

Up to the minute project summary:

We have been studying the involvement of the RNA binding protein HuR in the regulation of adipocyte differentiation. Our work demonstrated that siRNA suppression of HuR expression led to an inhibition of the 3T3-L1 differentiation program; an observation consistent with a critical/essential role for HuR in adipogenesis. HuR is ubiquitously expressed and localized predominantly to the nucleus but shuttles between the nucleus and cytoplasm. In the nucleus, recent data have supported roles for HuR in the splicing as well as in the regulation of polyadenylation. The later function mediated through competitive inhibition of the binding of the cleavage and polyadenylation specificity factor, thereby attenuating polyadenylation and nuclear export. In the cytosol there is compelling evidence to suggest that HuR functions to control the stability and translational efficiency of its ligand mRNAs. The accumulated data support an important regulatory role for HuR.

To date our studies have focused on one HuR ligand, the C/EBPβ mRNA. To identify other HuR ligand

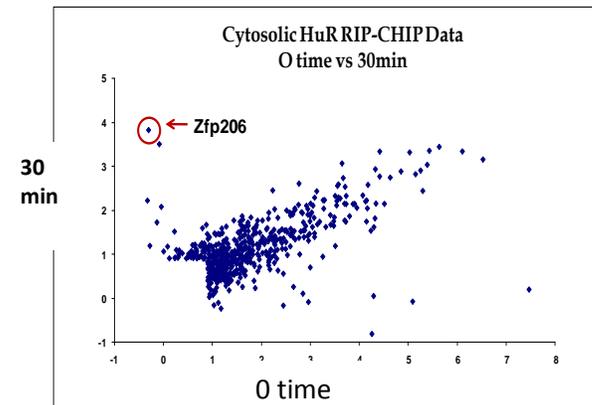


Fig. 1. Enrichment plot of HuR ligands, 0 vs 30 min after induction of differentiation.

mRNAs early in the differentiation program, we used antibodies directed against HuR to perform an RNP immunoprecipitation-microarray (RIP-Chip) analysis. This allows the identification of discrete subsets of RNAs associated with the multi-targeted HuR and provides information regarding changes in the intracellular composition of mRNPs in response to the differentiation program. From our RIP-Chip analysis, we identified 496 mRNAs that served as ligands for HuR. One of these mRNAs was not a ligand at 0 time but markedly enriched 30min after induction of differentiation. That ligand was identified as Zfp206 (Fig. 1). While the HuR binding site has not yet been localized in the Zfp206 mRNA 3'UTR, the nucleotide sequence ²⁶⁶⁸-aaauuguuuuaagu-²⁶⁸² is predicted to form the core of the binding domain. The data displayed in Fig. 1 have been verified by independent HuR immunoprecipitations and RTPCR analysis for Zfp206 mRNA.

As shown in Fig. 2, RNA was isolated from the 3T3-L1 cells during a differentiation time course and analyzed by RTPCR to characterize expression of Zfp206. The Zfp206 message was detectable at

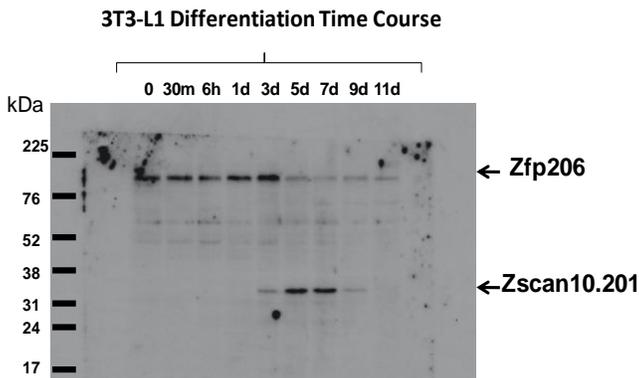


Fig. 2. RTPCR analysis of Zfp206 and PPARγ mRNA expression during a 3T3-L1 differentiation time course.

It may simply be a sampling error. The lower panel represents an analysis for PPARγ mRNA accumulation during the same differentiation time course.

