Xi'an ShiyouDaxueXuebao (ZiranKexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication:Online Open Access
Vol: 65 Issue 03 | 2022
DOI 10.17605/OSF.IO/V4S8M

THE ROLE OF NOVEL PROMOTERS IN *Igf2* GENE EXPRESSION REGULATION

Van Giang Tran[1] *, Guy Cathala[2] and Thierry Forné[2]

[1]University of Education, Hue University, Hue city, 49000, Vietnam
[2]Institut of Genetique Moleculaire in Montpellier, UMR 5535 CNRS-Montpellier II University, Montpellier, France.
Email: tranvangiang@dhsphue.edu.vn, tvgiang@hueuni.edu.vn

Abstract:

Genomic imprinting occurs when only one allele of a gene is expressed depending on its parental origin. The imprinted *Igf2* gene (Insulin-like growth factor 2) encodes a growth factor that plays an important role in embryonic development and formation of the placenta. The regulation and expression of *Igf2* are carried out by different promoters. The promoter expression is extremely complex in wild-type mice during development and is altered in several mutant mice that have deletions at the *Igf2*/*H19* locus. In this experiment, two newly discovered promoters were Pm and Pm2. The expression levels of these two promoters were investigated in different tissues, such as the tongue, kidney, muscle and liver of wild-type mice. We investigated the imprinting levels of the two promoters simultaneously, and they were both highly expressed on the allele derived from the father. All these RNAs were reversely transcribed to cDNA before quantifying the expression levels of the promoters by quantitative PCR (qPCR). Our results show that the transcriptions of the *Igf2* Pm and Pm2 promoters are the highest in epidermal tissue. That is why they are named Pm and Pm2 (mesoderme).

Key words: gene expression, genomic imprinting, *Igf2*, promoter.

INTRODUCTION

A typical human cell expresses only approximately 20% of the total number of genes at a determined time, from which only 1.5% are coded for proteins [2]. Gene expression refers to the process that translates the genetic information of genes into mRNAs and proteins. The expression of each gene is controlled and tightly regulated by various mechanisms within the cell. The main elements controlling the expression process are gene promoters. A promoter is a region of DNA that initiates the transcription of a particular gene. Promoters are located around transcription start sites (TSSs) of genes, on the same strand and upstream of the coding DNA sequence. Promoters can be approximately 100 - 1000 base-pairs long [17]. A gene can be controlled by one or several promoters. Studies on the mechanism of the expression and regulation of gene expression in eukaryotes, and more particularly in mice, are particularly complex, especially for imprinted genes [3, 7]. The expression of the Insulin-like growth factor 2 (*Igf2*) gene is quite complicated because it is carried out by several promoters and occurred only on the paternally inherited allele. The expression levels of different promoters are completely different depending on the tissue and the mouse strain [4, 5, 19]. In mammals, embryonic growth and development are controlled by a number of genes, among them is *Igf2*. This imprinted gene is located on the chromosome 11p15 in humans and the chromosome 7 in mice. It is paternally expressed both in humans and in mice. The regulation of *Igf2* gene expression is

Xi'an ShiyouDaxueXuebao (ZiranKexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication:Online Open Access
Vol: 65 Issue 03 | 2022
DOI 10.17605/OSF.IO/V4S8M

extremely complex. The abnormal expression, loss of imprinting or hypermethylation of this gene lead to Beckwith-Wiedemann syndrome, Silver-Russell syndrome or Wilms' tumors in children. In adults, it is also involved in some pathways leading to cancers [1, 14, 20]. When the *Igf2* gene was inactivated in mouse embryos, the weight of the offspring was 40% lower than that of the normal offspring issued from the same litter [6, 15]. Knockout of endodermic specific enhancers, located at the *Igf2*/*H19* locus, results in fetuses lacking *Igf2* expression in the endoderm [9, 18]. Many subsequent studies have confirmed that the *Igf2* gene plays an important and direct role in the proliferation, differentiation and differentiation of cells in mammals [6]. There are 4 promoters that drive *Igf2* expression levels; among them, P1-P3 are transcribed in all tissues, while P0 is expressed specifically in the placenta [4, 11, 12]. There are some significant differences in the size of the resulting transcripts [10, 13]. Furthermore, this gene also plays an important role in the development and differentiation of the placenta. In *Igf2* knockout mice, the size of the placenta was reduced, resulting in a body weight at birth that was only 60% compared to wild-type mice [6, 8]. 5'RACE experiment from the capped fraction of non-polyadenylated 7-day-old mouse liver RNAs then mapped the Transcription Start Site (TSS) of the promotor more precisely to the position of novel promotors. However, the tissue-specific expression levels of the different promoters in wild-type and mutant mice have not yet been extensively explored.

MATERIALS AND METHODS

Generation of mouse crosses: *Mus musculus* mice were fed at the Institute of Molecular Genetics of Montpellier (France). Several crosses were generated. In the SD7/Dom and Dom/SD7 crosses, the paternal chromosomes were either wild type or issued from the Mus spretus strain (SD7). The *H19*Δ3 mutant strain was provided by Luisa Dandolo's laboratory (INSERM Paris, France) [15, 20]. Δ3/Dom is a cross where the maternal allele of the *H19* gene is removed. *H19* is an imprinted gene, repressed on the paternal allele and located at the same locus as *Igf2*. It encodes a noncoding RNA that participates in the regulation *of the Igf2* gene (Figure 1). U2/Dom is a mutant mouse cross that removes the U2 exon from the maternal *Igf2* allele. The primers used in the experiments are shown in table 1.

DNA extraction: tissues were homogenized at room temperature in 2 mL of buffer. 40 µl proteinase (20 µg /ml) dissolved in pK 2X buffer was then added. After 4 hours of incubation at 50°C, the reaction was extracted with 4 ml of phenol/chloroform (1:1) and centrifuged for 30 minutes at 20°C at 6000 rpm. The supernatant was precipitated with 8 ml of EtOH and 300 µl of 5M NaCl at -20°C for 12 hours or overnight. The reaction mixture was centrifuged to collect the RNA pellet that was washed with 500 µl 70% EtOH. The DNA was then dried at room temperature.

Extraction of total RNA: Total RNA extraction was similar to DNA extraction with a few differences: after adding PK, we performed DNase I treatment to remove DNA. During extraction (by acidic phenol), centrifugation was carried out at a low temperature (4°C). After total RNA was obtained, total RNA was extracted through a column containing

Xi'an ShiyouDaxueXuebao (ZiranKexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication:Online Open Access
Vol: 65 Issue 03 | 2022
DOI 10.17605/OSF.IO/V4S8M

oligo dT to separate the mRNA from the mixture. The mRNA was then recovered by elution.

