Insulin-like growth factor antisense gene (Igf2as) expression was investigated in different mouse tissues during development, in differentiating C2C12 cells and in a ΔDMR1-U2 knockout mouse model. The expression levels of Igf2as were high in fetal and newborn liver and muscle tissues compared to adults. The Igf2as gene was also expressed in placenta and in brain. The expression data suggests that the Igf2as gene plays a role in early development of the mouse and in placenta. There was no consistent evidence for an interaction between Igf2 and Igf2as transcripts. Furthermore, in knockout placentas lacking Igf2as transcription, Igf2 expression was comparable to that in wild type. These results indicate that Igf2as does not regulate Igf2 sense transcripts. In previous studies, it was suggested that the ΔDMR1-U2 knockout mouse showing intrauterine growth restriction was caused by the absence of placenta-specific Igf2 P0 transcription. We conclude that the ΔDMR1-U2 deletion phenotype should be reconsidered in the light of a functional Igf2as gene.

1. Introduction

The insulin-like growth factor 2 gene (Igf2) is an imprinted gene, paternally expressed and encodes for the insulin-like growth factor II peptide [1, 2]. In mice the Igf2 gene has five promoters, from which three main transcripts, a placenta specific transcript and a newly described mesoderm-specific transcript, originate [3]. Moore et al. [4] described multiple imprinted sense and antisense transcripts from the Igf2 locus. The Igf2as gene located within Igf2 is transcribed from the complementary DNA strand. We recently found that Igf2as transcripts are located in the cytoplasm and associated with polysomes indicating a protein coding function [5].

To further elucidate the function of Igf2as, we investigated the ΔDMR1-U2 knockout mouse [6]. This mouse has a 5 kb deletion within the Igf2 gene comprising DMRI, an adjacent repeat sequence mostly embedded in exon U2 of the placenta specific Igf2 P0 transcript and Igf2as transcripts. The maternal transmission of the ΔDMR1-U2 deletion results in loss of Igf2 imprinting in heart, kidney, and lung without affecting H19 gene expression [6]. The authors conclude that their result demonstrates that DMRI plays a role in Igf2 imprinting regulation and gene expression independent of H19 [6]. In that study, paternal transmission of the ΔDMR1-U2 deletion was associated with intrauterine growth restriction (IUGR) manifested by mutants birth weight being 71% that of normal mice. Later, Constância et al. [7] reported that the 5 kb ΔDMR1-U2 deletion abolishes expression of the Igf2 P0 transcript in the labyrinthine trophoblast of the placenta where it is specifically expressed. Noteworthy, placental growth deficiency in ΔDMR1-U2 placentas resembled that of Igf2-null mice lacking IGF-II peptide in all placental layers and the fetus which was unexpected since P0 transcripts constitute about 10% of total Igf2 transcripts in placenta [8]. Constância et al. [7] suggest that differential translatability of Igf2 transcripts may explain this observation. However, the lower birth weight of mutant ΔDMR1-U2 pups was compensated during the first three months of life. They could also
demonstrate with inert hydrophilic molecules that passive permeability of the mutant placenta was reduced compared to the wild type. However, they showed that the mutant placenta actively transferred more than wild-type placenta indicating compensatory effects to the passive permeability and the smaller placenta size.

The permeability of the mouse placenta to hydrophilic solutes was further studied in ΔDMRI-U2 mice versus wild-type mice [9]. In their study they found that the permeability for hydrophilic solutes was significantly reduced in the ΔDMRI-U2 knockout placenta at E19. Stereological analysis showed a reduction in surface area and an increase in thickness of the exchange barrier in the ΔDMRI-U2 knockout labyrinthine layer of the placenta. This suggests that labyrinthine P0 Igf2 expression has a function in the development of normal diffusional exchange characteristics of the mouse placenta influencing fetal growth [9]. Constância et al. [10] further investigated the placental nutrient supply and the fetal demand in the ΔDMRI-U2 deletion mouse model (Igf2 ΔP0/−) and an Igf2 null mouse model [10]. Constância et al. [10] could further show by using Igf2 P0+/− and Igf2 null mice models that placental nutrient transfer occurs in response to fetal nutrient demands and involves Igf2 mediation. Additional evidence for the placental adaption was provided by showing lower expression of the transplacental calcium transfer protein calbindin-D9K in Igf2 P0+/− at E17 but not at E19 compared to that of wild types [11]. More recently, it was shown that adult Igf2 P0+/− mice but not Igf2 null mice exhibited behavioral phenotypes [12]. These mice were exposed to a whole range of emotion-related behaviors tests and Igf2 P0+/− mice showed increased reactivity to acute anxiety-including stimuli. It was further found that anxiety associated genes in the hippocampus of these mice were altered. These long-term behavioral effects were attributed to the imbalance between fetal demand and placental supply of nutrients during gestation [13].

