherlands) was received (METC 13-4-043). Patients who underwent an abortion for known non-psychological medical reasons were excluded from this study, as this could affect the morphology and gene expression of the notochord. The procedure was performed in an abortion clinic in Maastricht (Centrum voor Anticonceptie, Seksualiteit en Abortus (CASA), Maastricht, The Netherlands). All patients were given enough time to consider signing a written informed consent and could withdraw their initial approval until just after the procedure. The abortion procedure, performed by the medical doctors in the abortion clinic, was exactly the same for patients that were included in the study as the patients that did not sign the informed consent, with

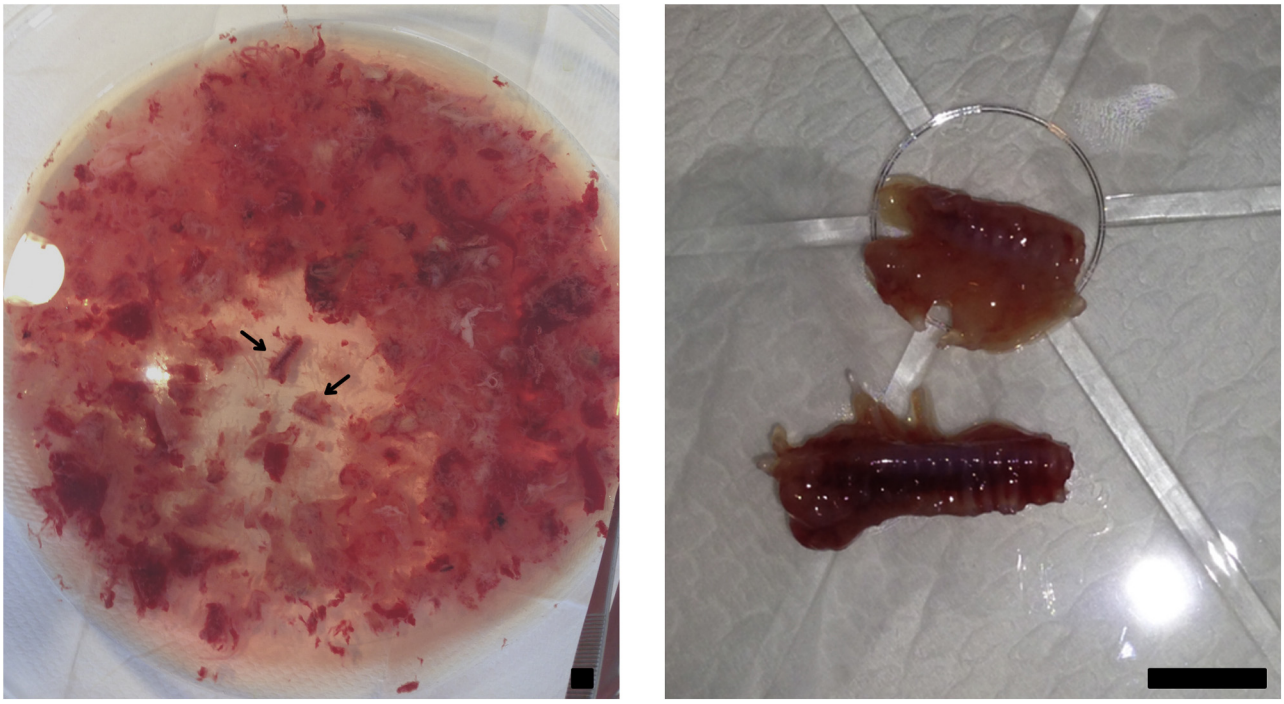

Fig. 1. A: Aborted material in a glass tray. This material was extracted with suction aspiration. Most organs and limbs can be identified in this tray aside to placental material. The spine is visible in two pieces in the middle of the tray (arrows). B: Picture of the two pieces of the spine that was found in the material after the abortion procedure. The scale bars represent 1 cm.

the only difference being that the fetal spine was preserved from the aborted material of included patients. These patients were asked to fill in a questionnaire about lifestyle, medication use, and hereditary diseases, to minimize the possibility of an abnormal foetus to be included in this study. The data and tissue were stored and processed anonymously and could not be lead back to the patient by anyone.

