PhD Programme

Complexity in post genomic biology

Progress Report: XX Cycle

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I.R.C.C.

Program

Chairpersons: Michele Caselle & Valentina Margaria

10-10 25: Claudio Isella
10.25-10.50: Matteo Cesaroni
10.50-11.15: Adrien Fauré

11.15-11.30: Break

Chairpersons: Raffaele Calogero & Alessia Mira

11.30-11.55: Cristiana Tomatis
11.55-12-20: Simona Pavan
12.20-12.45: Raffaella Bagnod

12.45-14: Lunch

Chairpersons: Michele DeBortoli & Carlo Baldassi

14-14.25: Ina.Berniakovich
14.25-14.50: Lucia Napione
14.50-15.15: Gabriele Seles
15.15-15.40: Alessio Noghero

Genomic analysis of RAS pathway activation in vitro an in vivo.

Claudio Isella

Over 20 years ago molecular biologists defined with the term “proto-oncogene” those genes that, when mutated or inappropriately activated, convert normal cells into transformed cells, i.e. *in-vitro* they proliferate in the absence of growth stimuli and grow in conditions that are limiting for normal cells, like soft agar in vitro and subcutaneous tumor growth in vivo; *in-vivo o*ncogene perturbations, such as mutation, expression alteration or amplifications, are typically observed in human cancers. Often oncogenes’ alterations are associated with specific tissue and/or tissue-histotype, suggesting different molecular sensitization for different pathway, depending on tissue origin.
Kirstein-RAS (KRAS) represents an archetypal proto-oncogene, for which mutations were found to occur very frequently in cancer (at codons 12 and 13) leading to constitutive activation of its signaling. *In-vitro*, as a consequence of KRAS mutation, normal fibroblasts are transformed.

We previously applied gene expression profiling to define transcriptome changes induced by KRAS activating mutation (G12D) in MLP-29, a normal mouse liver progenitor cell line. The mutation was introduced in MLP-29 by homologous recombination, generating an isogenic model (“knock-in”) in which the only difference between the parental and the mutated cells is a single nucleotide change at the desired position in the RAS coding sequence. This approach is substantially different from exogenous addition of a mutated KRAS coding sequence by transduction with an expression vector, which is much less similar to somatic mutation occurring during natural cancerogenesis.
Gene expression analysis was carried out in two microarray platforms, Affymetrix and Illumina, with systematic cross-validation leading to the identification of a “KRAS Ki signature” composed of 345 genes either induced or suppressed by KRAS KI in both microarray platforms.
Because of the occurrence of KRAS mutations in lung adenocarcinomas, we subsequently interrogated gene expression of our KRAS signature in a human lung cancer dataset composed by adenocarcinomas wild type or mutated for KRAS in 12, 13 residues.

To assess the class prediction ability of the signature in human cancer, we mapped the KRAS-KI signature on microarray data generated by Bhattacharjee and colleagues including 94 human lung adenocarcinoma samples for which they annotated the mutational status of codons 12 and 13 of KRAS. Also in this case, we observed a significant enrichment for genes with high SNR ($p < 0.0005$; Supplementary Figure 4). Notably, the KRAS-KI signature was found to have a less significant SNR when used to discriminate normal lung tissue from lung cancers of various types ($p = 0.0056$; Supplementary Fig. 3), indicating that it specifically discriminates KRAS mutational status rather than neoplastic progression. We then used the Nearest Mean classifier approach (22) to classify each lung adenocarcinoma based on the expression of the signature genes in all the other samples. Briefly, average expression was

calculated for each gene in the KRAS mutated and WT group, excluding the sample undergoing classification. The results of this classification are presented in Figure 6A. Interestingly, in most cases our KRAS-KI signature was able to correctly predict the wild-type status of the KRAS locus, as only 5 out of 36 cases classified as WT carried mutated KRAS sequences. Conversely, a large fraction (29 out of 58) of cases classified as "mutant" displayed a wild-type KRAS. At least two hypotheses can explain these results. The first option is that the results reflect misclassification of the samples, possibly due to poor specificity. The second possibility is that the signature detects a functional status of KRAS hyperactivation, possibly deriving from mutations at KRAS codons other than 12 and 13 or involving activation of signaling molecules acting in the same pathway.