In this study, we performed an expression analysis of Igf2 and Igf2as transcripts in ΔDMRI-U2 and wild-type placentas from different development stages. The expression of these genes is studied in wild type placentas and in ΔDMRI-U2 placentas which are lacking Igf2 P0 and Igf2as expression. Our results from this comparison are challenging previous findings from this mouse model. We also followed the expression patterns of this sense/antisense pair in different tissues during development and measured Igf2/Igf2as expression in differentiating C2C12 cells for functional characterization of the putative protein coding Igf2as gene.

### Table 1: Primers pairs used for genotyping. Forward (F) and reverse (R) primers are indicated. WT refers to the RT-PCR product size in wild-type samples and KO refers to the product size in knockout ΔDMRI-U2 transgenic mouse.

| Primer | Sequence (5’ > 3’) | Product |
|--------|--------------------|---------|
| F_K0   | AAGTTCCCTCGGGTTAGGG | 374 bp (WT) |
| R_K0   | CTAAGACGATGTGGGGGTGT | 582 bp (WT) |
| R_K0_control | TTGGCTAGAAGGCCGAAAGAA | 374 bp (KO) |

flasks were harvested separately, RNA isolated, and used for quantification by real-time PCR.

2.2. Animals. The experiments were carried out in strict accordance with Swiss Federal Law on Animal Protection of 16 December 2005 (Tierschutzgesetz TSchG, SR 455), Art. 32, Absatz 1; Ordinance on Animal Protection of 23 April 2008 (Tierschutzverordnung TSchV, SR 455.1). Brain, muscle and liver tissues were collected from wild-type C57BL/6 fetuses at E18 (embryonic day 1 (E1) = day of plug), from newborn and adult mice. Also brain samples were collected from embryos at E14. ΔDMRI-U2 transgenic male mice carrying the mutation on the paternal allele were donated from M.R. Dilworth and colleagues [6, 11]. The males containing the mutation were crossed with wild-type C57BL/6 females. Mice resulting from these crossings were sacrificed at E12, E14, E16, and E19 and placentas were collected. DNA from each mouse tail was extracted to genotype for the ΔDMRI-U2 deletion. The primers used for this PCR test are shown in Table 1. From the crosses between male carriers of the ΔDMRI-U2 deletion and C57BL/6 females, three mutant placentas and three wild-type littersmates were used for the analysis.

2.3. RNA Extraction and Quantitative Real-Time PCR (qRT-PCR). RNA extraction was performed using Trizol reagent according to manufacturer's protocol (Invitrogen). All RNA samples were treated with DNase I (Ambion). The RNA was further purified with the RNaseasy Mini Kit (Qiagen). The cDNA was synthesized by reverse transcription of 1 μg total RNA using the QuantiTect Reverse Transcription kit according to the manufacturer's protocol (Qiagen). Each sample was quantified in triplicates for all Igf2 variants (V1, V2, V3, and Vm) and the Igf2as transcript by qPCR using TaqMan probes or conjugated minor groove binder (MGB) probes to measure Igf2as transcription (Table 2) (Figure 1). The Ct values of the target were normalized by subtracting the mean of the Ct values from Actb and Gapdh. The normalized ΔCt values were converted to relative expression. The relative quantifications were calculated relative to the first value which was set to 1 in each respective figure. The calculations were performed for each of three samples measured in triplicate, averaged, and had the standard deviations calculated. The normalized data was analyzed by using two-tailed Student’s t-test.
Table 2: Primer pairs and probes used in TaqMan qPCR experiments. Forward (F) and reverse (R) primers are indicated.