2.2. Notochord visualization and immunohistochemistry

For determining the best age of embryo to be used, three aborted foetuses of 9, 11, and 13 weeks of gestation were collected at the abortion clinic. Immediately after the abortion procedure, the aborted material was washed in phosphate buffered saline (PBS) and transferred in a glass tray. From this material, the spine could usually be found in one or two pieces (Fig. 1). The spines were fixed in 3.7% formalin for 48 hours, paraffin embedded by standard procedure, and cut on a microtome (Leica RM 2245, Nussloch, Germany). Five-micrometer sections were cut from paraffin-embedded samples, dewaxed in xylene and rehydrated. Histological staining with haematoxylin and eosin was performed. Some adjacent sections were immunohistochemically stained with an anti-brachyury antibody, to confirm the notochord-identity of tissue (Vujovic et al., 2006). In these slides, antigen-retrieval was performed in 0.01 M sodium citrate (pH = 6.0) in a 99.9 °C water bath for 15 min. The endogenous peroxidase activity was inhibited with 0.3% H_2O_2 in Tris-buffered-Saline with Triton (TBS-T) and blocking of nonspecific binding with 3% normal donkey serum in TBS-T for 30 min at 37 °C. The sections were incubated overnight at 5 °C with primary anti-brachyury antibody (Santa Cruz, CA, USA) diluted 1:100 in TBS-T. After washing three times in TBS-T an incubation of one hour with secondary antibody (Donkey-anti-rabbit; Jackson Immunoresearch Laboratories, West Grove, PA, USA), diluted 1:400 in TBS-T was performed. The signal was amplified with avidin-biotin complex. To visualize the horseradish peroxide reaction product, the sections were incubated with 3,3′-diaminobenzidine tetrahydrochloride with nickel chloride enhancement after further washing. The specificity of the primary antibody was tested with chordoma samples as positive control tissue, and chondrosarcoma and vestibular schwannoma samples as negative control tissue. Also, immunohistochemical reactions with chordoma samples without primary antibody were used as negative control.

2.3. Notochord isolation

Five spines of aborted fetuses with a gestation between 9 and 10 weeks were flash frozen in liquid nitrogen at the abortion clinic, immediately after the abortion procedure. The gestational age was estimated by measuring the fetal crown-rump length, because most patients did not know the first day of their last menstruation. Ultrasound alone has been proven to be even more accurate than a "certain" menstrual date for determining gestational age in the first and second trimesters (≤23 weeks) in spontaneous conceptions (Butt and Lim, 2014). The gestational age was quantified as the duration of the pregnancy since the day of the fertilisation. The tissue was stored at −80 °C. Pieces of the spine was fixed in Tissue Tek FSC22. (Leica biosystems, Maarn, the Netherlands; #3801480) Sagittal, 14 μm sections of the fetal spine were cut on a cryostat (Leica Microsystems; CM3050S), mounted on normal glass slides (Superfrost Plus microscope slides, WVR international, Leuven, Belgium; #631-0108) and stained with toluidine blue (TB), to determine the position of the section and to check for notochord clusters. At first, ten sections were mounted and stained with TB. On these sections, vertebrae and intervertebral discs were visible. When approaching the centre of the spine, every time, two sections were made and stained with TB, until the notochord was visible. The next ten sections were mounted on a RNase free glass PEN membrane slide (Leica Microsystems, The Netherlands; #11505189), which is suitable for laser capture microdissection (LCM). Subsequently, one section was mounted on a normal glass slide, and stained with TB. If there were still notochordal clusters visible, the next ten sections were mounted on one RNase free glass PEN membrane slide. Again, one section was cut and mounted on a normal glass slide, and stained with TB. This continued until there were no more notochordal clusters visible. The RNase free glass PEN membrane slides containing the notochord sections where kept at −20 °C all the time and stored for a maximum of 4 weeks at −80 °C till LCM.

