Differential expression of fibromodulin mRNA associated with tendon fibril growth: isolation and characterization of a chicken fibromodulin cDNA

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A 450 bp cDNA fragment similar to that encoding bovine fibromodulin was isolated using a screening procedure to isolate genes differentially expressed between the pre- and post-growth phases of fibril growth in the developing chicken embryo metatarsal tendon. Using this fragment, a 2.4 kb cDNA clone for chicken fibromodulin was isolated from a λ ZAP library, and the 5' rapid amplification of cDNA ends technique was employed to clone the 5' end of the fibromodulin cDNA. The full-length cDNA contained an open reading frame coding for a 380-amino-acid protein. There was ~80% similarity with human, rat and bovine fibromodulins, which confirmed its identity as fibromodulin. Structural features of the deduced sequence include an 18-amino-acid signal peptide, cysteine residues in conserved positions in the N- and C-terminal regions, and a central leucine-rich domain containing eleven repeats of the sequence LXXLXXNXL/I. Features unique to chicken fibromodulin

include an additional glycosylation site as well as a decreased number of tyrosine residues that could be sulphated, and therefore potential changes in the charge of the molecule. In addition, there was little similarity among the untranslated regions. When compared with chicken decorin and lumican, fibromodulin showed greater similarity to the other keratan sulphate-containing proteoglycan, lumican. Northern blot analysis revealed a 6–8-fold increase in the fibromodulin mRNA level from day 14 to day 19 of development. In the chicken tendon, collagen fibril growth is a process characterized by a precipitous increase in length during a short developmental period. The necessary changes would require the expression of different genes regulating fibril formation and growth, and interactions between fibromodulin and collagen fibrils may participate in the regulation of collagen fibril growth and matrix assembly.

INTRODUCTION

Collagen stabilizes the structure of most organs and is the major component of tissues such as tendon, cornea, dermis, bone and cartilage. Fibrillar collagens are the most abundant and have major roles in the stabilization structure. Fibrils assemble and are organized into fibres. The fibres are further organized into tissue-specific aggregates such as cables (tendons and ligaments), regular layers (cornea and bone), irregular layers (dermis) or meshwork (cartilage) [1,2]. Collagen fibrillogenesis and matrix assembly are multistep processes. The interaction of the small interstitial proteoglycans (SIPGs) with collagen fibrils has been implicated in the regulation of several of these assembly steps [3].

The SIPGs form a gene family consisting of fibromodulin, lumican, decorin, biglycan and PG-Lb. The core proteins are similar in size (36–42 kDa). These core proteins have a central domain containing leucine-rich repeats (LRRs) and terminal domains with cysteine residues in conserved positions. Fibromodulin and lumican are keratan sulphate-containing proteoglycans (PGs), while the others are dermatan sulphate-containing PGs. The members of this class of proteoglycans are found in a wide variety of connective tissues [3].

We have used the chicken metatarsal tendon as a model system for the study of extracellular matrix assembly [4–6]. Fibrils assemble and are organized into a tissue-specific extracellular matrix during development. Associated with development of the

tendon architecture is a precipitous increase in fibril length between 16 and 18 days of development [4]. We hypothesize that this is due to a post-depositional growth of short fibrils (segments). This growth would require: (1) a stabilization of fibril segments during the periods of rapid tissue development; (2) a destabilization followed by fibril growth; and (3) a final stabilization of the mature fibrils. We propose that growth into fibrils is regulated at each of these steps. One class of potential regulatory macromolecules are the fibril-associated SIPGs. These PGs have been implicated in modulating matrix assembly at a number of different steps (reviewed in [3]). Decorin, lumican and fibromodulin have all been shown to alter the kinetics of fibril formation *in vitro* and in some cases alter the resulting fibril diameter [7–10]. In addition, the disruption of these PGs using inhibitors can alter matrix assembly *in vivo* [11–13].

A study of components present or absent before and after fibril growth will contribute to an understanding of the regulation of fibril growth. We have used subtractive hybridization to identify gene expression associated with the different stages of fibril growth in the developing tendon. Chicken fibromodulin was identified as one of the up-regulated molecules. This PG has been identified in tendons from other species, but no inferences regarding a developmental role or potential regulatory roles have been described. To investigate the role of fibromodulin using the readily accessible developing chicken embryo required the isolation of a full-length cDNA. We have characterized this SIPG

Abbreviations used: PG, proteoglycan; SIPG, small interstitial proteoglycan; LRR, leucine-rich repeat; RACE, rapid amplification of cDNA ends; TGF, transforming growth factor.