To discriminate between these two hypotheses, we adopted the same classification procedure using genes from the two previously mentioned RAS signatures obtained in the mouse lung model ("Mouse Lung signature") and in HMECs ("HMEC-HRAS signature"). Both signatures displayed a performance very similar to the KRAS-KI signature, with good specificity for the samples classified as WT and high occurrence of WT RAS sequence in samples classified as mutated. We then verified how many of the samples with WT KRAS sequence were concordantly called mutated by all three signatures. The results of this analysis, shown in Fig. 6B, highlighted a striking concordance, with 15 samples called mutated by all three classifiers (hypergeometric p-value <0.000001). Notably, these make up a fraction of 25% of all samples with WT KRAS sequence at G12/13. Moreover, 13 additional WT samples (22%) were called mutated by 2 out of 3 classifiers. These results strongly support the possibility that the three signatures capture a functional status of active KRAS pathway even in the absence of mutations at codons 12 or 13. These results have obvious implications as they indicate that a "functionally activated" KRAS signaling pathway might be in place in lung tumors lacking the G12/13 KRAS mutations.

Activation of the Ras pathway takes place not only during cancer onset, but also in some hereditary syndromes. As an example, recent work pointed out misregulation of the RAS pathway in the dominant autosomic Noonan syndrome, characterized by specific symptomatology that predisposes patients to myeloid proliferative disorders. In particular PTPN11 and SOS1, two direct activators of RAS were found to have gain of function mutation respectively in 40% and 15% of the cases. Moreover with lower frequency also RAS mutations were found in different patients, with preferential mutation near to the typical oncogenic mutational hotspot observed in cancer. These genetic screenings, taken as whole, support the idea of a mild, but systematic activation of the RAS pathway in the Noonan Syndrome.
To investigate this opportunity we interrogated the expression profiles of peripheral blood mononuclear cells (PBMCs), and compared a cohort of Noonan patients versus non symptomatic individuals. In a preliminary analysis it was possible to define a strong intrinsic signature subdividing the Noonan patients in two subgroups, one with Noonan-specific gene expression and the other more similar to normal controls. These results indicate that PBMCs are a good reference to predict Noonan phenotype.
Further analysis will be performed in order to unveil association between expression profiles and clinical features as well as genetic alterations.

Higher-order chromatin changes induced by an oncogenic transcription factor

Matteo Cesaroni

Gross modifications in chromatin texture and global changes of gene expression are constant features of cancer cells. The underlying mechanisms, however, as well as their mechanistic links remain largely unknown. We report here the effects of the PML-RAR oncogenic transcription factor on higher-order chromatin structure and their consequences on gene expression. We found that 30% of the >1000 transcriptional targets of PML-RAR are distributed in the genome as gene clusters, do not possess PML-RAR - specific DNA-recognition elements (RAREs) within their promoters, and are flanked by one or more clusters of 30-40 Alu repeats each containing one RARE (Alu-RAREs). Chromatin analysis revealed that PML-RAR is first recruited at clusters of Alu-RAREs, where it accumulates at high density, and then at the transcription start sites (TSSs) of adjacent genes, through the formation of multiple DNA loops. High-resolution *in vivo* imaging showed co-localization of PML-RAR with clusters of co-regulated genes and long-range chromatin changes of the corresponding chromosomal regions. Treatment with drugs that target PML-RAR and induce tumor regression (RA and TSA) reverted higher-order chromatin changes and transcriptional repression of clustered genes. These findings demonstrate that higher-order chromatin changes are early events following PML-RAR oncogene expression and that they cause global changes of gene expression. Since also wild-type RARs bind Alu-RAREs and Alu repeats contain DNA-recognition elements for different transcription factors, specific recruitment to transposable elements and induction of higher-order chromatin changes might function as a general mechanism of transcriptional regulation.