The slides were transported on dry ice to the laser microdissection system. (Leica LMD7000). This system is a contact- and contamination-free method for isolating small areas of tissue from glass slides. Prior to the dissection, the slides were stained with 0.5% toluidine blue, washed twice with DEPC-treated water and once with 70% alcohol in DEPC-treated water. A Leica LMD7000 laser microdissection system was used to dissect the notochord. The dissected tissue was collected in a 0.5 ml Eppendorf tube with 40 μl RLT buffer containing 1% beta-mercaptoethanol from an RNeasy micro plus kit (Qiagen GmbH, Hilden, Germany, #74034). RNA was extracted with the RNeasy micro plus kit according to the manufacturer's instruction. The quantity was tested with a nanodrop ND-1000 spectrophotometer (Isogen Life Science). cDNA conversion was performed with a commercially available cDNA synthesis kit (RevertAid first strand cDNA synthesis kit, Thermo Scientific, #K1622), using oligo $(dT)_{18}$ primers according to the manufacturer's instructions.

2.4. qRT-PCR

Reverse transcriptase quantitative PCR (RT-qPCR) was performed in 96-well plates with a lightcycler 480 Real-Time PCR system (Roche applied science, Rotkreuz, Switzerland, serial 20504). Two housekeeping genes were used (ACTB and YWHAZ). The primer sequence, primer efficiency, and amplicon length are listed in Table 1. One twentieth of the converted cDNA was used per

Table 1
Genes and primers used for quantitative PCR.

Abbr.	Primer	Primer sequences 5' -> 3'	Amplicon length (Bp)	Primer efficiency (%)	Annealing temperature (°C)
ACTB	Beta-Actin	FW: GCACTCTTCCAGCCTTCCTT RV: CGTACAGGTCTTTGCGGATG	106	102	60
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	FW: ACTTGACATTGTGGACATCGGA RV: CAAAAGTTGGAAGGCCGGT	86	103	60

reaction, with 250 nM forward/reverse primer and 1x sensimix (SYBR No-RCX; #SMT-N-314304) in a 20 μl reaction. The reaction conditions were set as: pre-incubation for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 60 °C, and 15 s at 72 °C. Finally, a melting curve was analysed with a ramp rate of 0.29 °C/s. Three chordoma sample were used as a positive controls. 'No template' and 'no reverse-transcriptase' negative controls were used for each primer.

3. Results

Haematoxylin/Eosin staining was performed on three spines of 9, 11, and 13 weeks of gestation (Fig. 2). The dimensions of the spine are significantly increased in this period. The intervertebral disc almost doubles in diameter and the vertebral body shows central ossification from 11 weeks. However, the notochordal dimensions remained more or less the same. The notochordal clusters are connected by an acellular sheath, which is better visible in the earlier stages of the gestation. To further characterize the notochord, an immunohistochemical staining with brachyury was performed (Fig. 2D–F). There was a specific staining of the notochord. Unexpectedly, also the surrounding tissue was weakly stained with this marker. The immunohistochemical staining was validated by positive and negative control tissue.

Fig. 3 shows an example of a slide before and after the removal of the notochord with LCM. All extracted tissue per fetus was pooled and subsequently, total RNA extraction was performed. Nanodrop spectrophotometre analysis measured an average of 99.88 nanogram of total RNA (65.89 ng–171.6 ng; Table 2). qRT-PCR with beta-Actin primers resulted in an average cycle treshold (Ct) value of 27.72 (24.24–34.45), and qRT-PCR with YWHAZ primers resulted in an average Ct value of 28.83 (25.99–33.06). Three chordoma samples were used as a positive control for these genes, with Ct values of beta-actin between 25.03 and 28.50, and YWHAZ between 28.15 and 31.92. Results of individual samples are shown in Table 2. With the exception of notochord sample #3, all samples are considered good enough for qRT-PCR analysis of genes with lower gene expression. The amplification curve and gel electrophoresis of the notochord samples and 1 chordoma sample (Fig. 4) demonstrate that a single product is formed.

