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Development of an improved protocol to analyse gene expression in temporomandibular joint condylar cartilage of rats using DNA microarrays

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ABSTRACT

Purpose: During recent years, gene expression analyses based on DNA chip technologies have allowed for the genome-wide identification of genes potentially associated with growth processes in a variety of organs. The present study aims to identify genes differentially expressed in the growing temporomandibular joint cartilage by means of transcriptome analyses.

Material and Methods: In total, the condylar cartilage of 32 rats comprising 4 age groups (newborn, 10 days, 21 days, 8 weeks) were used for analysis. Transcriptome analyses were carried out using Affymetrix Expression Arrays (Rat Genome 230 2.0 Arrays). The availability of high-quality RNA preparations from homogeneous tissue samples is a fundamental precondition of successful transcriptome analyses using DNA arrays. An optimised preparation protocol allowed RNA isolation of sufficient quality which was validated using capillary electrophoresis. RNA collected from 8 test animals of the 4 age groups respectively was mixed in equimolar RNA pools which served for the transcriptome analyses using Affymetrix arrays.

Results: Statistical analysis of the gene expression data indicated the existence of genes differentially regulated in the growing temporomandibular cartilage. This evidence, however, requires validation by RT-PCR using individual animals' RNA. Preliminary candidate genes belong, among others, to the groups of matrix-degrading proteases, protease inhibitors and genes involved in cell growth, apoptosis and bone remodelling.

Conclusion: These differentially expressed genes in TMJ growth identified using DNA array technology may possibly contribute to a better understanding of growth biology and provide an approach to necessary therapy.

Key words: microarray, temporomandibular joint, condylar cartilage, condylar growth.

INTRODUCTION

The cellular changes occurring during temporomandibular joint development exhibit a complex nature and are subject to a variety of factors [1,2]. The strongest shape-forming influences originate from genetic, exogenous, neurological and hormonal factors. Their individual effects on temporomandibular joint growth vary during the course of development [3].

Genetic factors act as a guide rail during developmental processes. The respective gene groups are switched on and off during different phases of growth. Some genes undergo only short-term expression, while others are expressed permanently.

Molecular analysis of single gene expression patterns is based upon established techniques such as Northern Blotting, RNA in-situ hybridisation or RT-PCR which are characterised by high sensitivity and reliability. However, their use is restricted, as they fail to permit simultaneous expression analysis of multiple genes. For several years, methods have been established that enable global analysis of gene expression. Detection of the total mRNA molecules present at a given point of time is referred to as transcriptome analysis

[4,5]. These technologies comprise serial analysis of gene expression (SAGE) [6] and the use of DNA array technologies such as macroarrays or microarrays [7].

The condyle with the condylar cartilage is among the most active growth areas of the mandible. The superior objective of the study starting with the present work is the analysis of differential gene expression in the growing temporomandibular joint cartilage. Histological analysis was introduced to TMJ diagnostics as the so-called standard for demonstrating structural changes [8,9]. Subsequent to histological analysis, however, tissue specimens are uneligible for further analyses on the mRNA level. The latter require RNA preparations of superior quality which cannot be obtained but by very quick processing of the sample material. Therefore, a primary aim of the present study was the establishment of a comprehensive protocol for transcriptome analysis using Affymetrix DNA arrays for the analysis of gene expression in the condyle cartilage including a protocol for isolation of applicable high-quality RNA preparations.

MATERIAL AND METHODS

Experimental animals

Owing to numerous shared morphologic and physiologic properties, the rat temporomandibular joint serves as a model of growth processes occurring in the human temporomandibular joint. Hence, LEW1W rats were chosen as the animal model for the present studies (Permission of the Ethics Commission M-V /TSD/7221.3-2.3-023/06). The dams inbred in the animal test house of Greifswald university were kept under standardised conditions at a light-dark regime (12 h light from 6 a.m. to 6 p.m. respectively) in K3 cages with feed and drinking ad libitum at a humidity of 50-60%. The animals were kept under breeder's and veterinary control at hygienic conditions permitting sufficient movement and were fed breeding food pellets. The condylar cartilages were collected from animals of 4 different age groups of 8 animals each. Collection of cartilage was performed using a surgical microscope. In group 1, samples were taken from newborn animals with a pronounced sucking reflex, but largely underdeveloped motor function. Group 2 comprised animals at the age of 10 days. At this time, the animals first open their eyes and are characterised by a more mature state of total motor function despite still being in the sucking period. Group 3 animals were 21 days old weighing about 35 gr and were freshly separated from the mother. Food ingestion comprised both sucking and chewing of the solid breeding food with a distinct masticatory mechanism. Group 4 animals were 8 weeks old with a live weight of about 150 gr. They displayed mature gnawing and chewing activity as well as fully developed masticatory muscles. Moreover, the animals are sexually mature at this age, but not yet ready for breeding. While the peak protein retention has been attained, merely the animals' fat percentage will still increase up to a body weight of 210 gr.