Table 1. Primer sequences.

Amplicon names	Forward primer sequence	Reverse primer sequence
P0 mRNA	5'-ATTGACCCAGCCAGCGGATC-3'	5'-CTGTACTCTAGTCGCTTCGTAG-3'
P1	5'-CTCGTCACTTCTCCTACGGTG-3'	5'-CCCAGTCGTTTTCCTGGACAC-3'
P2	5'-GTTCTGTCCCGTCGCACATT-3'	5'-GGTATGCAAACCGAACAGCG-3'
P3	5'-CTGGACATTAGCTTCTCCTG-3'	5'-CTGAAGTTGGGTAAGGAGGC-3'
*Igf2 total**	5'-CATCGTCCCCTGATCGTGTTAC-3'	5'-GGAACTGTCCCTGCTCAAGA-3'
5'Race	5'-CGACTGGAGCACGAGGACACTGA-3'	
GeneRacer RNA oligo	5'CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA3'	

This amplification allows quantification of Igf2 mRNAs issued from all known promoters.

Preparation of cDNA: To 2 µg of DNase treated RNA, 1 µl of random primer or specific primer and 10 µl of sterile H2O were added. The samples were incubated at 70°C for 10 minutes and then placed on ice for 5 minutes. Add 4 µl of buffer 5X (FS), 2 µl of DTT 0.1 M, 1 µl of dNTP 2.5 mM, and 0.7 µl of reverse transcriptase. The mixture was stabilized for approximately 10 minutes at room temperature and incubated at 42°C for 1 hour. The cDNA was purified by glass balls and washing solution. cDNA was diluted 10 times for quantifying PCR or for storage at -20°C.

PCR Quantifying: In this work, the Gapdh reference gene was used to calculate the relative quantities of mRNAs of the target gene (*Igf2*) expressed in the samples. The reaction mixture consisted of 1 µl of cDNA (5 ng/µl), 1 µl of qPCR mix (containing dNTP, MgCl2, qPCR 10X buffer), 0.5 µl of each primer (10 µM stock) and 7 µl of sterilized water. The positions of the primers used in the experiments are shown in figure 2 (black arrowheads). PCR program: 95°C, 2 minutes and then 41 cycles (95°C/5 s; 52°C/15 s; 72°C/30 s).

The 5' RACE method: This method is used to screen new putative promoters. To 2 µg of DNase treated ARN, add 1 µl of Buffer CIP (Calf Instestine Alakine Phosphatase) 10 X, 1µl of RNAseOut (40U/µl), 1µl of CIP (10U/1µl) and 5µl of DEPC. Incubate the samples at 50°C for 1 hour, then add 90 µl of DEPC and 100 µl phenol:chloroform. Strongly vortex the samples during 30 seconds and centrifuge at 16000rpm for 5 minutes at room temperature. Collect the supernatant then add 2 µl of glycogen (10mg/ml), 10 µl of 3M sodium acetate (pH 5.2) and mix well. Add 220 µl of 95% ethanol, vortex, and put on ice for approximately 10 minutes. Centrifuge at 4°C for 20 minutes (16000rpm) and wash the pellet with 500 µl of 70% ethanol. Centrifuge, dry and add 7 µl of DEPC. Add 1 µl of TAP (Tobacco Acid Pyrophosphate) buffer 10X, 1 µl of RNaseOut (40U/µl) and 1 µl of TAP (0.5 U/µl) (total volume is 10 µl). Gently shake and incubate the mixture at 37°C for 1 hour. Centrifuge to precipitate the RNA and dissolve it in 7 µl of DEPC. Next, add this reaction into a tube containing a 0.25 µg of GeneRacer RNA Oligo, incubating at 65°C for 5 minutes, cool the tube in ice for 2

Xi'an ShiyouDaxueXuebao (ZiranKexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication:Online Open Access
Vol: 65 Issue 03 | 2022
DOI 10.17605/OSF.IO/V4S8M

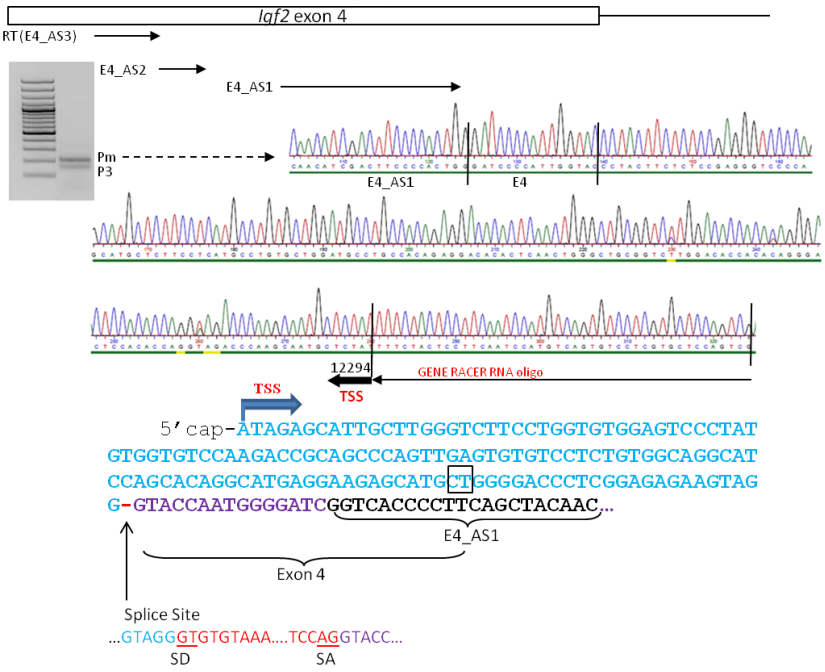
minutes then add 1 µl of 10X ligase buffer, 1 µl of ATP (10 mM), 1 µl of RnaseOut (40 U/µl), and 1 µl of T4 RNA ligase (5 U/µl). Incubate the mixture at 37°C for 1 hour and then centrifuge it to collect RNA and add 10 µl of DEPC water. Store the reaction mixture at -20°C.