Logical Modelling and Analysis of the Budding Yeast Cell cycle

Adrien Fauré

The budding yeast cell cycle core engine has been modelled in great detail, most notably by the groups of Béla Novak and John Tyson, using a differential formalism [1, 6]. Several models focusing on different regulatory modules have been developed. In this respect, the use of a logical formalism facilitates the development of more integrated models, through the articulation of control modules to the core engine. Such integrated models are difficult to build with the differential formalism due to the lack of quantitative data, as well as to numerical instabilities inherent to large non linear systems. Relying on the logical framework defined by Thomas and Kaufman [5], we use *GINsim*, the modelling software developed in our team [4] to integrate the morphogenesis checkpoint module to the core model of the yeast cell cycle (see also [3] for an application on the Mammalian cell cycle). Our current logical model recapitulates the wild type succession of events as presently characterised. We are now adjusting the model parameters to account for all mutant phenotypes described together with the original differential model [1]. At this point we focus on Knock Out (KO) mutants and on strong gene overexpressions. Our logical simulations provide consistent results for two third of the 90 mutants already tested. The remaining problematic cases involve regulatory
genes such as Sic1 and Cdh1, whose parameterisation is very complex. We are currently implementing in GINsim the possibility to define the parameters in the
form of logical formulas to overcome this problem. In parallel with our model of the budding yeast cycling core, we are developing a logical model of the morphogenesis checkpoint, inspired by the work of Ciliberto *et al*. [2]. This module is expected to produce a stable state with active Clb2 when the checkpoint is off, and when the checkpoint is on, Clb2 activation should be delayed, to prevent the formation of dinucleate cells. Still, the arrest is not complete, and after a while the checkpoint can be overcome. In our model, this
relates to an increase of mass. Clb2 is then activated and the cell can complete nuclear division, becoming dinucleate. This module has been designed to fit the wild-type behaviour, as well as that of the swe1Δ, mih1Δ and hsl1Δ mutants. Interestingly, the double mutants mih1Δ hsl1Δ and mih1Δ swe1Δ also exhibit the expected behaviour. Other mutant phenotypes listed or predicted in [2] have still to be tested. The two models have then been connected together. Preliminary stable state analysis shows that the coupled model retains the properties of the two modules. A more thorough analysis should confirm this result. At the present time, we still have to tune these models to fit all the mutant phenotypes. In the longer term, we plan to model other checkpoints and connect the resulting modules to our cell cycle core model. Finally, we also plan to systematically apply regulatory circuit analysis and model-checking approach to the resulting model.

References

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Calcium signaling and angiogenesis

Dr. Cristiana Tomatis

Growth-factors-induced intracellular calcium signals in endothelial cells regulate cytosolic and nuclear events involved in the angiogenic process. Among the intracellular messengers released after proangiogenic stimulation, arachidonic acid (AA) plays a key role and its effects are strictly related to calcium homeostasis and cell proliferation. The mechanisms responsible for the specificity of the biological effects regulated by intracellular calcium are still partially elusive: nevertheless, temporal and spatial properties of calcium signals could provide relevant informations. Using confocal calcium imaging we studied the potential role of caveolae and lipidic rafts in the generation and control of localised AA-induced calcium increases following store-independent calcium entry from extracellular medium. Another point was to study the role of AA-induced intracellular calcium signals in tumor endothelial cells derived from human breast carcinomas (B-TECs). AA promotes B-TEC proliferation and organization of vessel-like structures in vitro. In particular AA-activated long-lasting component of calcium entry in B-TECs is associated to the progression through the early phases of angiogenesis, mainly involving proliferation and tubulogenesis, and it is downregulated during the reorganization of endothelial cells in capillary-like structures. Finally we performed preliminary proteomics analysis on BTECs in order to identify calcium-dependent ex novo synthesis of proteins.

VEGF-A_{165} induced intracellular signaling pathways: how cell confluency influences protein activation