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and compared it with fibromodulins from other species as well as with other members of this class of PGs from the chicken.

MATERIALS AND METHODS

Preparation of RNA and cDNA

Metatarsal tendons were dissected from staged 14- and 19-day chicken embryos [14]. Isolation of mRNA from ~ 0.5 g of tissue was done using a Fast Track Kit (Invitrogen), and cDNAs were obtained by reverse transcription of 5 µg of poly(A)⁺ RNA with oligo(dT) as the primer using a cDNA synthesis system (Amersham). A cDNA library with an average insert size of 2–3 kb was constructed using a λZAP cDNA cloning kit (Stratagene) for both stages of tendon development.

Subtractive hybridization

This procedure was performed using a modification of the protocol described by Wang and Brown [15]. The cDNAs were digested completely with *Sau3A*I. Each digest was then ligated with 8 nmol of specific linker. The first linker was prepared by annealing the synthetic oligonucleotide dGACACTCTCGAGACATCACCGTCC with its complement, dGATCGGACGGTGATGTCTCGAGAGTG [16]. The second linker was prepared in an identical manner using the oligonucleotide dGACACTCTCCAGCCTCTCACCGCA and its complement, dGATCTGCGGTGA [17]. The cDNA fragments were amplified using the appropriate linker strand as a primer: dGACACTCTCGAGACATCACCGTCC for the 14-day cDNA amplification and dGACACTCTCCAGCCTCTCACCGCA for the 19-day cDNA. Amplification was performed in *Taq* polymerase buffer (Perkin-Elmer) with 2.0 mM MgCl₂ (94 °C, 1 min; 50 °C, 1 min; 72 °C, 2 min with 25 s of autoextension per cycle; 30 cycles). The amplified cDNAs (~ 30–50 µg of each) were then labelled with Photoprobe Long-Arm Biotin as recommended by the manufacturer (Vector Laboratories). Removal of cDNAs common to 14- and 19-day tendons was accomplished by two rounds of subtractive hybridization. An excess (35 µg) of biotinylated 14-day cDNA was mixed with 1.5 µg of non-biotinylated 19-day cDNA, precipitated, redissolved in 10 µl of water, denatured at 100 °C, mixed with 10 µl of 2× hybridization buffer (1.5 M NaCl, 50 mM Hepes, pH 7.5, 10 mM EDTA, 0.2% SDS) and hybridized at 68 °C either overnight or for 4 h. After hybridization, the mixture was diluted to 0.5 M NaCl and biotinylated molecules were removed after incubation with streptavidin followed by phenol extraction repeated four times [18]. The remaining subtracted cDNA fragments were amplified and cloned into the pCRII plasmid using the TA cloning kit (Invitrogen).

Screening the cDNA library

To obtain a full-length fibromodulin cDNA, the 19-day tendon λZAP library was screened with the 450 bp fibromodulin cDNA fragment. Approximately 10⁵ phages were grown on three 150-mm plates, lifted in duplicate on to nitrocellulose membranes (Schleicher & Schuell) and hybridized with the labelled cDNA. Several positive plaques were rescreened twice and the inserts were analysed by PCR using the internal primers for the fibromodulin clone (dCTGGATGGCGGTGAT and dCGTTAGCCATGTAAGTTC). One of the chosen plaques contained a 2.4 kb insert. A clone in pBlueScript was excised using the EXAssist/SOLR system (Stratagene) and the 2.4 kb *Bam*HI/*Kpn*I insert was cloned into a pSP72 vector for sequencing.

5' Rapid amplification of cDNA ends (RACE) cloning

To isolate the 5' end of the fibromodulin cDNA, 5' RACE was performed using the Marathon kit (Clontech). For the PCR amplification, the internal fibromodulin primer was designed based on the known sequence for the 450 bp cDNA fragment (dCTGGATGGCGGTGATTG) and used in conjunction with the AP1 primer from the kit. The amplification was performed for 25 cycles (94 °C, 30 s; 58 °C, 30 s, 68 °C, 4 min). The resulting 600 bp product that overlapped with the 2.4 bp cDNA fragment was cloned into the plasmid pCRII (Invitrogen) and sequenced from both strands.