4. Discussion

The first part of this study focused on visualizing the notochord of human fetuses from 9 to 13 weeks of gestation to establish which age of gestation is best used for dissection. Sagittal sections of spines from aborted fetuses from 9, 11, and 13 weeks of gestation were made and stained with haematoxylin/eosin. In these stages, the notochord was clustered in the center of each intervertebral disc and connected to each other with an acellular sheath. These results are similar to previous studies (Pazzaglia et al., 1989; Babic, 1991; Cotten et al., 1994). Immunohistochemical staining of these sections with anti-brachyury antibody, identified the clusters of densely stained notochordal cells. However, the surrounding tissue

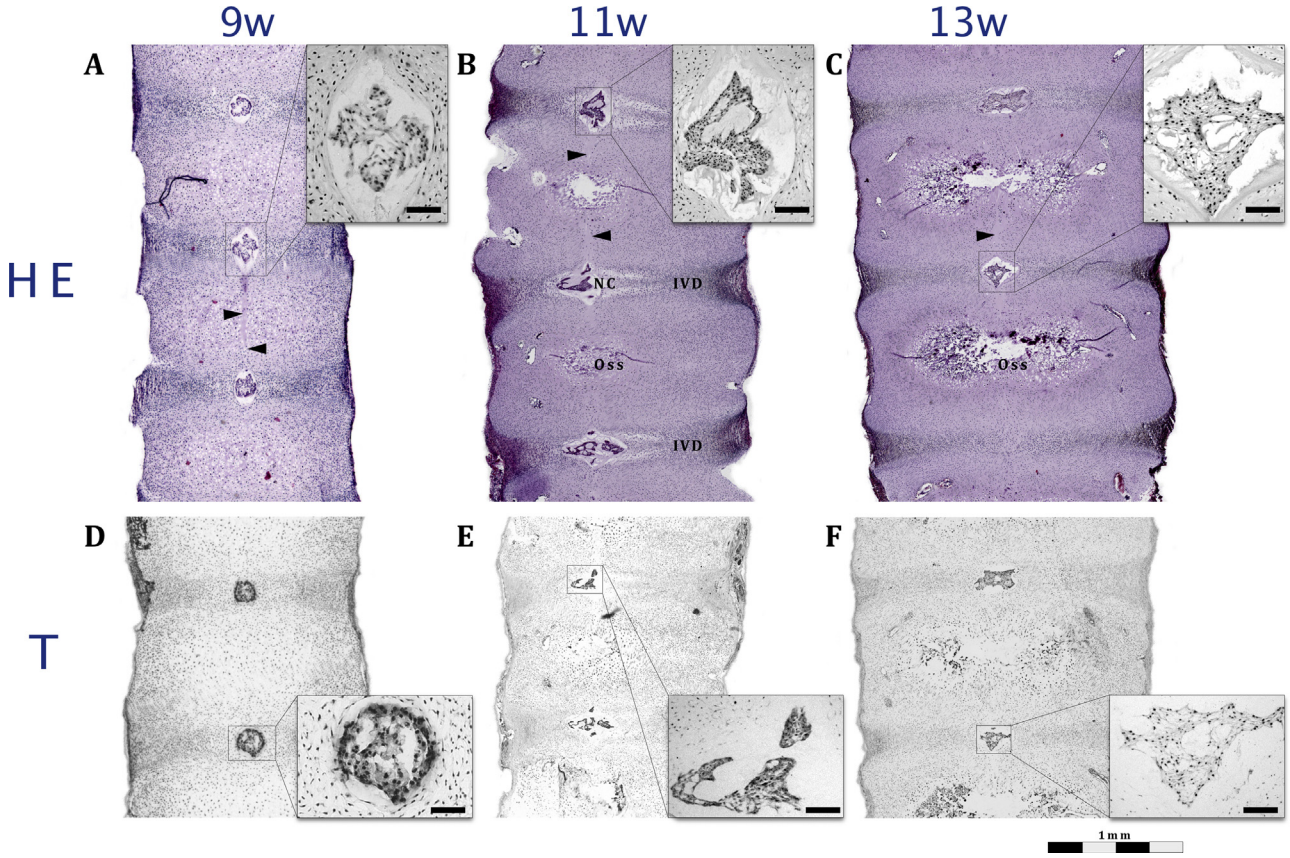

Fig. 2. A–C: Coronal sections of spines of respectively 9, 11 and 13 weeks of gestation. The notochord is located in the centre of the intervertebral disc. Even though the vertebral body and intervertebral disc grow significantly in this gestational period, the volume of the notochord stays more or less the same. The notochordal clusters are connected by an acellular sheath (arrowhead), which is best visible in figure A. In figure B and C, the start of the ossification of the vertebral body is visible. The scale bar represents 1 mm and is the same in all figures, the scale bar in the insets represents 60 μm. D–F: immunohistochemical staining with brachyury (T) of an adjacent section of respectively A, B and C. The nuclei of the notochordal clusters are stained with the highest intensity. However, there is also staining of the intervertebral disc and vertebral body.
Abbr.: NC = notochord; IVD = Intervertebral disc; Oss = Ossification.