RESULTS AND DISCUSSION

Characterization of a new promoter: the Pm promoter

Highlight: Ectopic expression of *H19* RNA and its antisense in *H19* KO-/- myoblasts therefore re-established *Igf2* RNA expression despite the presence of a cis-demethylated ICR. In addition, through an RT-qPCR study, we have shown that this re-expression does not involve transcripts from classical *Igf2* promoters (P0, P1, P2, P3). It was, therefore, necessary to look for one or more alternative promoters likely to be activated by the *H19* gene.

A



Xi'an ShiyouDaxueXuebao (ZiranKexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication:Online Open Access
Vol: 65 Issue 03 | 2022
DOI 10.17605/OSF.IO/V4S8M

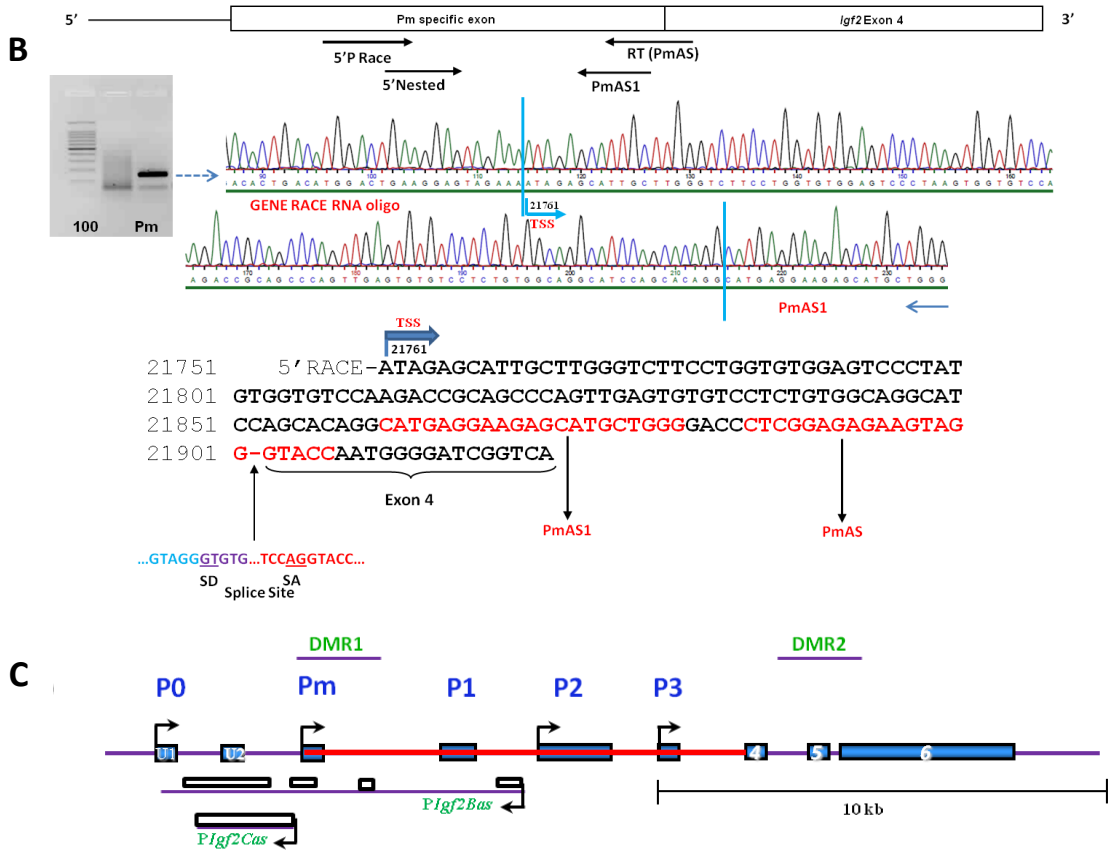


Figure 1. Characterization of the TSS of RNAs derived from the new Pm promoter in clone 4 and in mouse liver. A and B. Represents the same TSS and the same Pm splicing identified in the liver of 7 days old mice. The top the diagram indicates the strategy we followed: after ligation of the 5'RACE RNA, we performed RT-qPCR with a primer complementary to the splice site found in A and followed by nested qPCRs. Genomic coordinates are given from the Poly-A site of insulin (+1), SD = Splice Donor; SA = Splice Acceptor. C. Map of the Igf2 gene showing the Igf2 Pm promoter. The sense and antisense Igf2 exons are shown as black and white rectangles respectively. The first intron of the Pm Igf2 mRNA is indicated in red.

Figure 1. Characterization of the TSS of RNAs derived from the new Pm promoter in clone 4 and in mouse liver. A and B. Represents the same TSS and the same Pm splicing identified in the liver of 7 days old mice. The top the diagram indicates the strategy we followed: after ligation of the 5'RACE RNA, we performed RT-qPCR with a primer complementary to the splice site found in A and followed by nested qPCRs. Genomic coordinates are given from the Poly-A site of insulin (+1), SD = Splice Donor; SA = Splice Acceptor. C. Map of the Igf2 gene showing the Igf2 Pm promoter. The sense and antisense Igf2 exons are shown as black and white rectangles respectively. The first intron of the Pm Igf2 mRNA is indicated in red.

We investigated the actual initiation of *Igf2* gene transcripts in clone 4, which exhibited the strongest re-expression of *Igf2* using a 5 'RACE experiment. In addition, from the expected transcriptional initiations, which correspond to the known promoters, we have demonstrated a new *Igf2* transcript, initiated between the P0 and P1 promoters,

Xi'an ShiyouDaxueXuebao (ZiranKexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication:Online Open Access
Vol: 65 Issue 03 | 2022
DOI 10.17605/OSF.IO/V4S8M

which begins with a first specific exon of 141 nucleotides linked to exon 4 , common to all known *Igf2* transcripts, thanks to a conventional splicing site (Fig. 1A). This transcript could also be demonstrated in *Igf2* RNAs obtained from the liver (Fig. 1B). The Pm promoter is activated in transfected clones: the Pm transcripts could be quantified using a primer in its first specific exon (like all other different *Igf2* transcripts), the other being located in exon 4 common to all *Igf2* messenger RNAs. Compared to its level in *H19* KO-/- myoblasts, the Pm promoter is activated in *H19* KO -/- myoblast clones, transfected with the *H19* gene, except in clone 11. Therefore, it is named Pm. The Pm promoter is induced during muscle differentiation: We then analyzed the level of Pm promoter expression during the differentiation of C2C12 myoblasts into myotubes after 3 days of serum deprivation (Fig. 4B). Pm expression is significantly increased during differentiation; the quantity of Pm transcripts is multiplied by 23. The trend is, therefore, the same as that for the P3 transcript of *Igf2,* which is the major transcript of *Igf2* under normal conditions. The Pm promoter is expressed in tissues of mesodermal origin: as we demonstrated Pm RNA in myoblasts, it was important to look for its expression in mouse tissues. We found it was mainly expressed at 6 days after birth in tissues of mesodermal origin (especially in the kidney) and expressed at low levels in the liver (endodermal tissue) or brain (ectodermal tissue) (Tran et al. 2012). However, at the e15 embryonic stage, a stronger expression of Pm was observed in the muscles and tongue (Fig. 4C). Experiments subsequent to the publication of our article have shown that this promoter is also very well expressed in the placenta (Fig. 4A).

