A New Small Nuclear RNA Gene-Like Transcriptional Unit involved in cell proliferation and differentiation

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In a previous work we have identified a new set of about 30 new non-coding transcriptional units transcribed by RNA Polymerase III. One of these novel transcriptional units, called 29A, map in the chromosomal location 11p15.3. 11p15.3 is a human chromosomal region to which some peculiar very important activities has been associated. It has been demonstrated that to this chromosomal region is associated a tumor suppressor activity, but at now remain unknown the gene responsible of this activity. In this work starting with a non-coding sequence as 29A we explore this region also in the non-coding part of the genome, and not only in the ORF (Open Reading Frame) that are present in the protein-coding fraction of the human genome. First of all we have verified the effective transcription and Polymerase III dependency of the 29A transcriptional unit, and we achieved at that by three different techniques, we performed an in vitro transcription in cell nuclear extract, a primer extension and a luciferase based assay to test the promoters efficiency. (Fig.2 e Fig.3)

29A expression seems to have a correlation with the proliferation rate of the different cellular type in witch we have measured the 29A expression, for this reason we have analyzed more in details the involvement of the 29A transcriptional unit in cellular proliferation. By measuring the cells doubling time (DT), and the 29A expression, we have found, in different cells type, an inverse correlation between the DT and 29A expression (Fig.5). Than we have transiently transfected different construct, obtained taking different portions of the 29A transcriptional units, in two different cell lines (HeLa e SKNBE), these experiments conducted measuring the [3H] thymidine incorporation, showed that 29A overexpression is related to a significant inhibition of cell proliferation. (Fig.5)

We have than checked in details the relationship between the increase of 29A expression and the decrease of proliferation rate. We have created a construct for the establishment of a stable cell line that overexpress 29A in order to check the effect on cells proliferation at longer time and at higher percentage of transfection. After the selection, we have obtained three stable cell lines one of control (that express only the GFP reporter gene) and two that overexpress 29A. (stab 29A 1, stab 29A 2). We have analysed these cells for the 29A expression (stab 29A 2 increase of 2,2 fold, stab 29A 1 increase of 5,4 fold in relation with the stab MOCK) and for the DT (30 hrs for stab MOCK, 39hrs for 29A stab 2, 48hrs for 29A stab 1) (Fig.6) After this first characterisation we have the interesting confirmation of the inverse correlation between the value of the 29A expression and the doubling time (DT) of each cell line.(Fig.6). The cell line that we have selected for the establishment of the stable cell line (SKNBE 2) is a neuroblastoma derived cell line, that it’s known from literature to be differentiated in neuronal-like cell, by adding retinoic acid at 10 μM in the culture medium. We have also investigated the level of expression of a neuron-specific differentiation marker, the matrix metalloproteinase 9 (MMP-9). MMP-9 is known from the literature to have an increased expression in differentiated SKNBE cells. We notice that in our stable cell lines the expression of MMP-9 correlate with the increase of DT and 29A expression. Through 29A secondary structures predictions, it was found a significant homology of sequence, secondary structure and some conservative mutations needed for the secondary structure maintenance, with the 7SL RNA, the ribonucleic component of the SRP ribonucleoprotein complex.
SRP (SignalRecognitionParticle). Also the binding sites of two proteins present in the SRP complex (SRP9, SRP14) were conserved in 29A RNA, so we perform some experiments to investigate this possible binding between 29A RNA and SRP9/SRP14. After the transfection of HeLa cells, with 29A in association with two constructs encoding for SRP9 and SRP14 proteins, we have noticed that the proliferation inhibition, revealed by a [3H] thymidine incorporation assay, was enhanced in the samples co-transfected with the SRP proteins together with 29A RNA. These last results highlight a possible molecular mechanism for 29A as a ribonucleoproteic complex. In an ongoing experiment we are performing an in vitro protein/RNA binding assay, to confirm the effective binding.

29A lies in a tumor suppressor region partially unexplored, 11p15.3, this region is associated to a significant tumor suppressor activity but it is still unknown the single responsive gene.

(a) To test their transcriptability we in vitro transcribed the 29A constructs taking advantage of HeLa cell nuclear extracts in presence and absence of alpha amanitin and Tagetin two specific inhibitors of RNA Polymerase II and III respectively.

(b) Primer extension
Than we have evaluated by Real Time quantitative PCR the expression of 29A in differents cellular types, evidencing a previously unknow RNA Polymerase III tissue specificity. (Fig.4)

c) To check for 29A active transcription in cultured cells, a 29A-specific cDNA was also amplified from total RNA samples, extracted from skin fibroblasts and four tumor cell lines (293, LAN5, HCT, HeLa), by random hexamer-based RT-PCR.

d) To assess 29A transcription we fused its promoter to a luciferase silencer hairpin and co-transfected this construct with a plasmid expressing luciferase. Results showed a halved luciferase activity 48 hours after transfection thus demonstrating an efficient transcription directed by 29A promoter. In the same experiment a canonical U6 promoter-driven construct was transfected as a control.

e, f) The same experiment was repeated after 24 hours of cell treatment with ML-60218, a cell-permeable indazolo-sulfonamide compound that displays broad spectrum inhibitory activity against RNA Polymerase III. Results showed an efficient luciferase-silencing activity in the absence of the Pol III inhibitor (as evidenced by a decreased luciferase emission) while after treatment with ML-60218 the luciferase signal was increased. This result suggests a decrease in hairpin synthesis as consequence of the reduced Pol III activity according with a Pol III specificity of transcription.

g) Real-time RT-PCR analysis of the RNA level of two known pol II–transcribed (c-Myc and
Real-Time RT-PCR Analysis of 29A Endogenous RNA in Different Cell Types with Respect to the Less Abundant Samples. Analysis also of ASCL3 and 5S rRNA.

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**Fig. 4**

- Relation between 29A expression and cells doubling time.
- Proliferation inhibition of HeLa and SK-NBE cells after 48 h of 29A constructs transfection.

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**Fig. 5**

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**Fig. 6**

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Relation between doubling time (DT) of stable expressing 29A cell lines, and expression (revealed by real-time quantitative PCR) of 29A and MMP-9 (neuronal differentiation marker)

Fig. 7

Secondary structure of 29A RNA. Blue letters indicate the changes in primary structure as compared to Alu Y RNA. Red letters indicate the binding sites of SRP9/14 by analogy to SRP RNA.

(A) Thymidine incorporation of transfected HeLa cells transfect. Cotransfection of vector encoding for SRP protein SRP9 and SRP14 increase the inhibition of proliferation compared to 29A transfection alone, a rescue of inhibition is obtained with a vector transcribing a hairpin that silence one of the two protein (SRP14) or with the transfection of construct that express mutated protein (pSRP14k, pSRP9k).