| Primer/probe      | Sequence (5' > 3') | Product |
|-------------------|--------------------|---------|
| F_Actb            | GCTTTCTTTGCAGCTCCTTCGT | 71 bp   |
| R_Actb            | GCCGACCGATATCGTACATC |         |
| Probe_Actb        | CCGGTCCACACCACGGCCACC |         |
| F_Gapdh           | CGGCCGACATCTTCTTGTG  |         |
| R_Gapdh           | TACGGCCAAATCGGTTCACT |         |
| Probe_Gapdh       | AGTGCACAGCTCTGTCGGACCA | 78 bp   |
| F_Igf2 V1         | CGGCTTCCAGTACATC     |         |
| R_Igf2 V1         | GCAGCGATGCAGCACAAG   |         |
| Probe_Igf2 V1     | ATGTTGTGCTTTCATCTTCCTGCTT | 90 bp   |
| F_Igf2 V2         | GCTTTCTCTCTCGGATCCT  |         |
| R_Igf2 V2         | ATGAGAAGCACAATCAGCTT |         |
| Probe_Igf2 V2     | CGACCTTCCGTGCTTGCACAA | 98 bp   |
| F_Igf2 V3         | CGAGCTTTTCCTGTCTTCATC |         |
| R_Igf2 V3         | CATTCTCTCAGCGGATCCTGAA |         |
| Probe_Igf2 V3     | TTCCAGCCCAGCGGCTC    | 68 bp   |
| F_Igf2 P0         | TGAACCCCGGTGCTCTTTT  |         |
| R_Igf2 P0         | TGTTTTTAATTCTCTGTCCCTGACTC |         |
| Probe_Igf2 P0     | TCCACGCTCCGGAACATTCCAGG | 84 bp   |
| F_Igf2 Pm         | TCGGAGAAGTAGGTGGTACCAATG |         |
| R_Igf2 Pm         | GCCGAAGCCACAGAGATG    |         |
| Probe_Igf2 Pm     | AAGTCTGGTTGTGCTCTT   | 77 bp   |
| F_Igf2as MGB      | TGCAACCAAATCGTCAACACAA |         |
| R_Igf2as MGB      | AAAGGCAGGTCTCAGATGA   |         |
| Probe_Igf2as MGB  | CACACAAAAATCTCTTTC   | 62 bp   |

Figure 1: Structure of Igf2 gene, Igf2as, and ΔDMR1-U2 mutation. The arrows indicate the five promoters (P0, Pm, P1, P2, and P3) for Igf2 and (P) for Igf2as. DMR1 indicates the position of the DMR1 regulatory region. V1, V2, V3, P0, and Pm indicate the five variants of Igf2 gene. The 5 kb deleted region in ΔDMR1-U2 mice is indicated.

3. Results

3.1. Igf2 and Igf2as Expressions in Different Tissues during Development. We found relatively high Igf2 and Igf2as gene expression in fetal and newborn liver and muscle tissue samples and a significant downregulation of these genes in adult tissues (Figures 2(a) and 2(b)). All three Igf2 variants followed similar pattern of expression at fetal, newborn, and adult stages of development. In muscle, we found comparable expression levels of Igf2as transcripts in fetus and newborn whereas the expression of Igf2 variants increased in newborn (Figure 2(a)). Igf2as and Igf2 variants were significantly downregulated in adult tissues (Figures 2(a) and 2(b)). Igf2as levels were not detectable after 40 qPCR cycles in adult muscle tissue. In liver, the level of Igf2as and Igf2 expressions is similar in fetus and newborn, except for variant 3 which significantly increased in newborn (Figure 2(b)). Transcripts originating from the Pm promoter were expressed at very low levels (data not shown). In brain we found significantly higher expression of Igf2 variant 2 and Igf2as in embryos compared to fetus with a continuous down-regulation towards the adult stage (Figure 2(c)). Igf2 variants and Igf2as followed this pattern, except variant 3 (Figure 2(c)). Pm transcripts in brain were not detected after 40 qPCR cycles.