The Pm promoter is expressed throughout late development: We performed a systematic analysis of the expression of Pm transcripts during the perinatal period in mice (Fig. 4C). The Pm expression levels were compared here with those of the RNAs derived from the majority promoter P3. In kidneys, the level of Pm transcript expression remains stable after birth. In tongue, its expression declines faster than the set of P3 transcripts. In muscles, the transcription rate of Pm RNA is very high at the embryonic stage e15, where it is almost as strong as that of P3. It is clear that at the earliest stages analyzed, the expression of these transcripts is far from negligible. The Pm promoter is subject to parental genomic imprinting: Finally, it was important to know whether this Pm promoter, capable of being activated despite a demethylated ICR, remained under the influence of the parental genomic imprint. This promoter is almost exclusively expressed from the paternal allele. It is, therefore, subject to parental genomic imprinting (Fig. 2).

Xi'an ShiyouDaxueXuebao (ZiranKexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication:Online Open Access
Vol: 65 Issue 03 | 2022
DOI 10.17605/OSF.IO/V4S8M

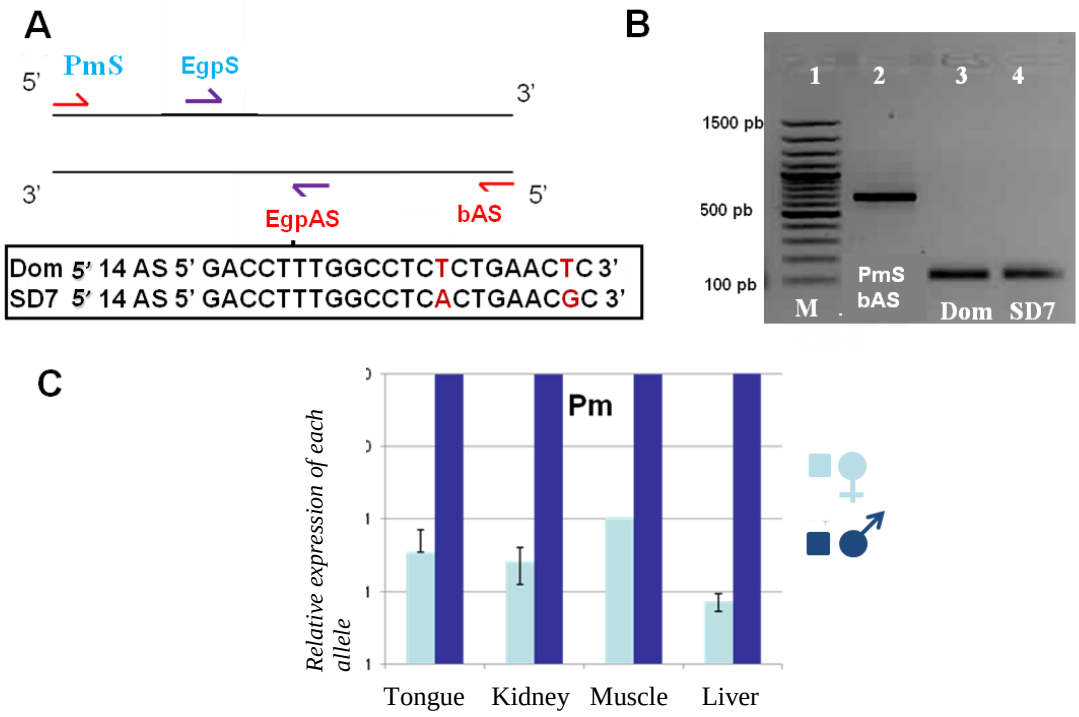


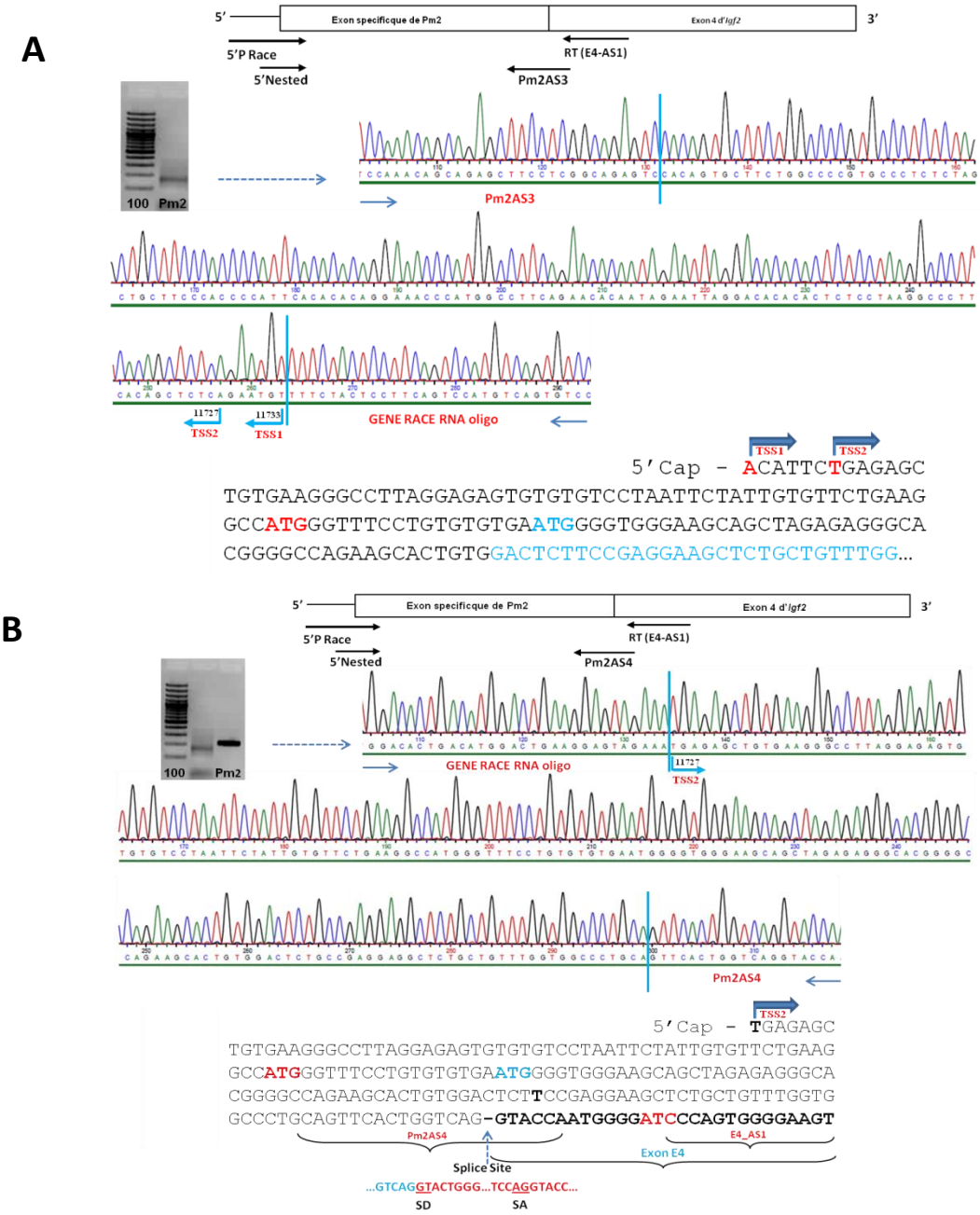
Figure 2. Determination of the allelic expression of RNAs derived from the Pm promoter in dom/SDP711 mice. Allele of maternal origin: ♀ dom, *allele* of paternal origin: ♂ SDP711. A. Strategy. A large Pm-specific fragment is amplified first (using a PmS primer from exon Pm and another common to all bAS transcripts in exon 6). Then, the product of this PCR (B line 2) is subjected to a nested PCR with the pair of specific primers of each parental *allele* (EgpS with either Egp domAS or Egp SD7AS), the products of which are presented in B lines 3 and 4, agarose gel electrophoresis of the PCR products. C. Relative rates of transcription of each *allele* in the 4 tissues studied.