Northern analysis

RNA samples [1 µg of poly(A)⁺ mRNA] were electrophoresed through 1% agarose/formaldehyde gels and transferred to a nylon membrane (Hybond-N[®]; Amersham). The membranes were baked under vacuum and hybridized to the random-primer labelled probes as described previously [4].

DNA sequencing

A set of gamma-delta transposon insertions [19] was obtained for the fibromodulin clone in the plasmid pSP72. This enabled the double-stranded sequencing of the cDNA clone with 3-fold redundancy. Sequencing was performed using the Dye Primer Cycle Sequencing Kit (Applied Biosystems) and an ABI 373A DNA sequencer. The results were analysed with Sequencer software from Genecodes Corp.

RESULTS

Identification of up-regulated mRNAs by subtractive hybridization

We identified a number of up-regulated genes associated with the period of rapid fibril growth in the tendon by subtractive hybridization. The cDNA fragments common to both 14- and 19-day tendon were removed from the 19-day library. The cDNA fragments obtained by subtractive hybridization were analysed by sequencing. A total of 62 clones were randomly chosen for sequencing, and one of these clones showed a similarity to bovine fibromodulin. However, the identity of the fibromodulin clone was not clear since fibromodulin is a member of a family of SIPGs which are structurally related. The isolated chicken clone had 72% identity with a region localized in a bovine fibromodulin cDNA at positions 330–480. This codes for the first two of eleven LRRs that are similar in all members of the SIPG family [20]. Therefore the chicken cDNA fragment could code for a novel protein from this family. Its identity was determined by cloning and sequencing the full-length cDNA of chicken fibromodulin.

Cloning and characterization of chicken fibromodulin

The 2.4 kb cDNA clone for chicken fibromodulin isolated from the 19-day tendon cDNA λZAP library, as well as the overlapping 0.6 kb clone obtained by extending the 2.4 kb clone by 5' RACE, were sequenced. The compilation of both sequences is shown in Figure 1. The complete 2657 bp cDNA corresponds to the 2.7 kb fibromodulin mRNA seen by Northern analysis (Figure 2). It contains a 1140 bp open reading frame (positions 197–1337) followed by a 1320 bp non-coding region. The poly(A) sequence on the 3' end of clone is preceded by the polyadenylation signal AAATAAA, located 17 bp upstream of the polyadenylation site.

The open reading frame codes for a protein of 380 amino acids. The first ATG codon in the coding sequence is preceded by the consensus sequence for translation initiation and followed by a typical signal peptide sequence of 18 amino acids. The deduced