Since the temporomandibular joints are very small-sized structures, the operation was performed using a surgical microscope. After collection from the right- and left-hand side, the condylar cartilages were shock-frozen with liquid nitrogen and stored at -70°C until further analysis.

Preparation of total RNA

RNA was isolated from the frozen condylus samples using a mechanical disruption protocol. To this purpose, 250 µl of Trizol (Invitrogen) was added to a disruption Teflon vessel which was filled and precooled with liquid N_2. Then, the frozen condylus sample was dropped in. The vessel was closed and inserted in a Mikro-Dismembrator S (B. Braun Biotech International, Melsungen, Germany). Mechanical disruption was carried out for 2 x 2 min at 2,600 rpm. The resulting powder was dissolved in 750 µl of Trizol and was refrozen in liquid nitrogen. For RNA isolation, samples were thawed and incubated for 5 min at room temperature. Then, 200 µl of chloroform was added, mixed and incubated for further 3 min at room temperature. The upper phase was transferred to a fresh tube, 500 µl of isopropanol was added and the RNA was left for precipitation at -20°C overnight. Next day, the RNA precipitate was centrifuged for 30 min at 19300 × g and 4°C. The resulting pellet was washed with 1 ml 70% ethanol and centrifuged for 5 min at 19300 × g and 4°C. Finally, the resulting pellet was air-dried and dissolved in 50-100 µl RNase-free water.

Elimination of contaminating DNA and purification of total RNA

To remove contaminating co-purified DNA traces which might still be present in the RNA preparation, a DNase treatment using the RNase-Free DNase Set (Qiagen) was performed. The diluted RNA was mixed with 10 µl buffer RDD and 2.5 µl Qiagen DNase I to a final volume of 100 µl and was incubated for 10 min at room temperature. Afterwards, the RNA was purified using the RNeasy Mini Kit (Qiagen) to remove the DNase I as well as Nucleotides generated by the DNase treatment. The RNA solution was mixed with 350 µl RLT and 250 µl absolute Ethanol by pipetting and the resulting mixture was added on an RNeasy spin column. The RNA was bound to the column by centrifugation for 1 min at full speed in a table-top centrifuge. After two washing steps with 500 µl RPE buffer, the column was dried by an additional centrifugation step. The purified, DNA-free RNA was eluted by adding RNase-free water and, after incubation at room temperature for 5 min, a final centrifugation step.

RNA quantification and quality control

Quantification of the total RNA was performed using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies), which minimised the loss of RNA material during the measurement procedure (only 2 µl RNA solution were used per sample). The average amount of DNA prepared from one condylus sample varied between 20 to 30 µg (data

not shown). Additionally, a first quality control was performed as the Nanodrop ND 1000 determines the ratio 260/280 nm, indicating protein contamination. For high quality RNA, the ratio has to be around 2. Measured ratios of all RNA preparations varied between 1.9 and 2.1 (data not shown).

Quality control of prepared RNA was carried out using RNA 6000 Nano Chips and an Agilent 2100 Bioanalyzer. The system is based on capillary gel electrophoresis. After loading the gel matrix to the RNA 6000 Nano Chip, 1 µl RNA solution (100 ng/µl) was added per well (up to 12 samples can be tested using one chip) for measurement. The result of the electrophoresis was visualised using the expert software as a virtual gel image or as a graph.