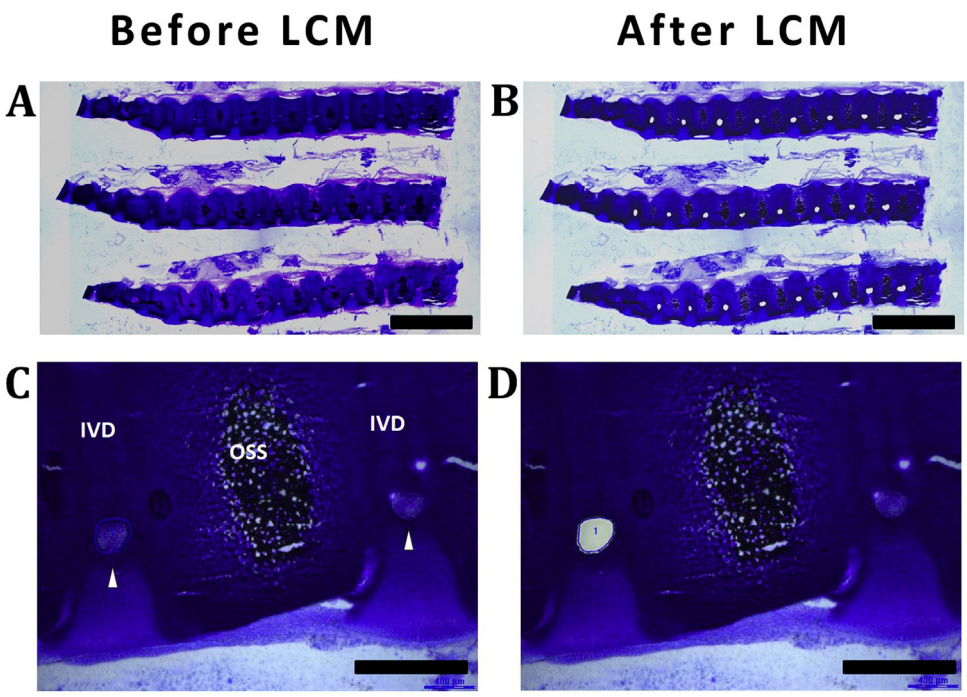

Fig. 3. Toluidine blue staining of a spine before (left) and after (right) LCM. It is clearly visible that nothing but the 8 notochord clusters has been cut out of the sections. The cut-out parts seem a little bit bigger than the notochordal clusters, which is caused by the intensity of the laser, which has a cutting thickness of a few micrometre (D). The scale bars represent 10 mm in the upper figures and 1 mm in the lower figures.
Abbr.: IVD = Intervertebral disc; Oss = Ossification. Arrowhead: notochord.

Table 2
Results of quantitative PCR.

			Nanodrop data				qRT-PCR data	
Sample #	Gestational age (days)	Clusters dissected	ng/ul	260/280	260/230	Total RNA (ng)	Ct value (beta-Actin)	Ct value (YWHAZ)
1	70	7	15,6	1,62	0,51	171,6	28,33	30,76
2	60	11	5,99	1,59	0,64	65,89	26,79	28,25
3	66	14	6,3	1,32	0,65	69,3	34,45	33,06
4	66	18	8,44	1,49	0,76	92,84	24,24	25,99
5	70	18	9,07	1,82	0,64	99,77	24,77	26,07
Average		13,6	9,08	1,568	0,64	99,88	27,72	28,83

was also weakly stained with antibody. This is in contrast to an earlier study (Vujovic et al., 2006), in which only the notochord of embryos of 6–8 weeks of gestation stained positive for this marker, without the surrounding tissue. In the current study, we used the same methods and identical primary antibody as this study. One difference is that our specimens were 2–4 weeks older than in the study by Vujovic et al. (Vujovic et al., 2006). Another study (Shen et al., 2013) immunohistochemically stained fetal spines of 12–40 weeks of gestation with brachyury, with no staining of notochord or surrounding tissue. Possibly, the notochord is only positive for this marker in the early and not in the later stages. Furthermore, this antibody may not be specific enough for brachyury, although there was no staining in all negative control tissue samples.