Determination of Zfp206 protein levels during a differentiation time course by western blot led to the detection of two protein species. As shown in Fig. 3, an 88 kDa protein, corresponding to the full length gene product was detected at day 0 with expression

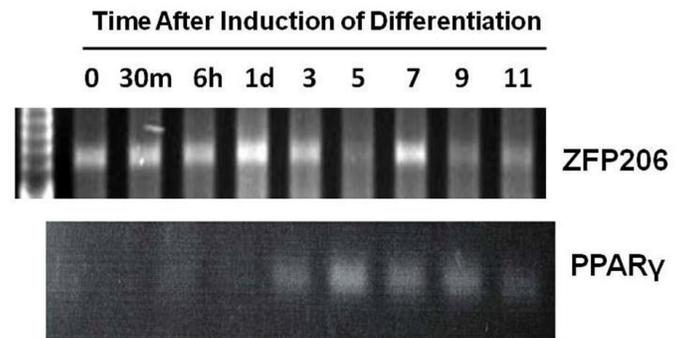


Fig. 3. Western blot analysis of Zfp206 and the dominant negative splice variant, Zscan expression during a differentiation time course.

maintained through day 3. At which time transient expression of a 32 kDa protein was detected. The antibody used in this study was generated against an N-terminal 134 amino acid fragment containing the full SCAN domain (Fig. 4). We note that the protein expression follows the mRNA accumulation data displayed in Fig. 2 very closely.

Examination of the annotation for the gene using the E! Ensembl Gene Browser http://www.ensembl.org/Mus_musculus/Transcript/ProteinSummary?db=core;q=ENSMUSG00000023902;r=17:23737823-3747986;t=ENSMUST00000095595) indicated that: 1. Zfp206 is also known as Zscan 10 and 2. there is sequence evidence for the existence of a potential splice variant of approximately 32.5 kDa referred to as Zscan 10.201. To date there has been no description of the expression of this variant, but then again there are only four published papers on the expression/function of Zfp206, a recent study did however identify the expression of at least seven splice variants in ES cells. Inspection of the sequence alteration in

formation of Zscan demonstrates that the zinc-finger domain as well as the domain encoded by exon 1 have been lost (Fig. 4). Interestingly *we note that the 3'UTR containing the HuR binding site remains intact*. The impact of the alternative splicing is to create a dominant-negative variant of Zfp206 that no longer binds DNA, yet maintains the

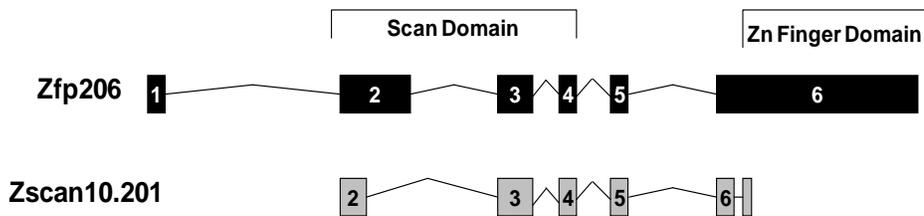


Fig. 4. Diagrammatic representation of the intron-exon structure of the Zfp206 gene and its splice variant, the dominant negative Zscan10.201

SCAN domain for interaction with protein ligands, potentially co-activators or co-repressors. .

With respect to expression of Zfp206, our array data indicated that at 0 time, the Zfp206 mRNA was not a ligand for HuR, yet at 30 min after induction of differentiation the complex was detected. The western blot (Fig. 3) clearly demonstrates expression of the Zfp206 protein at 0 time. The data suggest that the HuR binding site may initially (0 time) be occupied by another protein complex. Indeed, differential occupation of an adenylate-uridylylate rich site in other mRNAs has been demonstrated for HuR and AUF-1. Alternatively, with the Zfp206 message, HuR may mediate splicing and not

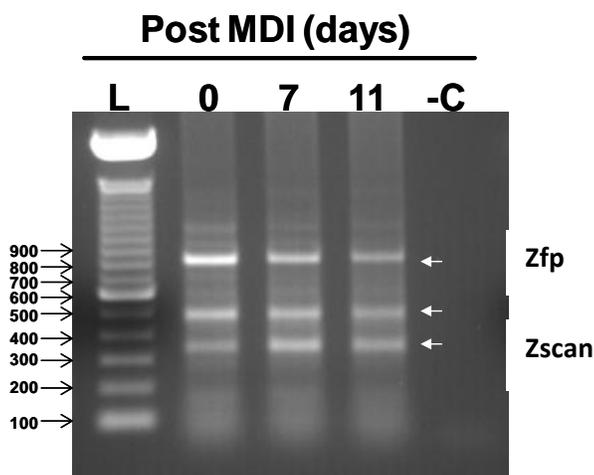


Fig. 7. RTPCR analysis was carried out on RNA isolated at days 0, 7, & 11 after induction of differentiation to confirm expression of Zfp206 and Zscan.

bind to the message until receiving the appropriate signal after induction of differentiation.

Since the primers used to generate the data displayed in Fig. 2 would not distinguish between mRNA for Zfp206 and Zscan, we designed primers to differentiate between the two mRNAs by yielding amplification products of 850 and 350 respectively. As shown in Fig. 5, both forms were found to be present during a 3T3-L1 differentiation time course.