The expert software calculates the ratio between the 28 / 18 S rRNA, which for high RNA quality is again supposed to be around 1.8 to 2. Additionally, the degree of degradation is described by a RIN (RNA integrity number). A RIN of 1 indicates complete degradation, whereas a RIN of 10 is typical for high quality RNA. In our laboratory, RNA preparations exhibiting a RIN $\leq$ 8.3 are generally discarded, because it turned out, that this quality is not sufficient for microarray analyses. The RNA preparations which were obtained using the method described here exhibited RIN between 7.8 and 9.8. Interestingly, there was an inverse correlation between the age of the rats and the RIN, which means that the quality of the prepared RNA decreased with age. However, all preparations used in the microarray experiments did exhibit a RIN ≥8.3.

Transcriptome analysis using Affymetrix DNA arrays

Gene expression profiling was performed using the Affymetrix Rat Genome 230 2.0 arrays as recommended by the manufacturer. The RNA samples of 4 animals each from age-matched groups were pooled in equimolar amounts. The experiments were performed with 5 µg pooled total RNA. Reverse transcription was carried out using a Thermo Cycler GeneAmp PCR System 9700 (Applied Biosystems). Subsequently, the resulting double-stranded cDNA was purified using the Sample Cleanup Modul. This cDNA was used as the template in the following in-vitro transcription reaction which generated biotin-labeled cRNA. The cRNA was purified using the Sample Cleanup Module. Subsequently, the concentration of the resulting cRNA solution was determined photometrically using the NanoDrop Spectrophotometer and 400 ng of cRNA was analysed using the Agilent 2100 Bioanalyzer to test the efficiency of the in-vitro transcription reaction.

Fragmentation of 20 µg of the cRNA was carried out in fragmentation buffer at 94°C for 35 min. To analyse the size of the generated cRNA fragments, an aliquot was removed and again analysed using the Agilent 2100 Bioanalyzer. The size of the generated fragments should be between 35-200 bases. Next, hybridisation of the cRNA samples with the Rat Genome 230 2.0 Array was prepared. To this purpose, the array was pre-hybridised for 10 min at 45°C with 200 µl 1 × hybridisation buffer in the Affymetrix Hybridization Oven 640. Altogether 15 µg fragmented cRNA was added to a hybridisation mixture resulting in 300 µl total volume. This hybridisation cocktail was denatured for 5 min at 99°C, subsequently equilibrated for 5 min at 45°C, and finally centrifuged at full speed in a table-top centrifuge for 5 min. After removing of the 1 × hybridisation buffer, the array was filled with 200 µl hybridisation cocktail and incubated for 16 h in the hybridisation oven at 60 rpm and 45°C. Washing and staining of the array was performed in the Fluidic Station 450, using the GCOS (GeneChip® Operating Software) program and the protocol EukGE-WS2v5. Scanning of the array was carried out with the Affymetrix GeneChip® Scanner 3000.