3.2. Igf2 and Igf2as Expression in Differentiating C2C12 Cells. We used C2C12 myoblast cells differentiation to monitor the sense/antisense Igf2/Igf2as gene pair for potential interac-
Figure 2: Quantification of expression levels of Igf2 variants and Igf2as transcripts in different tissues. Igf2 variants and Igf2as were quantified in muscle (a), liver (b), and brain (c) tissues of fetus, newborn (NB), and adult and included embryonic brain. V1, V2, V3, and Igf2as stand for Igf2 variants 1, 2, 3, and Igf2 antisense, respectively. The results are expressed in relative expression, calculated relative to the first value. Each bar represents the mean of three samples each analyzed in triplicate. The standard deviations are indicated as error bars. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

3.3. Igf2 and Igf2as Expressions in ΔDMR1-U2 and Wild-Type Mice. We measured Igf2 and Igf2as expressions in placenta of ΔDMR1-U2 mice and their wild-type litter mates (Figure 4). We found no differences in the expression of Igf2 variants 1-3 between mutant and wild-type placenta except for Igf2 variant 3 at E16, which was more highly expressed in the knockout placenta (Figure 4(c)). Igf2as expression levels in wild-type placenta significantly increased from E16 to E19 (Figure 5). The expression levels of Igf2 P0 in wild-type placenta were constant during E12 to E16 and augmented significantly from E16 to E19 (Figure 5). In ΔDMRI-U2 placentas no Igf2as and, as expected, no P0 transcripts were detected.

4. Discussion

The aim of this study was to further contribute to ascertaining the function of Igf2as. We studied Igf2 and Igf2as gene expression in different tissues during development, in differentiating C2C12 cells, and in placenta of a ΔDMR1-U2 deletion mouse model. All Igf2 variants and Igf2as followed a similar expression pattern in different tissues during development indicating a common regulation. In brain, we found a more continuous downregulation of Igf2 and Igf2as transcription compared to distinct downregulation of these genes in adult liver and muscle. Most relevant is, however, that we did not find any consistent evidence for an interaction between Igf2 and Igf2as transcripts although they are both present in the cytoplasm and contain complementary nucleotide sequences [5]. Additionally, we observed that the lack of Igf2as transcripts in placenta did not produce significant changes in...
Igf2 variants expression levels compared to wild type with the exception for Igf2 variant 3 at E16. Igf2 variant 3 does not contain complementary sequences which could directly interact with Igf2as transcripts to form double-stranded RNA molecules. In addition variant 3 expression at E12, E14, and E19 was not different compared to wild-type mice and thus the interpretation of this event remains elusive. The comparison of Igf2 and Igf2as expression between ΔDMRI-U2 and wild-type mice argues against the hypothesis that Igf2as transcripts have a role in Igf2 gene regulation. The increase of Igf2 variant 3 at E16 in mutant mice may have compensatory effect on Igf2 protein content in labyrinthine trophoblast challenging the notion that the ΔDMRI-U2 phenotype is solely caused by the lack of P0 transcripts [7, 10]. During C2C12 differentiation, we observed different expression patterns for Igf2 variant 3 and Igf2as expressions arguing against an interaction between the two genes. Noteworthy is the increased expression of Igf2 variant 3 and Igf2as transcripts between 24 h and 48 h of differentiation when myotubes start to form.