We took advantage of the fact that in clone 4, the expressions of the classical strong *Igf2* promoters were largely repressed to make an inventory of weak promoters likely to be functional in certain circumstances (tissues, pathologies, age, etc.). To do this, we analyzed on agarose gels the various products resulting from a 5 'RACE experiment on the RNAs extracted from clone 4, treated with alkaline phosphatase CIP (Calf Intestinal Phosphatase), pyrophosphatase TAP (Tobacco Acide Pyrophosphatase) and reverse transcripts. Then, we amplified them by PCR the cDNAs obtained using an oligonucleotide complementary to the 5 'end of exon 4, common to all the *Igf2* transcripts, and the PCR primer of 5' RACE (5'P RACE). Each amplification band isolated by gel cutting was subjected to nested PCRs, which were sequenced after cloning. Accordingly, all of the transcripts that will be described in this section are capped at their 5' end. They are, therefore, not pieces of RNA resulting from degradation.

Highlight: Reproductionally, we have characterized a new initiation of the transcription of the *Igf2* gene that begins with a new specific exon of 186 or 178 nucleotides linked

Xi'an ShiyouDaxueXuebao (ZiranKexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication:Online Open Access
Vol: 65 Issue 03 | 2022
DOI 10.17605/OSF.IO/V4S8M

to exon 4, common to all *Igf2* transcripts, by a site of classic splicing. The 3' terminus of this exon is located 389 nucleotides upstream of the Pm promoter (Fig. 3A and C). We have verified that this promoter also exists in the liver (Fig. 3B).



Xi'an ShiyouDaxueXuebao (ZiranKexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication:Online Open Access
Vol: 65 Issue 03 | 2022
DOI 10.17605/OSF.IO/V4S8M

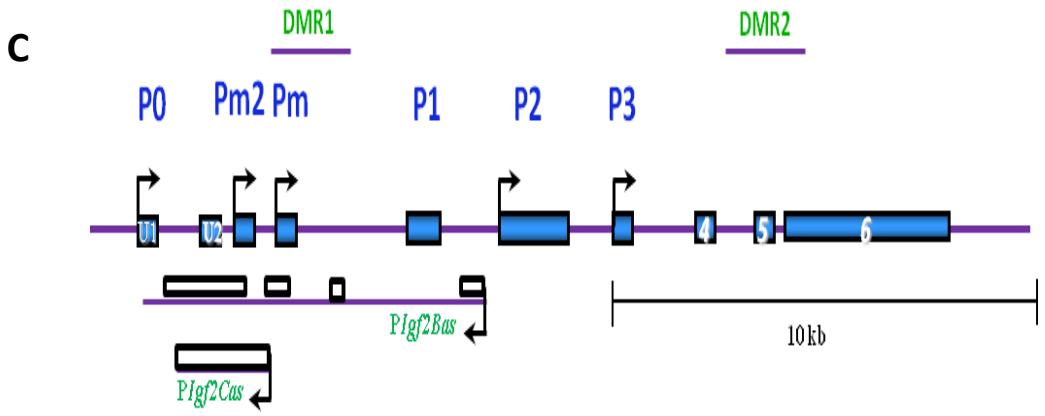


Figure 3. Characterization of the TSS of the RNAs derived from Pm2 in the transfected *H19* KO -/- myoblasts (clone 4) (A) and in the liver (B). The strategy used is presented at the top of each sequence. After ligation of the 5'P RACE oligoribonucleotide, we performed specific RT from exon 4 (primer E4-AS1) before performing nested PCR. Here, we were aided by the presence of a splice site identified in a preliminary experiment. We have indeed used a primer targeting the Pm2 and E4 junction (Pm2AS3 (A), Pm2AS4 (B)). Nested PCRs were analyzed on agarose gel, and bands were cut and cloned for sequencing. The position of the Pm2 promoter is reported in the diagram presented in C.