RESULTS

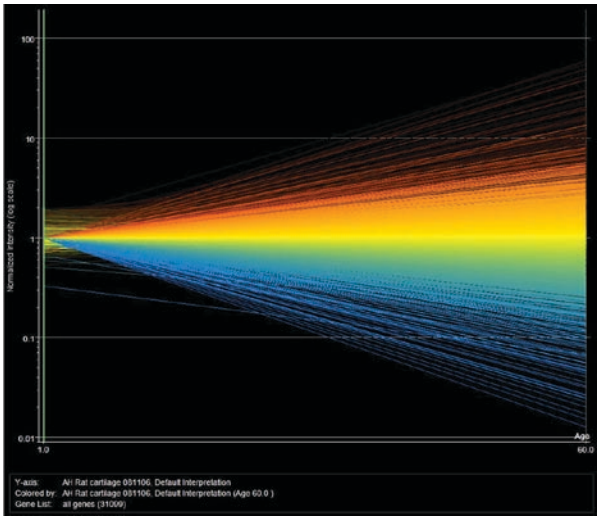
Primary data analysis

From each scanned array, GCOS creates image and data files. Generally, the interpretation of the generated expression data is performed in three steps: Raw data analysis producing the report for quality control, statistical data analysis and filtering to identify significantly expressed and regulated genes, and finally the functional association to known cellular pathways. GCOS was used for raw data analysis and quality control. For statistical data analysis and filtering of the data as well as for functional analyses, the GeneSpring software (Agilent) was used. First, all genes which did not exhibit an at least marginal expression value in both replicates for at least one of the four time points (newborn/day 1, 10 days, 21 days, 8 weeks) were excluded from further analyses. Then, gene specific expression values of the two replicates were averaged for each time point. To check for a statistically significant increase or decrease of mRNA amounts between one time point and the others, we calculated the ratios of "newborn/day 1" against the remaining three time points. This was also done for "10 days", "21 days" and "8 weeks", respectively. For each of those groups a two-sided one-sample t-test on the log ratios against zero was performed at significance level of 5%. Subsequently, probe sets exhibiting a significant p-value in at least one of the four t-tests were chosen to represent significantly regulated genes. Only genes represented by probe sets exhibiting an at least twofold up- or downregulation between at least two time points were included in the final list.

Detection of differentially regulated genes

The rigorously performed statistical analysis of the generated transcriptome data revealed that, at the present state of the study, significant expression values were available for two of the analysed time points: the 1-day and the 60-day sampling time point. Several genes did show an altered expression level when these two time points were compared (Fig. 1). The strongest up-regulation after 60 days as compared to 1 day was observed for the Hemgn gene (around 13fold) whose product hemogen is synthesized in bone marrow cells and osteoblasts

Figure 1. **Courses of expression levels of all genes present on the AffyMetrix rat genome array 230 2.0 in tissue prepared from eight weeks old rats (relative expression values shown on the right) compared to one day old rats (relative expression values which were set to 1 for all genes shown on the left). The image was generated using the GeneSpring software. Red colour indicates increased gene expression, blue colour indicates decreased gene expression, yellow colour indicates no change. The intensity of the corresponding colour reflects the intensity in up- or downregulation.**



and might be involved in osteoblast recruitment [10] (Fig. 2), and for Best5 (around 7fold) which has been described to play a role in the regulation of bone formation [11]. Three further genes also exhibited relatively strong up-regulation after 60 days: Mepe (5fold) encoding an extracellular matrix phosphoglycoprotein with ASARM motif which may play a role in the regulation of bone remodeling, Ca2 (4fold) encoding carbonic anhydrase 2 involved in bone resorption [12], and Omd (3.6fold) whose gene product osteomodulin is involved in the regulation of biomineralization processes. The remaining genes whose mRNA was present in higher amounts at the later stage of development did exhibit a rather moderate extent of up-regulation, between 2- and 3 fold. This group includes four genes encoding proteins described to be involved in ossification processes: Calcr encoding a calcitonin receptor [14], Mmp8 which encodes the matrix metallopeptidase 8 [15], Dmp1 encoding dentin matrix protein 1 [16], and Bglap2 encoding the bone gamma-carboxyglutamate protein 2 [17]. The products of two further genes belonging to this moderately up-regulated group were described to be involved in skeletal development: Ank encoding the progressive ankylosis (Ank) protein [18] and Tnfrsf11b which encodes osteoprotegerin, the member 11b of the tumor necrosis factor receptor superfamily. Finally, four additional genes exhibited moderate up-regulation: Gpnmb encoding a transmembrane glycoprotein which may play a role in bone matrix production and mineralization [19], Zbtb7a encoding the zinc finger and BTB domain containing protein 7a which acts as a transcriptional repressor and may play a role in osteoclastogenesis [20], Acp5 which encodes the Acid phosphatase 5, an iron containing glycoprotein described to be involved in bone resorption [21], and, besides Mmp8, a second matrix metallopeptidase: Mmp9, which is also involved in extracellular matrix remodeling and bone resorption [22].

Figure 2. **Up-regulation of the Hemgn (Edag1) gene encoding hemogen in tissue prepared from eight weeks old rats (relative expression value shown on the right) compared to one day old rats (relative expression value which was set to 1 shown on the left). The image was generated using the GeneSpring software.**