The knockout mouse carrying the ΔDMRI-U2 deletion was extensively studied in regard to placental nutrient supply and fetal demand [6, 7, 9–11]. Here, we argue that the previously observed effects of the placenta specific Igf2 P0 transcript may be confounded with the effects of the putative protein-coding Igf2as gene. Our alternative or additional explanation for the observed ΔDMRI-U2 deletion effects is therefore the lack of a functional Igf2as gene. The knockout of paternally expressed genes results in IUGR [13]. Igf2as is an imprinted and paternally expressed gene and therefore the IUGR phenotype described in ΔDMRI-U2 mice agrees with common functions of paternally expressed genes [13]. Assuming that the lack of a functional Igf2as gene contributes to the phenotype of IUGR, expressed as smaller placentas and lower birth weight, it might have similar functions as the Igf2 gene. Interestingly, the recently described long-term behavioral effects found in ΔDMRI-U2 mice could also be, at least in part, due to the absence of Igf2as function [12]. This is suggested because we observed considerable Igf2as expression in brain during development. We think that the presented results indicate that previous studies have to be interpreted in the light of potential Igf2as gene effects. In summary, Igf2as is most expressed during prenatal development followed by a pronounced downregulation after birth in most tissues similar to the Igf2 gene. In brain, we found also
highest Igf2as expression in early development with a gradual downregulation from embryo brains to adult brains.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

**Acknowledgments**

The authors would like to thank Dr. Miguel Constância and Dr. Wolfgang Reik who generated the ΔDMR1-U2 knockout mouse and Dr. Mark Dilworth for providing them with two male ΔDMR1-U2 mice. This research was supported by the Swiss National Foundation (Project 31003A_127564).
References

[1] T. M. DeChiara, A. Efstratiadis, and E. J. Robertson, "A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting," *Nature*, vol. 345, no. 6270, pp. 78–80, 1990.

[2] T. M. DeChiara, E. J. Robertson, and A. Efstratiadis, "Parental imprinting of the mouse insulin-like growth factor II gene," *Cell*, vol. 64, no. 4, pp. 849–859, 1991.

[3] V. G. Tran, F. Court, Duputié et al., "H19 antisense RNA can up-regulate Igf2 transcription by activation of a novel promoter in mouse myoblasts," *PLoS One*, vol. 7, no. 5, Article ID e37923, 2012.

[4] T. Moore, M. Constancia, M. Zubair et al., "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*, vol. 94, no. 23, pp. 12509–12514, 1997.

[5] C. Duart-Garcia and M. Braunschweig, "The Igf2as transcript is exported into cytoplasm and associated with polysomes," *Biochemical Genetics*, vol. 51, pp. 119–130, 2013.

[6] M. Constância, W. Dean, S. Lopes, T. Moore, G. Kelsey, and W. Reik, "Deletion of a silencer element in Igf2 results in loss of imprinting independent of H19," *Nature Genetics*, vol. 26, no. 2, pp. 203–206, 2000.

[7] M. Constância, M. Hemberger, J. Hughes et al., "Placental-specific IGF-II is a major modulator of placental and fetal growth," *Nature*, vol. 417, no. 6892, pp. 945–948, 2002.

[8] J. Baker, J. P. Liu, E. J. Robertson, and A. Efstratiadis, "Role of insulin-like growth factors in embryonic and postnatal growth," *Cell*, vol. 75, no. 1, pp. 73–82, 1993.

[9] C. P. Sibley, P. M. Coan, A. C. Ferguson-Smith et al., "Placental-specific insulin-like growth factor 2 (Igf2) regulates the diffusional exchange characteristics of the mouse placenta," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 21, pp. 8204–8208, 2004.

[10] M. Constância, E. Angiolini, I. Sandovici et al., "Adaptation of nutrient supply to fetal demand in the mouse involves interaction between the Igf2 gene and placental transporter systems," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 52, pp. 19219–19224, 2005.

[11] M. R. Dilworth, L. C. Kusinski, E. Cowley et al., "Placental-specific Igf2 knockout mice exhibit hypocalcemia and adaptive changes in placental calcium transport," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 8, pp. 3894–3899, 2010.

[12] M. A. Mikaelsson, M. Constância, C. L. Dent, L. S. Wilkinson, and T. Humby, "Placental programming of anxiety in adulthood revealed by Igf2-null models," *Nature Communications*, vol. 4, article 2311, 2013.

[13] W. Reik, M. Constância, A. Fowden et al., "Regulation of supply and demand for maternal nutrients in mammals by imprinted genes," *Journal of Physiology*, vol. 547, part 1, pp. 35–44, 2003.