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CGCCCGAGCAGGTCAAAAACCTGCACAGCCCGTGACCTGCAAGCACAGTGTGATCTGGGATCTAGGGCGGGCACCAG 80
CCTTGACACCTTGGGAGACGCACTCACATGCTGCTTGACACACGGGAGATCCAGAGCAGCAGCGAGGCTTTGGAAAG 160
GAGGCTCTCTGTTGGTCCCTCCAGTGCCAGCAGCATGCGTTGGGCCAACATCCTGCTGTTGCCGGGCTCTGCAGAGCC 240
      M R W A N I L L V A G L C R A
TCCCTGGGGCAGTACAATGAGGAGGAGACCTGGCGTGCGTACTACATGCGGCAGTCCCGGATGTCCTCTACAA 320
S L G Q Y N E E E D L A W L Q Y Y M R Q S R M S S Y N
CTACATGCCCTACTACGAGGATGAGAACAGCCCTACGTTTACTCTTACGTCCCGGCCCGGACACTGAAGCAGAGCCTG 400
Y M P Y Y E D E N S P Y V Y S Y V P A P D T E A E P
TCCCTGAAGCTCAGCAGGCCTCATCTGGCAATGTCCCCAGGAGTGTGACTGTCCCCCAACTTCTCGTCAGCCATGTAC 480
V P E A T Q Q A S S W Q C P Q E C D C P P N F S S A M Y
TGCACACGCGCAACCTGAGGTATCTGCCCTTCTGTCACCCCGGATGAAGTACGTCTACTTCCAGAACAACCAATCAC 560
C D T R N L R Y L P F V P T R M K Y V Y F O N N O I T
CGCCATCCAGGAGGGAGCTTTCGACAACGCCACCGAGCTGGAGTGGCTGCGACTGCACAACAACAGATCAGCAGCGAGA 640
A I Q E G A F D N A T E L E W L A L H N N O I S E
AGATGGGCAAGAGGCTCTTTGCCAAGCTCAAAAACCTGGAGCGGTTGTACATGAACAACAACCTGACCAAGATGCCC 720
K M G K R V F A K L K N L E R L Y M N N N N L T K M P
AGCCCTTGGCCCGTCCCTGAGGGAGCTCCACTTGTCTTACATCAGATCTCAAGGTTCCCTCCAATGCTCTGGAGGG 800
S P L P R S L R E L H L S Y N O I S K V P S N A L E G
TCTGGAGAACCTCAGCCCTGTACTCTAGCCACAACCTATCTTTTGGAGTGGGAGCATCCCTCAAAGGGCTCAAGTCT 880
L E N L T A L Y L S H N Y I F E M G A S L K G L K S
TGATCCTTGTCTGATCTGAGCTACAACACCTCAGGAAAGTCCCTGATGGCTCCCAATGGCTCTGGAACAGCTCTACCTA 960
L I L A D L S Y N H L R K V P D G L P M A L E O L Y L
GAGTACAACCTACATCAACGCCATCCCTGATGACTATTTCAAGGTGTCTCCCAAGCTGTCTATGTAAGGATGTCCACAA 1040
E Y N Y I N A I P D G A T K V S P K L L Y V R M S H N
CAGCCTGACAAACCAAGGGCTCTCTACCAACACTTTCAACAGCAGCAGCATCTCGAGCTGGACCTCTCTACAACCGGC 1120
S L T N Q G L S T N T F N S S S I L E L D L S Y N R
TGCAGAAGATCCCCCGGTCAGCACCACCTGGAGAACCTCTACCTTCAAGGGAACCAATCAACGAATTCTCCATCAGC 1200
L Q K I P R V S T N L E N L Y L O G N O I N E F S I S
AGTTTCTGCACCGTCTGGATGTCATGAACCTACTCCCGCTCCAGGTGCTGCGGTTGGATGGGAATGAGATCAAGCGCAA 1280
S F C T V V D V M N Y S R L O V L R L D G N E I K R N
CGCGATGCCCCCTGAGCCCCGCTGTGCTGCGGCTGCCACCGTCATCGAGATCTAGCCTGCTGCCAGCCCCGCTCTC 1360
A M P P D A P L C L R R A T V I E I
AGGACTCTGCTCCCTCCTTCCAGGGAGACACTCACTCCCATTTTATGCAGCTTGACAGTTTGACTTGGCTTCTGCAAT 1440
AGTGCAATAAGGGTGTCCAATGCCAGCCACACAGGGGGCAGGATCTGCTCATCCCTAAATCCCCCCCCCCCCGGGC 1520
TCTCCGGCTCAGAGATCCCATGTAGCCATGCCATGGTCCACACAGGAGGAGTGGGGAAGGAGAAAGGGCTCATCCCATC 1600
TCTGAAACGTGATGCTGAGCGTCCGGCTCCAGGTGTGGGTCTGTCAGAACACCCCAAGTGGCTCCCCCTGGGCCAGGGTT 1680
TGGTGATCACTTGGAGCTCAACAGGGAAGGGTTACAGATTTCCCAACCAACAGCATCCCTCTCTCTAAGATGGGAATAA 1760
GCAAAATCTTTATCTTACTTCCCACTTTTCCCTGAAGCTTCTGCTCCAAACATCAAGCCAAGAGGAAAGAAATCTTTC 1840
CTGCTGCTGCCAGCAGGCCTCCAGCTGCTGAGCTCCCGAGGAGCAGCATGGTGAGAGGGGACCCCAACCTCTCAG 1920
GTTTTGGGACAGGACTGGGGCCAAGAGATGCACATTGCAGTGCATCACCCACAGTGTCCAGGTCTATGCCAGGAGCA 2000
ACCCCTCTGTCCCTGCTGTCCCTGCCAGGCACAGGTGTCTGTGTCACATCTTGGTGCAACTGGGCCACACTCTGT 2080
GTCTGCACACAGCAGTGTCCCTCCACTGTGCCAACACCCCTGTGTGTGTGCCACTCAGCTGCAGCTGCCCCAGCTT 2160
AAGGGGAGCAGCCCCCAGCAGGTGAGGTTTGAACCCAGGTGGGACCCCAAGGTTTAAACAGGGGAGGGTTTCACTGCT 2240
CCCAAAATCTTGGTCTCACTGCAGTGCAGGATCTGCCCTACCCCTCTACCCCTCCACTGTGTGCCAGCTCTGGGC 2320
TTCCACCCAGGGAAGTGAAGCTCTCCCACTAAGAATAACGATGCAGCAGTAATGTCTGCATGGGCAGCAGCATCTCGATT 2400
TATCGAGAGCTGAACACCCGCTTGGTTCTTGGACAGGTTTACTGAGAACCAAGGGGAGCCAGCTTCGAAGATGAC 2480
CTGCAAGGCCGAAGAGGTATCCAGACCCCTCCACATTTAACTGCTATGCTAAACACTATAGAAACAAAAACCAAAAAAC 2560
CTGCACTATCACCAGCGCATGAAGTGTAGATGTATACCGACAAATAAGCCCTTTCTGTAACGTGCAAAAAA 2640
AAAAA