Level of expression of the Pm2 promoter in tissues, during muscle differentiation and during late development: RNA levels from the Pm2 promoter were compared to those from the Pm promoter in the tissues of 6-day-old mice in embryos and even in the placenta (Fig. 4A & C). They were also compared with those of the P3 transcript during differentiation of myoblasts (Fig. 4B) or during its development (Fig. 4C). With the exception of muscles, RNAs from both Pm and Pm2 promoters showed similar levels of expression. In addition, they were strongly expressed in the placenta (Fig. 4A). Regarding the differentiation of myoblasts into myotubes, a very high activation of the Pm2 promoter was observed, greater than that of the Pm and P3 promoters (Fig. 4B). The results obtained during late development are shown in Figure 4C with respect to the activity of the P3 promoter. The RNAs from Pm2 and Pm decrease overall during the perinatal period. Their level of expression depends on the stage of development (Fig. 4C).

Xi'an ShiyouDaxueXuebao (ZiranKexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication:Online Open Access
Vol: 65 Issue 03 | 2022
DOI 10.17605/OSF.IO/V4S8M

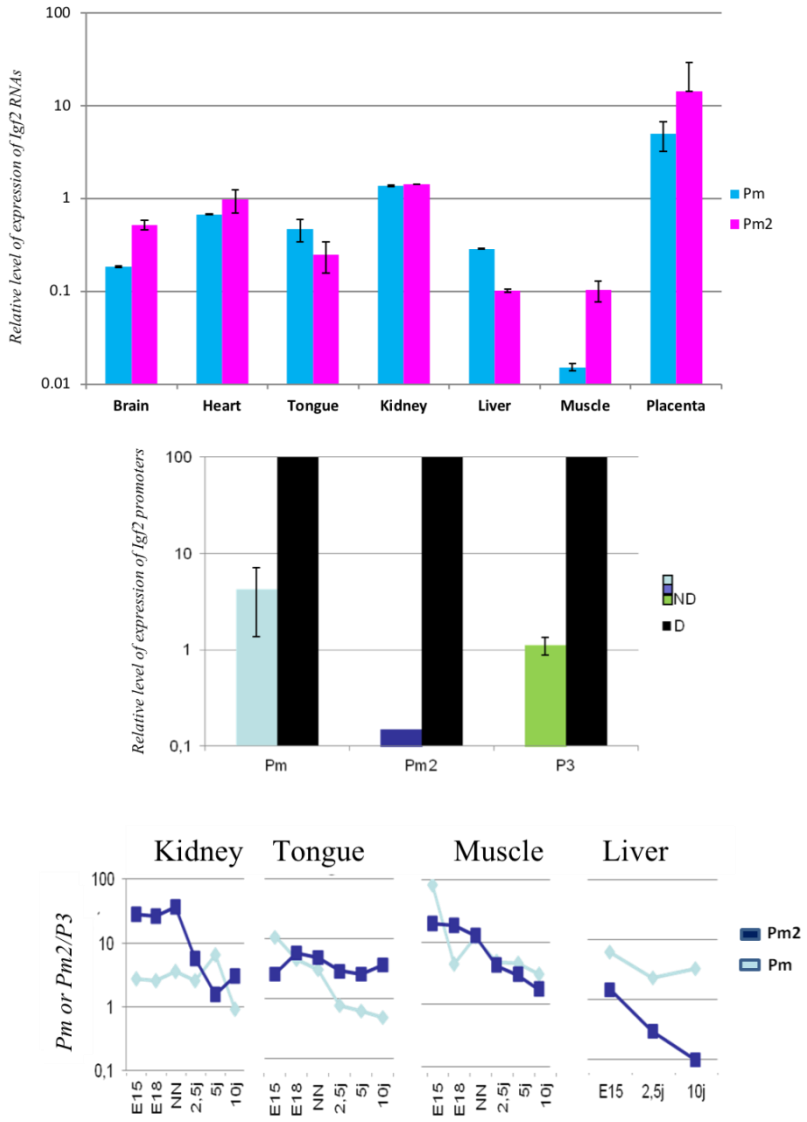


Figure 4. Expressions of RNAs from the Pm and Pm2 promoters. The RT-qPCR measurements in A come from amplifications carried out with a primer located in the first specific exons of Pm or Pm2, and another in exon 4, common to all transcripts (E4-AS1). These quantifications therefore cannot be normalized with a range of genomic DNA to be rigorously compared. However, we have verified, from dilutions of a standard range, that the values of the amplification parameters a and b (slope and "intercept") are completely comparable. B, represents the relative level of expression during differentiation of C2C12 myoblasts. Undifferentiated cells (ND) and cells after 3 days of differentiation (D). C. represents the relative expression levels during the perinatal period in mice.

The Pm2 promoter is subject to parental genomic imprinting**:** We analyzed the level of expression of the Pm2 promoter (with the same strategy as for Pm) on each parental allele in several tissues, including the placenta. Like all *Igf2* promoters, this promoter is expressed only on the paternal allele and therefore is subject to parental genomic imprinting (Fig. 5).

Xi'an ShiyouDaxueXuebao (ZiranKexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication:Online Open Access
Vol: 65 Issue 03 | 2022
DOI 10.17605/OSF.IO/V4S8M