Simona Pavan

In endothelial cells (EC), Vascular Endothelial Growth Factor Receptor 2 (VEGFR2 also known as Flk-1 or KDR) has a key role in promoting EC adhesion to extracellular matrix, migration and proliferation by activating different intracellular signaling pathways in response to its ligand VEGF-A_{165}. The two main responses mediated by VEGFR2 are cell survival and proliferation, which depend on PI3K/Akt and PLCγ-Raf-MAPK molecular cascades respectively [(1)]. Recently it has been demonstrated that cell confluency is responsible for the switching between survival and proliferation, since in confluent cells (*in vivo* stabilized quiescent vessels) VEGFR2 preferentially promotes survival and in sparse cells (*in vivo* sprouting vessels) it leads to cell proliferation [(2)]. The aim of our study is either to underline the differences existing in the activation of the two signaling pathways or to give a quantitative annotation to complex network of these protein interactions or to create a mathematical model which can describe our experimental observations and be predictive for potential biological behaviours. To get preliminary data, we focused our attention on PLCγ, Erk, p85 and Akt, as main effectors of the survival and proliferative pathways. As experimental model, HUVE (Human Umbelical Vein Endothelial) cells were plated so as to obtain *in vitro* cellular conditions which resembled both the *in vivo* stabilized and sprouting vessels. HUVECs were stimulated with VEGF-A_{165} at different time points and then lysed. Cell lysates were so used to perform immunoblot analysis. The results we obtained are those relative to PLCγ, Erk, p85, Akt and their modulation subsequent to VEGF-A_{165} stimulation. They all show different behaviours depending on cell confluency. These results were propaedeutic for new experimental approaches consisting in quantitative immunoblot technique, involving samples randomization, normalization and quantification. To date, the results we got were used to draw curves for every data set to highlight the activation differences. This kind of analysis will be extended to all the proteins we have to consider in our project and it will prompt us to find something which can explain such differences.

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Development of HIV-1 infectivity peptide inhibitors

Raffaella Bagnod

Tat (Trans Activator of Transcription) is a small protein of 86 to 101 amino acids (depending on viral strain) produced by HIV-infected cells and conserved in the genomes of all primate lentiviruses. Tat main function is to enhance the transcription of viral RNAs, thus allowing the production of new viral particles and the consequent spreading of the infection. Due to its peculiar amino acid sequence and accordingly to the principle of viral economy, Tat has been demonstrated to modulate several other processes: activation of host gene transcription, invasion and modulation of surrounding cell gene expression, and growth factor mimicking. All the above listed biochemical effects have been related to clinical outcomes: a large number of data suggest that Tat could contribute to the onset of disorder associated with HIV-1 infection. When extracellularly released Tat is concentrated on cell surface proteoglycans and protected from proteolytic degradation, thus remaining in a biologically active form. Tat has been shown to bind the surfaces of both HIV-1- infected and surrounding uninfected cells. We provide evidence for a specific interaction (K_d = 8 nM) between Tat and the HIV-1 gp120 envelope protein, which enhances virus attachment and entry into cells. A series of phage display selected peptides able to bind Tat has been prepared and tested for their ability to inhibit HIV-1 entry and propagation of the infection. One of these peptides is represented by our candidate lead, CT319, a fully owned 15 L-amino acid peptide mimicking the gp120 V1/V2 loop. CT319 gp120-like region appears to be involved in target molecular recognition that drives the formation of a Tat/CT319 covalent bond. To confirm in vitro inhibitory activity, human PBMCs of two health donors were infected with HIV-1 R5 or X4 and CT319 was administered either at day 3, 6, 9 and 12. The comparison of RT activity in the absence/presence of CT319 reveals a weak and not reproducible inhibitory activity.

The metabolic syndrome.

I.Berniakovich

The metabolic syndrome (MS) is a world-spread disease which consists of cluster of single symptoms. According to IDF definition, *sine qua non* of MS is obesity with two or more other components: glucose intolerance, insulin resistance, raised TG level, raised blood pressure, reduced HDL cholesterol.

The $p66^{Shc}$ gene product is known to be localized within mitochondria and to produce hydrogen peroxide (1). Deletion of $p66^{Shc}$ decreases oxidative stress both *in vivo* and *in vitro* (2-5). Previously we established how the deletion of this gene could influence fat tissue development. Brown and white fat tissues of KO animals have defects in lipid accumulation because of decreased insulin sensitivity of preadipocytes. Notably, ROS productive function of $p66^{Shc}$ has been involved in the regulation of this phenomenon. We also reported resistance of KO animals to body weight gain both upon normal and high fat diet (6).

Also, we demonstrated higher total body insulin sensitivity and glucose tolerance of $p66^{Shc}$ KO animals. From our experiments and published data (7) we can conclude that $p66^{Shc}$ KO animals may represent an animal model for resistance to MS.