The other aim of the study was to isolate RNA from notochordal tissue, to use as control tissue for chordoma studies. In our experimental setup, notochord from fetuses of 9–10 weeks of gestation appears to be the best stage for dissection with this technique. Before the age of 9 weeks, the spine is too small to be distinguished within a sufficient time span adequately from other abortion material. This tissue could be obtained by using aborted fetuses that have been extracted by using an abortion pill. However, this tissue is deceased for at least a few hours, which leads to significant gene expression changes. Also, this tissue is usually not brought back to the abortion clinic. Notochord in a later stage (>13 weeks of gestation) is also more difficult to find, due to legal restrictions to gestational age in abortion. Also, in this stage, it is more difficult to distinguish the notochord from nucleus pulposus tissue.

Spines of human fetuses between 9 and 10 weeks of gestation were collected and flash frozen within 20 min after the procedure, which preserved the RNA quality. The biggest strength of this technique is that almost every notochordal cell in the spine, with minimal contamination with surrounding tissue, can be dissected. This improves the quality of the control tissue significantly. The notochord in this stage is clearly distinguishable from the surrounding tissue, so there will be nearly no contamination with other cells. Nanodrop analysis resulted in an average yield of 99.88 nanogram of total RNA. However, the 260/280 and 260/230 ratio's are very poor, which makes these results less reliable. Nonetheless, qRT-PCR resulted in low Ct values in 4 out of 5 cases, which makes this RNA suitable for analysis, also for genes with low expression levels. Because only 10% of the cDNA was used for these reference genes, at least 10 genes can be tested with qRT-PCR per notochordal sample.

This is the first study that describes a method that can be routinely used for dissection of pure fetal notochordal tissue to use as a control for chordoma research. In an earlier study (Duan et al.,