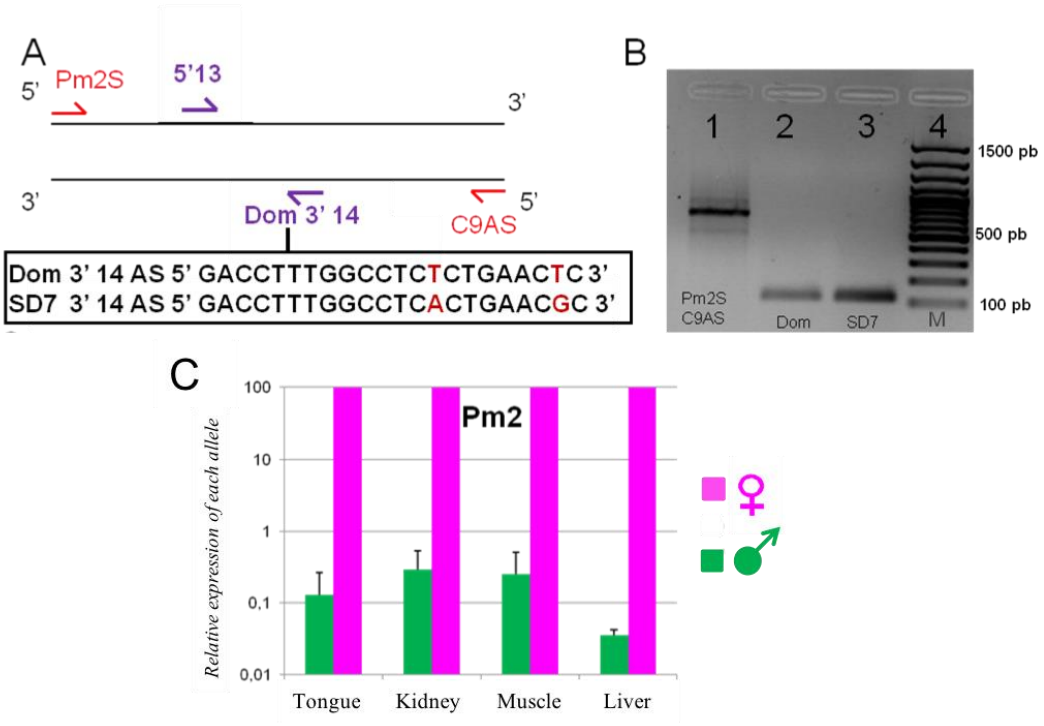


Figure 5. Parental genomic imprinting of the Pm2 promoter. Allele of maternal origin: ♀, allele of paternal origin: ♂. A. Amplification strategy : first, a large specific fragment of Pm or Pm2 is amplified (amplification with the oligonucleotide C9AS). The products of this PCR are then analyzed with the pairs of primers specific to the parental alleles (the antisense oligonucleotides dom and spretus are indicated). B. Electrophoresis of PCR products for Pm2 is shown by way as an example. 1 amplification Pm2C9AS, 2 specific amplification dom, 3 specific amplification spretus, 4 molecular weight markers. C. Relative transcription rate of each allele, from Pm2 or Pm, in the 4 tissues studied (Tongue, Kidney, Muscle, Liver).

The Pm2 promoter is not activated in transfected clones: We observed that transfected clones expressed Pm2 transcripts of the *Igf2* gene at a level similar to that seen in untransfected cells. Furthermore, using clone 4, we showed that this Pm2 promoter, unlike the Pm promoter, was not reactivated by the ectopic sense and/or antisense RNA expressions of the *H19* gene (Fig. 6).

Due to the importance of the *Igf2* gene, and unlike the negative regulations described above, the cell has been secured, during evolution, the possibility of initiating transcription of the *Igf2* gene by multiplying the number of its promoters and its potential enhancers. It, therefore, seems that one of the concerns of the mammalian cell is to control the expression of the *Igf2* gene as closely as possible by a complex network of regulations involving the entire locus. My paper is mainly interested in the transcriptional aspect of this regulation in mice, in order to identify the complexity of this network, and in the longer term, to transfer the knowledge acquired to humans, in an attempt to open up new avenues in cancer treatment.

Xi'an ShiyouDaxueXuebao (ZiranKexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication:Online Open Access
Vol: 65 Issue 03 | 2022
DOI 10.17605/OSF.IO/V4S8M

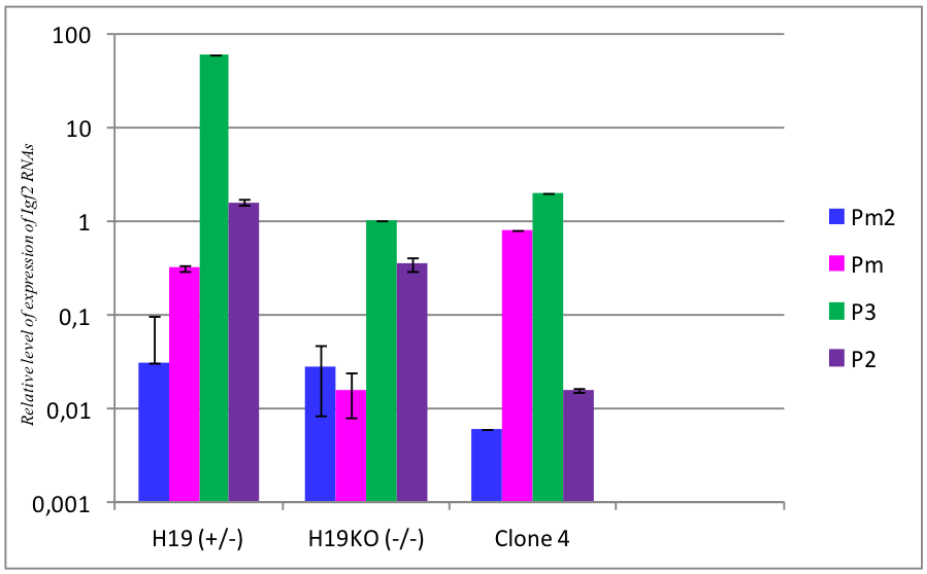


Figure 6. Comparison of RNA expression levels from different Igf2 promoters (P3, P2, Pm and Pm2) in myoblasts. In this experiment, the RNAs derived from the P3 and P2 promoters were measured using a sense primer specific for their first exon and the antisense primer located in exon 4 (E4-AS1) were compared with those derived from Pm and Pm2 (Fig. 4).

For more than a decade, qPCR methodology has replaced the Northern blot technique in RNA measurement. However, the two types of technique are not contrasting/in competition with each other; they are not equivalent, but complementary. To study Igf2 RNAs, it is clear that one cannot perform without a Northern blot analysis which measures not only the quantity, but also the size, of the RNAs and therefore their quality. This technique, is less precise and more tedious than RT-qPCR, but it is much more informative and less prone to misinterpretations. Indeed, the measurements by RT-qPCR in the 5' part of the *Igf2* gene are misleading. Due to multiple co- or post-transcriptional regulations, they absolutely do not give access to that of the functional messenger RNAs, which will make it possible to produce the IGF2 protein, although they give a good idea of the transcriptional level. This example constitutes a textbook case and deserves to be considered for other genes, especially those used in diagnostics: before placing all your confidence in a measurement by RT-qPCR, it is essential to know what the corresponding amplification makes it possible to account for.

CONCLUSION

In *H19* KO -/- myoblasts, re-expression of *Igf2* messenger RNA is partly or totally due to the initiation of a new endogenous *Igf2* (Pm) promoter activated by the ectopic antisense transcripts of the *H19* RNA from a plasmid introduced by transfection. The two new promoters have similarities. They are activated in the process of differentiation of C2C12 cells. They are subject to parental imprint and expressed during development. However, they are very different in the levels of their expression

ISSN: 1673-064X
E-Publication:Online Open Access
Vol: 65 Issue 03 | 2022
DOI 10.17605/OSF.IO/V4S8M

in muscles, and especially unlike Pm, Pm2 is not reactivated in *H19* KO -/- myoblasts after transfection of the *H19* transgene.