We confirm our data with transplantation experiment, where peadipocytes from KO and WT animals were injected subcutaneously into nude mice. In four weeks they gave origin to fat mass with different histology. Fat mass originated from KO preadipocytes consisted of high amount of small cells, whereas WT preadipocytes were developed into larger cells with higher amount of triglycerides.

Now we propose molecular mechanisms in which $p66^{Shc}$ can regulate lipid accumulation in adipocytes. Since it is well known, that stimulation of insulin receptor leads to the activation of MAPK and Akt signaling pathways, we checked if kinetics of MAPK and Akt activation upon insulin stimulation is altered in KO preadipocytes. Indeed, WT and KO cells respond in the same way to insulin in terms of MAPK phosphorylation, but Akt activation was significantly impaired in $p66^{Shc}$ KO ADPC. The steady state of phosphorylated Akt reflects the balance between the kinases and phosphotases activity. One of the most potent phosphotases which regulates insulin signaling is PTEN. PTEN exists in two forms, oxidized (not active) and reduced. We measured the amount of the two isoforms in brown preadipocytes. We found that the levels of reduced form in KO cells and of oxidized form in WT cells were significantly augmented upon insulin stimulation, suggesting that the increased level of dephosphorylation of Akt in $p66^{Shc}$ KO cells could be PTEN dependent.

One of the transcription factors regulated with Akt is FOXO1, known to be involved in ADP differentiation. Upon its phosphorylation by Akt it is excluded from the nucleus and translocates to the cytoplasm changing the pattern of expressed genes. Indeed, immunofluorescence studies revealed that insulin stimulation induced massive FOXO1 cytoplasmic translocation in WT brown preadipocytes, whereas this process was almost absent in KO cells. Notably, reintroduction of WT form of $p66^{Shc}$ but not its mutant form defective in ROS production ($p66^{Shc}$ qq) into KO cells rescued the FOXO1 translocation.

Hence, we propose that defect in fat accumulation in KO animals results from defect in lipid accumulation in adipocytes, due to alteration insulin-Akt-FOXO signaling in ROS dependent way.

SIGNAL TRANSDUCTION MODELLING APPLIED TO ENVIROMENTAL-INDUCED SWITCHING IN VEGF-A_{165} BIOLOGICAL PATHWAYS: ***Quantitative ImmunoBlotting (From the Theory to the Practical one) & Model Features***

Lucia Napione

Many important aspects of cell biology can be researched by a combined experimental and computational approach to develop models that yield useful results in order to understand the complex dynamical phenomena we observe in the living cells. In this context suitable experimental methods and appropriate data analysis, which allow the generation of high quality quantitative data, as well as mathematical concepts and tools are required. On the basis of these considerations, it is evident the need for a continuative an interactive collaboration between theoreticians and experimentalists, such that the modeller understands biological knowledge about the system and takes part in the definition of new experiments and that the experimentalist understands the principles of converting biological information into mathematical descriptions. This is the case of the present project.

The well-established multifunctionality of the Vascular Endothelial Growth Factor A_{165} (VEGF-A_{165}) make its signal transduction cascade an interesting subject of study using a system biology approach. VEGF-A_{165} cause a complex and integrated networks of signaling pathways which stimulate differentiation, survival, proliferation and migration of endothelial cells (ECs). Moreover, strong evidence from literature suggest that VEGF-induced signal pathways are under the influence of environmental conditions in terms of cell confluency; in EC population VEGF-A_{165} preferential induces cell survival, while in sparse ECs it triggers cell proliferation. The specific aim of this project is focused on the development of a modelling applied to environmental-induced switching in VEGF-A_{165} biological pathways. The experimental strategy provide for time-resolved quantitative data performed using Quantitative ImmunoBlotting (QI) methods {[1]; [2]} in order to develop mathematical modelling based on experimental data of high quality.

In the previous report, we described, from a theoretical point of view: 1) how advance the established techniques of immunoprecipitation and immunoblotting to more accurate and quantitative procedures using QI technique; 2) a QI-based experimental strategy designed for biochemical studies of environmental-induced switching in VEGF-A_{165} biological pathways. In the present report we show data analysis processes of signals obtained from our QI-based experiments, underlining the biological meaning of each analytical step. Moreover we describe the main features of the considered signal transduction model from a biological point of view; we talk about complementary experiments which are necessary for model development, and finally we suggest the involvement of a key molecule which might explain the presence of a sensible parameter in the model.