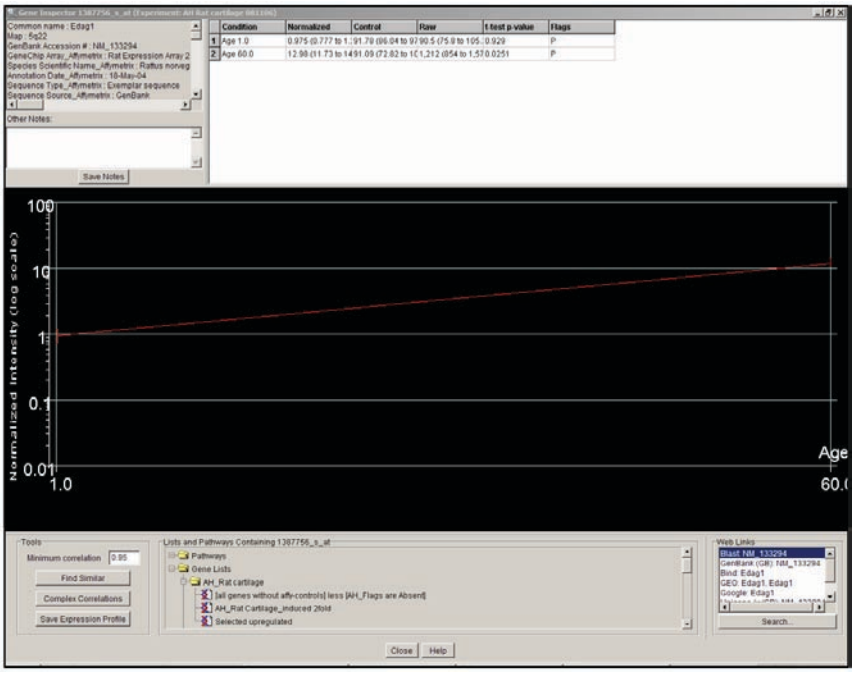
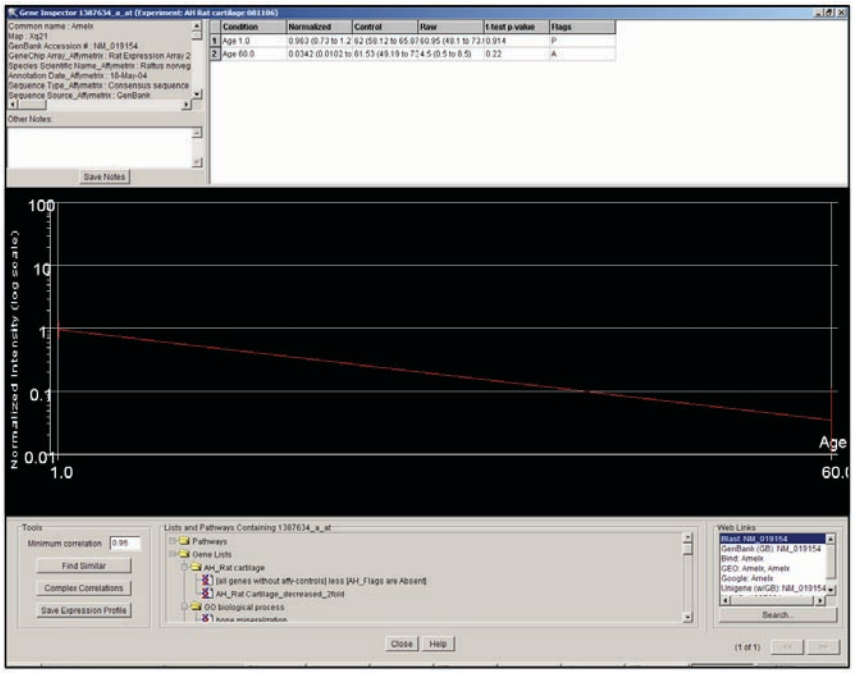


Figure 3. **Down-regulation of the Amelx gene encoding amelogenin in tissue prepared from eight weeks old rats (relative expression value shown on the right) compared to one day old rats (relative expression value which was set to 1 shown on the left). The image was generated using the GeneSpring software.**