Acknowledgments: We are very grateful to Thierry Forné's laboratory at the Montpellier Institute of Molecular Genetics, Luisa Dandolo (INSERM laboratory, Paris), for providing the *H19*Δ3 mutant strain, and the French Republic for providing the Gapdh primers for quantifying RNAs and several other chemicals. This work was supported by Hue University under the Core Research Program, Grant No. NCM.DHH.2022.07.

REFERENCES

[1] Bhusari S., Yang B., Kueck J., Huang W., Jarrard D. F. (2011) Insulin-like growth factor -2 (*IGF2*) loss of imprinting marks a field defect within human prostates containing cancer prostate. Prostate. 71(15): 1621 - 1630.

[2] Biressi S., Molinaro M., Cossu G. (2007) Cellular heterogeneity during vertebrate skeletal muscle development. Devel Bio. 308: 281 - 293.

[3] Butler, J. E. F., and J. T. Kadonaga. (2002) Regulation of Gene Expression The RNA Polymerase II Core Promoter: A Key Component in the Regulation of Gene Expression. Genes & development 16: 2583 – 2592.

[4] Chao, W., and A. D. Patricia. 2008. "*IGF2*: Epigenetic Regulation and Role in Development Disease." Cytokine Growth Factor Rev. 19(2): 111 – 120.

[5] Constância M., Hemberger M., Hughes J., Dean W., Ferguson-Smith A., Fundele R., Stewart F., Kelsey G., Fowden A., Sibley C., Reik W. (2002) Placental - specific IGF-II is a major modulator of placental and fetal growth. Nature: 417 (6892): 945 - 948.

[6] Dai, B., H. Wu, E. Holthuizen, and P. Singh. (2001) Identification of a Novel Cis Element Required for Cell Density-Dependent down-Regulation of Insulin-like Growth Factor-2 P3 Promoter Activity in Caco2 Cells. The Journal of biological chemistry 276(10): 6937 – 6344.

[7] DeChiara T. M., Efstratiadis A., Robertson E. J. (1990) A growth - deficiency phenotype in heterozygous mice carrying an insulin - like growth factor II gene disrupted by targeting. Nature. 345(6270): 78 - 80.

[8] Hardouin, S. N., R. Guo, P. Romeo, A. Nagy, and J. E. Aubin. (2011) Impaired Mesenchymal Stem Cell Differentiation and Osteoclastogenesis in Mice Deficient for *Igf2*-P2 Transcripts. Development. 138(2): 203 – 213.

[9] Joanne L., Thorvaldsen., Kristen L., Duran., Marisa S., Bartolomei. (1998) Deletion of the *H19* differentially methylated domain results in loss of imprinted expression of *H19* and *Igf2*. Gene & Development. 12(23): 3693 - 3702.

[10] Leighton, P. A., Saam, J. R., Ingram, R. S., Stewart, C. L. and Tilghman, S. M. (1995). An Enhancer Deletion Affects Both *H19* and *Igf2* Expression. Genes & Development. 9(17): 2079 – 2089.

[11] Meinsma D., Holthuizen P. E., Van den Brande J. L., Sussenbach J. S. (1991) Specific endonucleolytic cleavage of IGF-II mRNAs. Biochem Biophys Res Commun. 179(3): 1509 - 1516.

[1]2 Monk, D., R. Sanches, P. Arnaud, S. Apostolidou, F a Hills, S Abu-Amero, A. Murrell, H. Friess, W. Reik, P. Stanier, M. Constância, and G. E. Moore. (2006) Imprinting of *IGF2* P0 Transcript and Novel Alternatively Spliced INS-*IGF2* Isoforms Show Differences between Mouse and Human. Human molecular genetics 15(8): 1259 – 1269.

[13] Moore, T., M. Constancia, M. Zubair, B. Bailleul, R. Feil, H. Sasaki, and W. Reik. (1997) "Multiple imprinted sense and antisense transcripts, differential methylation and tandem repeats in a putative imprinting control region upstream of mouse *Igf2*." Proceedings of the National Academy of Sciences of the United States of America 94(23): 12509 – 12514.

ISSN: 1673-064X
E-Publication:Online Open Access
Vol: 65 Issue 03 | 2022
DOI 10.17605/OSF.IO/V4S8M

[14] Nielsen J, Christiansen J, Lykke-Andersen J, Johnsen AH, Wewer UM, Nielsen FC (1999) A family of insulin-like growth factor II mRNA-binding proteins represses translation in late development. Mol Cell Biol 19: 1262–1270.

[15] Ohta, M., T. Sugimoto, M. Seto, D. Mohri, Y. Asaoka, M. Tada, Y. Tanaka, Y. Yamaji, F. Kanai, T. Kawabe, and M. Omata. (2008) Genetic Alterations in Colorectal Cancers with Demethylation of Insulin-like Growth Factor II. Human pathology 39(9): 1301 – 1308.

[16] Ripoche M.A., Kress C., Poirier F., Dandolo L. (1997) Deletion of the *H19* trancription unit reveals the existence of a putative imprinting control element. Genes & Development. 11: 1596-1604.

[17] Sambrook J., Russel D. W. (2001) Molecular cloning: A laboratory manual, 3rd ed. Cold spring harbor labortory, cold spring harbor, NY.

[18] Sharan R. (2007) "Analysis of Biological Networks: Transcriptional Networks – Promoter Sequence Analysis". Tel Aviv University. 1-5.

[19] St-Pierre J., Hivert M. F., Perron P., Poirier P., Guay S. P., Brisson D., Bouchard L. (2012) *IGF2* DNA methylation is a modulator of newborn's fetal growth and development. Epigenetics. 7(10): 1125 - 1132.

[20] Tran V.G., Court F., Duputié A., Antoine E., Aptel N., Milligan L., Carbonell F., Lelay-Taha M.N., Piette J., Weber M., Montarras D., Pinset C., Dandolo L., Forné T., Cathala G. (2012) *H19* antisense RNA can up-regulate *Igf2* transcription by activation of a novel promoter in mouse myoblasts. PloS ONE 7: e37923.

[21] Ward, A. (1997) Beckwith – Wiedemann Syndrome and Wilms' Tumour. 3(2): 157 – 168.

[22] Wolfgang H., Andreas W., Arend K., Steffen A., Steven G., Gray T. J., Torsten P. (2011) Promoter - specific transcription of the *IGF2* gene: a novel rapid, non-radioactive and highly sensitive protocol for mRNA analysis. Virchows Arch. 439: 803 - 807.