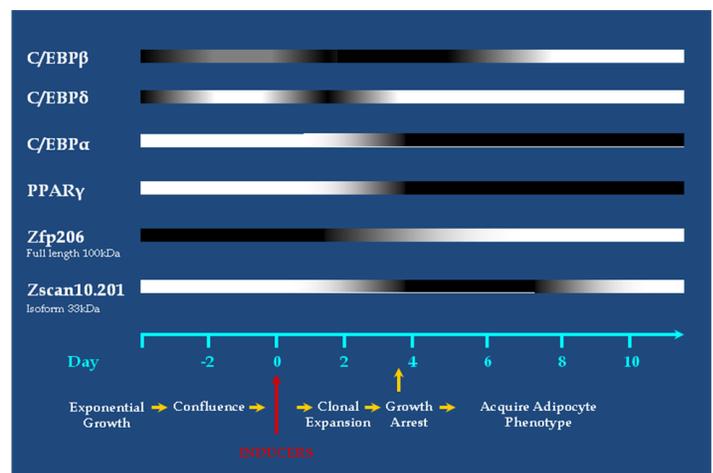


Fig. 6. Schematic representation of the time course of expression of Zfp206 and Zscan in the context of expression of the major adipogenic transcription factors. Color scheme: White – no expression; Grey – increased expression; Black – maximal expression.

Interestingly, while the Zscan protein cannot be detected until day 3, the message can be detected by RTPCR at day 0. A third unknown product of approximately 500 bases was also observed which we have not as yet sequenced. Eventually, sequence data will provide information on the function of this species.

The protein expression patterns of Zfp206 and Zscan are shown diagrammatically in the context of the 3T3-L1 differentiation program in Fig. 6. As stated earlier, Zfp206 has been suggested to function in the maintenance of embryonic stem cell pluripotency. The expression pattern for Zfp206 observed in the 3T3-L1 cells relative to C/EBP α and PPAR γ suggests that it may be functioning in a similar manner, maintaining the differentiation potential until the adipocyte phenotype is terminally locked in with the expression of PPAR γ and C/EBP α .

In embryonic stem cells, expression of Zfp206 is controlled by two other embryonic transcription factors, Oct4 and Sox2. While we believed it was unlikely that they would be expressed in the 3T3-L1 cells, we

isolated RNA from a differentiation time course and examined for their presence by RTPCR. As shown in Fig. 7 expression was not observed relative to that found in the D3 murine embryonic stem (ES) cell line. The data suggest that expression of Zfp206 must be controlled by other factors in the 3T3-L1 cells.

To further examine the regulation of Zfp206 expression we utilized the C/EBP β ^{-/-} MEFs obtained from Peter Johnson (NIH,

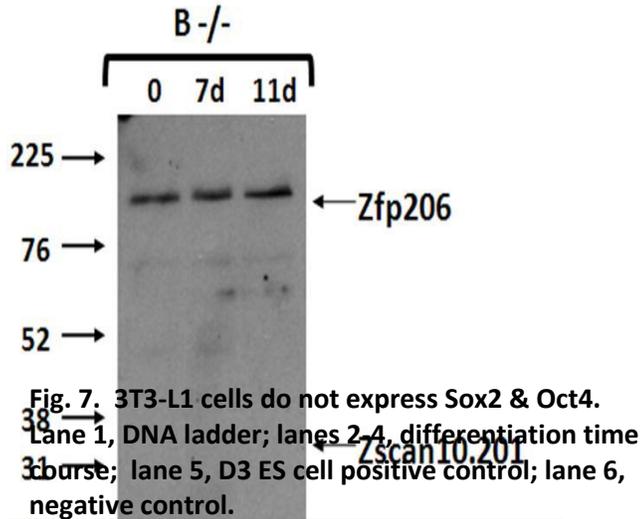


Fig. 7. 3T3-L1 cells do not express Sox2 & Oct4. Lane 1, DNA ladder; lanes 2-4, differentiation time course; lane 5, D3 ES cell positive control; lane 6, negative control.