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Figure 1 cDNA sequence and deduced amino acid sequence of chicken fibromodulin

Nucleotides of the cDNA are numbered on the right. Translation of the open reading frame is given below the DNA sequence. Cysteine (C) residues in conserved positions are given in bold. Potential sites for N-glycosylation (N-X-S/T) are double-underlined. The regions containing the consensus sequence LXXLXXNXL for LRRs are single-underlined.

protein sequence has six Asn-Xaa-Ser/Thr sequences (double-underlined in Figure 1), which represent potential sites for N-glycosylation. The locations of five of these sites are identical to those found in bovine, rat and human fibromodulin. There are six cysteine residues, all located in conserved positions in fibromodulins from different organisms and also when compared with another keratan sulphate-containing SIPG, chicken lumican (Figure 3). Chicken fibromodulin possesses an extensive LRR that contains multiple LRRs of the basic sequence LXXLXXNXL/I. These repeats are characteristic of the core proteins of the SIPGs. These features, coupled with the ~ 70 % identity (80 % similarity) with other fibromodulins, confirm that the identified clone is chicken fibromodulin.

Comparison of the chicken amino acid sequence with those of bovine, human and mouse fibromodulins reveals striking homology (Figure 3). Major variations exist only in the N-terminal

region. This is also true for decorin sequences when comparisons are made of core proteins from different organisms [21]. The N-terminal domain of chicken fibromodulin contains 10 tyrosine residues. Only three of these tyrosines are adjacent to acidic amino acid residues and thus are candidates for sulphation by tyrosyl protein sulphotransferase [22]. The N-terminal domains of other fibromodulins contain more tyrosine residues that could be sulphated. Bovine, human and rat fibromodulins contain five, six and seven tyrosine residues respectively with the consensus sequence for sulphation. When fibromodulins from these species are compared (Figure 3) only one of the tyrosine residues is in a conserved position. This region of the core protein is thought to be of importance for interactions with other matrix molecules and therefore the alterations in the negative charge are potentially important. The influence of such a decrease in charge on the functions of fibromodulin needs to be determined.

Figure 2 Comparison of fibromodulin mRNA in 14- and 19-day chicken embryo tendon by Northern blot analysis

The fibromodulin mRNA level increased 6–8-fold from day 14 to day 19 of tendon development. Poly(A)⁺ RNA (1 µg) was separated in 1% formaldehyde agarose gels and transferred to a Hybond membrane. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used to normalize the different mRNAs.