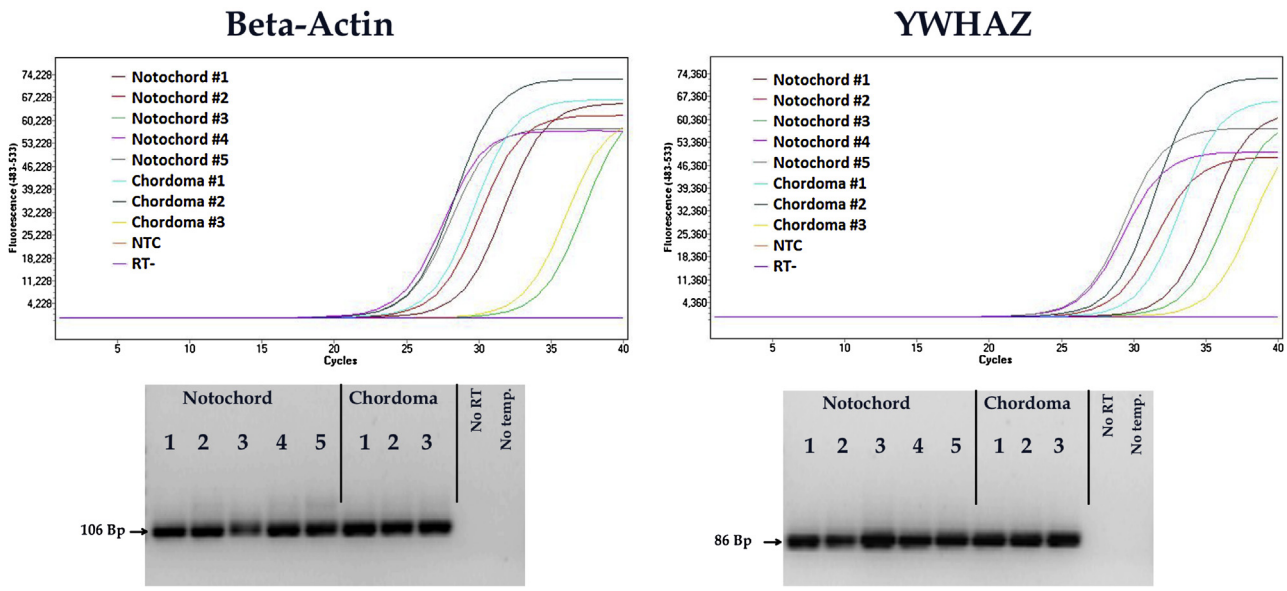

Fig. 4. Linear view of the amplification curve of notochord and chordoma samples in qRT-PCR for beta-actin (left) and YWHAZ (right). All samples show a good curve with a baseline, exponential and plateau phase. Gel electrophoresis of the PCR products of all five notochord samples and three chordoma samples show single bands at the correct height, implicating a single product has formed.

2010) notochord was used as control tissue for miRNA expression analysis, but it was not describe how and in what stage of gestation this tissue was collected. Furthermore the purity of tissue was vague. Another study (Long et al., 2013) used LCM to isolate notochordal tissue from aborted fetuses of 24–27 weeks of gestation. This method cannot be routinely used, as this gestational material is not available in a regular manner. In addition, the time between abortion and freezing of the spine is significantly longer, since the intervertebral disc has to be cut out of an intact foetus. Moreover, every intervertebral disc has to be cut out separately and only few sections can be mounted on one glass, considering the volume of the intervertebral disc. In current study design, one section can contain multiple notochordal clusters and many sections can be mounted on one glass membrane. This does not only reduce costs, it's also easier and less time consuming. Furthermore, this gestational period correlates with a viable human being, which might impede ethical approval. Finally, the notochord disappears with increasing age, and becomes less distinctive from the intervertebral disc (Pazzaglia et al., 1989).

The main purpose of the study was to establish a sound method to isolate control tissue for chordoma research. However, the question of the ideal stage of gestation that can be used as control tissue for chordoma remains unanswered. Even though 9–10 weeks of gestation may be the easiest to dissect with LCM, that this is the best control tissue for gene expression studies in chordoma was impossible to show. Furthermore, not only the stage, but also the location can influence the transcriptome. Possibly, notochordal tissue from the cervical vertebrae can serve as a better control tissue for skull base chordoma, and notochord in the lumbar region for sacral chordoma. Future research should investigate this hypothesis, as this technique could be used to collect all the clusters of the notochord separately. Finally, only one spine per age of gestation was investigated. There may be a variance in notochordal dimensions between fetuses, although we do not expect this to be a significant difference. In our opinion, the quantity and quality of total RNA can be improved in the LCM step. Immediately after the abortion procedure, the tissue is frozen. Also, cutting on the cryostat is in frozen condition. However, during staining with toluidine blue and LCM, the slides are at room temperature, facilitating RNA degradation by RNase. In current study, this period was around 30 min. If this step can be shortened in a way, we believe that the quality and quantity would improve significantly.

Working with aborted material requires ethical consideration. We emphasize that the abortion procedure was not altered in any way when patients were included in the study. The patients were informed of the study, only after they made their final decision to proceed with the abortion, so this decision could not be influenced by the study. The only difference between inclusion/exclusion in the study was that of the aborted material of participants the spine was collected and analysed. The remaining tissue was disposed of by standard human tissue disposal procedures.

We suggest this procedure as a gold standard for collecting control tissue for chordoma studies. Because abortions of fetuses in this stage of gestation can be planned, and the tissue can be frozen immediately after the procedure, this is an excellent method to preserve tissue quality. Because the total size of the fetal spine is relatively small, sections of a foetal spine with at least 7 intervertebral discs (i.e. notochordal clusters) can be cut and mounted on the same glass. LCM is a fast, user friendly, and state-of-the-art technique, which is perfectly suitable for dissection of this kind of tissue. Without an amplification step, RNA isolated from these spines can be used in qRT-PCR. Using the here presented method, also DNA, proteins and miRNA can be extracted.

Since there is insufficient knowledge about the driving factors behind the formation of chordoma from notochord, this high quality control tissue may play a key role in understanding these molecular mechanisms. Understanding these mechanisms can facilitate the development of new targeted treatment and improve the treatment outcome of patients with chordoma.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchemneu.2016.12.004.

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