From day 13.5 embryos the C/EBP β ^{-/-} cells will not be exposed to the differentiation induction cocktail (we note that they do express HuR). For the experiment shown in Fig. 8, cells were grown to confluency and two days later exposed to the differentiation inducers. Cells were maintained in culture for 11 days post induction of differentiation. During this period the cells maintained their preadipocyte morphology and no accumulation of triacylglycerol was observed. Western blot analysis at selected time points demonstrated that Zfp206 was expressed at 0 time and was not down regulated as observed during the differentiation program of the 3T3-L1 cells (Fig. 3). Interestingly,

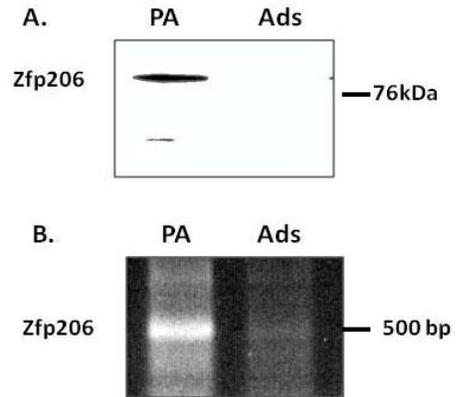


Fig. 8. β ^{-/-} cells neither down regulate Zfp206 nor express Zscan. Western blot analysis of a differentiation time course for Zfp206 & Zscan expression.

expression of the dominant negative splice variant Zscan was not observed (Fig. 8). These data should be interpreted in the context of the data displayed in Fig. 3, the time course of Zfp206/Zscan expression in differentiating 3T3-L1 cells. In the current experiment, the lack of C/EBP β expression correlates with the inability of the MEFs to down regulate Zfp206 and express the splice variant Zscan. The data are consistent with a role for Zfp206/Zscan in the differentiation program and suggestive of C/EBP β involvement (directly or indirectly) in its expression. We also believe that these observations provide support for our hypothesis. These cells express no C/EBP α or PPAR γ , Zfp206 is never down regulated and adipocyte phenotype is never established.

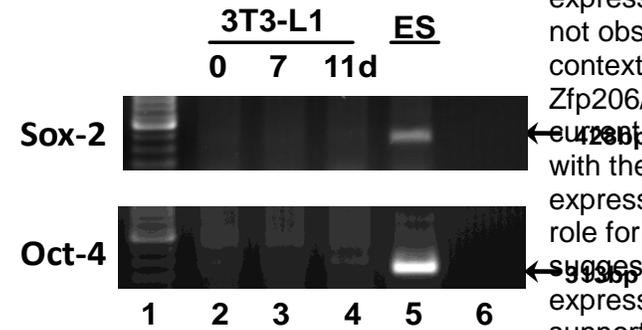


Fig. 9. Zfp expression in human preads and adipocytes. Panel A. Western blot for Zfp206. Panel B. RTPCR analysis for Zfp206 mRNA.

Finally, we obtained human preadipocytes from Zen-Bio, Inc. The cells were derived from 6 female donors of average age 40 with an average BMI of 27.9. The donors were neither diabetic nor smokers. We examined for Zfp206 expression in both the preadipocytes and those induced to differentiate to adipocytes. As shown in Fig. 9 (panel A) we were able to detect Zfp206 protein expression in the preadipocytes (day 0) and demonstrate that similar to the 3T3-L1 cells, it is down regulated as the cells differentiate (day 14). We note that we had limited sample material and could only examine the two time points. Thus at this time we have no information on the time course of Zfp206 expression or if the splice variant Zscan is expressed. RTPCR analysis indicated that the Zfp206 mRNA levels corresponded to the protein levels (Fig. 9, panel B). (We note that the human cells also express HuR.)

The demonstration that Zfp206 is expressed in human preadipocytes is consistent with involvement of this embryonic stem cell transcription factor in the differentiation program of these human cells. We suggest that involvement may be at the level of a critical regulator of the differentiation process. These data endorse our use of the 3T3-L1 cells as a model to obtain information that may eventually be applied to human adiposity/obesity.

Work in progress. We are initiating experiments designed to suppress Zfp206 expression with shRNA constructs. In addition, we are preparing expression vectors to over express Zfp206.

In Summary, our preliminary data demonstrate that:

- **Zfp206 mRNA is a ligand for the RNA binding protein HuR.**
- **Zfp206, an ESC transcription factor, is transiently expressed during the adipogenic differentiation program.**
- **At day 3, a splice variant Zscan which lacks the zinc finger domain is transiently expressed as Zfp206 is down regulated.**
- **In contrast to embryonic stem cells, Zfp206 expression in 3T3-L1 cells does not appear to be controlled by Oct4 or Sox2.**
- **Zfp206 expression is not down regulated in the β -/ β - cells and Zscan is not expressed; supporting a role for C/EBP β and/or early transcriptional events in the control of Zfp206 expression.**
- **Zfp206 protein and mRNA are expressed in human preadipocytes and down regulated as the cells terminally differentiate, similar to that observed in the 3T3-L1 cells. This is highly suggestive that Zfp206 may be performing a similar role during adipogenesis in humans. Additionally, this conclusively demonstrates that Zfp206 expression is neither an artifact of nor specific to the 3T3-L1 cells.**