The up-regulation of fibromodulin as determined by its isolation from a subtracted library was confirmed by Northern analysis (Figure 2). The Northern blot demonstrates a 6–8-fold increase in fibromodulin mRNA between 14 and 19 days of

tendon development when normalized to glyceraldehyde-3-phosphate dehydrogenase. Previous data from our laboratory [4] have shown that abrupt collagen fibril growth occurs between days 16 and 18 of tendon development. The 6–8-fold increase in fibromodulin mRNA expression is seen in the post-growth phase. Thus fibromodulin may play a role in the stabilization of fibrils within the mature tissue and/or facilitate interaction with other matrix components necessary for tissue integrity and function.

DISCUSSION

The amino acid sequence of chicken fibromodulin has been deduced from the full-length cDNA. The sequence predicts a protein containing 380 amino acid residues. The distinguishing feature of the putative protein is a 20–23-amino-acid-long leucine-rich sequence repeated 11 times. The 80% similarity of the deduced amino acid sequence with those of other known fibromodulins confirms the identity of the clone as chicken fibromodulin.

Fibromodulin, lumican and decorin are members of a subfamily of the SIPGs, based on their sequence identity [3,21]. All have been shown to alter the kinetics of collagen fibril formation *in vitro* [3,8,10,23,24]. *In vivo* these PGs show specificity with

Figure 3 Comparison of the chicken fibromodulin deduced amino acid sequence with those of bovine, rat and human fibromodulins

The Cys residues are in conserved positions (open boxes). Potential glycosylation sites are also conserved (shaded boxes). Tyr residues that could be sulphated are shown in bold. Only one of these is conserved in all species compared. Accession nos.: bovine (bov), X16485; rat, X82152; human (hum), X75546.

respect to binding to fibrils; decorin binds at the d and e bands, whereas fibromodulin and lumican both bind at the a and c bands [25]. The differences in fibromodulin and lumican (keratan sulphate PGs) compared with decorin (dermatan sulphate PG) interactions with fibrils may be the result of modulation of the core protein interaction by the different glycosaminoglycan chains. The regions of the core protein containing the LRRs have been implicated in the binding to fibrils [20,26]. Since the degree of identity among LRRs is comparable for fibromodulin, lumican and decorin, those that differ in modifications such as potential sites for glycosylation might influence the affinity for fibrils. It has been shown that LRRs 4–5 of decorin are the major binding sites for type I collagen. Additional sites located either N- or C-terminally may modify the affinity [27]. The decorin LRR 4 differs from the fibromodulin LRR 4 in that it is not glycosylated. This suggests that glycosylation of particular LRRs may determine the specificity of SIPG binding. Given this, candidate regions contributing to collagen binding by fibromodulin are LRRs 6–8.

The data presented here show a 6–8-fold increase in fibromodulin mRNA levels from day 14 to day 19 of tendon development. We previously reported that decorin mRNA levels increase 3–4-fold during the same stages. It has also been shown that growth of fibril segments occurs during days 17 and 18 of tendon development [4]. Thus we suggest that, *in vivo*, SIPGs may stabilize the fibrillar matrix, and PG–collagen interactions may regulate fibril growth and influence higher-order matrix assembly. It has been proposed that a function for these PGs on the collagen fibril surface may be to determine and modify the surface properties and, therefore, interactions with other matrix macromolecules as suggested from studies in cartilage [28]. Our demonstration of an increase in fibromodulin mRNA during the later stages of matrix assembly suggests a role for this PG in the organization of fibrils into fibres and tissue-specific matrices [12,29].

Another possible regulatory function of fibromodulin could involve interactions with growth factors. An increase in the synthesis of SIPGs was reported in some cell lines after addition of growth factors including transforming growth factor- β (TGF- β) [30,31]. Decorin can bind TGF- β through its core protein and may serve as a reservoir of the growth factor in the extracellular matrix [32,33]. Fibromodulin also binds different TGFs [34]. Thus these PGs might be effector molecules in a negative feedback loop regulating TGF- β activity. Our identification of up-regulated TGF- β , insulin-like growth factor and signalling molecule genes (M. V. Nurminskaya and D. E. Birk, unpublished work) during this period of tendon development suggests that fibromodulin may be implicated in growth factor regulation.

The question regarding the function of fibromodulin and the rest of this class of PGs in the extracellular matrix remains. The roles of interactions with fibrillar collagen, influence on fibril and matrix assembly as well as binding of regulatory molecules such as growth factors remain to be fully elucidated.

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