The comparison of the expression values obtained for day 1 and day 60 also allowed for the identification of several genes exhibiting significantly lower mRNA amounts at the later stage of development. The most pronounced down-regulation (≥33fold) was observed for Amelx encoding amelogenin which has been described to play a role in mineralization of tooth enamel matrix [23] (Fig. 3). The Matn1 gene encoding the cartilage matrix protein matrilin 1 which fulfills functions in cartilage development did also exhibit a relatively strong down-regulation (≥14fold). Two further genes belong to this strongly down-regulated group: Npr3 (≥8fold) encoding the natriuretic peptide receptor 3 involved in skeletal development [24] and Fgf18 (≥6fold) encoding fibroblast growth factor 18 which was described to play a role in ossification [25]. The following genes did show a less pronounced down-regulation (2-3fold): Alcam, which encodes the activated leukocyte cell adhesion molecule involved in osteogenic differentiation [26]; Egfr encoding epidermal growth factor receptor formerly described to play a role in ossification processes [27]; Nog, whose gene product noggin is involved in bone morphogenetic processes [28]; Thra, which encodes the thyroid hormone receptor alpha also involved in ossification processes [29]; Igsf10 encoding the member 10 of the immunoglobulin superfamily, which represents a marker of early osteochondroprogenitor cells which may be involved in the maintenance of the osteochondroprogenitor cell pool in bone [30]; Col5a2 encoding collagen type V, alpha 2, fulfilling functions in organization of bone morphogenesis [10]; Smad5 encoding the MAD homolog 5 also involved in bone morphogenetic processes [31]; and Smo encoding the smoothened homolog protein described to be involved in osteoblast differentiation [32]. It has to be emphasized that in all cases of observed differential gene expression, the obtained patterns still have to be validated by RT-PCR and thus represent preliminary results.

DISCUSSION

Nowadays, the number of fully sequenced genomes is growing almost daily. The methods of functional genome analysis based on the availability of such complete genome sequences enable comprehensive insight into developmental processes of tissues and organs on the mRNA and protein level. Parallel analysis of the differential expression of thousands of genes by one single experiment using the microarray technology permits analysis of the expression profile of a specific gene in the context of the total expression pattern of all genes of a defined cell assembly, thus providing essential information on the functional context.

The present study shows that despite the small amounts of cartilage available from rat temporomandibular joints, such expression analyses using Affymetrix microarrays are feasible in principle. They are based on an optimised protocol for RNA preparation and sample conditioning presented in this article.

A preliminary and still superficial analysis of the transcriptome data suggests that gene expression in the condylar cartilage is subject to temporal changes, i.e., in the different age groups, partly different genes are expressed in the growing tissue and/or the same genes are expressed variably.

Preliminary candidate genes belong, among others, to the groups of matrix-degrading proteases and genes involved in cell growth, bone morphogenesis and remodelling. A more comprehensive and detailed analysis of the gene expression data obtained with extensive consideration of the available data from literature is expected to allow more subtle conclusions regarding the underlying cell biological mechanisms, e.g., the identity of the transcription factors involved.

The degree of interindividual biological variation between the individual test animals was estimated as relatively high by various studies [33-35]. Pritchard et al. [33] analysed the normal variation of gene expression using DNA microarrays and found, for instance, a variation of 3.3% for the kidney tissue of healthy adult mice.

In the experiments described, therefore, RNA preparations of 4 rats respectively were mixed in equimolar concentrations. These RNA pools were subjected to transcriptome analysis. This approach has the advantage of levelling interindividual differences between the test animals. Thus, detection is restricted to genes exhibiting truly significant differential regulation in all four animals. On the other hand, there is the risk that an outlier, i.e., an animal that displays an extremely uncommon (e.g., pathogenous) gene expression pattern as compared to all other animals, may bias the results yielding an expression profile not really representative of the whole group. This risk was attenuated analysing samples from two subgroups each comprising 4 animals of one age group. The similarity of the expression profiles of these two pools from the same age group needs to be significantly greater than the similarity to the profiles of the other age groups.

Ultimately, it is necessary to validate a set of selected genes identified to be differentially regulated by transcriptome analyses with additional follow-up analyses using RNA preparations of the individual animals from the different age groups instead of using pooled RNA. The expression profile assessed in the respective pool needs to be detected in the individual animals as well. For such follow-up experiments, RT-PCR proves to be the primary method of choice; however, Northern analyses and in-situ hybridisation or immunohistochemical methods may be used as well. In addition, immunohistochemical techniques allow determining the localisation of the specific gene products in the respective tissue assembly.

CONCLUSION

In principle, the presented method of transcriptome analysis using Affymetrix DNA microarrays permits the assessment of gene expression profiles in the condylar cartilage and identification of significantly differentially regulated genes. The resultant increasing insight into functional associations in tissue growth may have a considerable impact on the development of diagnostic and therapeutic approaches.

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