In the last semester of work our attention has been focused on three main issues: a) the performance of our experiments using the suitable apparatus that allow us to process data using QI-based criteria analysis; b) the data analysis, looking for a perfect matching between analytic procedure and biological meaning; and c) the construction of a small-scale model of limited complexity, containing a reduced number of variables, and aiming at addressing the specific question of environmental-induced switching.

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Gabriele Seles

There are several kinds of interesting informations that one can mine performing alignments of genomic sequences. Alignment networks are an useful tool to organize those results: we took genomic regions as vertexes and we put a link between two of them if they were linked by a suitable alignment. We then built a database to store this kind of data structures, focusing our attention on self alignments because we were mainly interested in paralogy and in duplication events. We believe it's possible to exploit those informations to theoretically predict novel genes; in fact we found a number of retrotranscribed regions in the human and mouse genomes that have not yet been annotated as transcribed. We developed a pipeline to automatically retrieve clusters of alignments presenting evidences of splicing, as those are a typical signature of retrotrasposition events. We explored our dataset and fine-tuned the pipeline analysis using a combination of statistical and visualization tools. We relied in particular on the Comparative Genomics Viewer (CGV), a web application producing bidimensional plots of alignments (in a way closely resembling dot-plots) and integrating biological annotations from various public databases (including ENSEMBL and UCSC). We compared our pseudogene collection with published datasets and we found a strong match with VEGA manually curated annotations. This result indicates that our pipeline is tuned to produce very few
false positives. We believe that this aspect makes it an useful tool to perform ab-initio gene predictions on new genomes still lacking thorough EST libraries. Finally, we selected the three most statistically significant gene Predictions both in human and in mouse and we validated them using RT-PCR. In two cases for each genome the results were positive, confirming the presence of a transcript for the predicted gene.

Analysis of neuronal and endothelial differentiation pathways in a converging cell culture system derived from embryonic stem cells.

Alessio Noghero

Mouse embryonic stem cells (ES) are blastocyst-derived totipotent cells that can in vitro differentiate into multiple cell types. They represent a good tool for the analysis of the differentiation processes and a rich source of precursors of different cell types. A key challenge is to understand the cues that direct ES cells into particular developmental pathways.

Taking advantage of the potential given by ES cell system we focused on the connections between endothelial and neural differentiation. It has been shown that the interplay between vascular and nervous systems is based both on anatomical/morphological resemblances and on molecular basis. Blood vessels and nerves run in parallel during embryonic development and interact at some anatomical sites through some shared molecules such as Neuropilin-1 (Nrp-1) and its extracellular ligand Semaphorin3A, the vascular endothelial growth factor receptor-2 (VEGF-R2/Flk-1), or the molecular signalling system Slit-Robo.

Starting from undifferentiated embryonic stem cells, using a specific protocol of differentiation (Conti et al., 2005), it is possible to generate mature neurons easily. We set up a cell culture system in which both neuronal and endothelial cells arise and develop simultaneously upon the same culture conditions. The presence of neuronal (BIII-tubulinn, MAP2, Tyrosine Hydroxylase) and endothelial (Flk-1, CD31, VE-Cadherin) markers was detected by immunofluorescent staining and RT-PCR. In this system we observed that the presence of growth factors is necessary for the formation of endothelial cells, while it is not for neurons.

To better characterize which factors are responsible for endothelium formation we tried different combinations of several growth factors (FGF, EGF, VEGF, BDNF, NGF) and different times of exposure. Then, the gene expression level of many known genes involved in neural and endothelial differentiation was analyzed. To do this, Taqman Low Density Arrays were used. This real-time PCR technique allowed us to study up to 96 genes at the same time, and to compare different samples that received different growth factors stimulation.

We started to analyze the gene expression level of the cells coming from the condition without growth factor, compared to that stimulated with FGF for the first three days from the day of the seed. As expected, the FGF stimuli induced an up-regulation of some representative endothelial genes, such as Angiotensin-1 Converting Enzime, angiopoietin-1, endoglin, CD-31, Tie-2 and many others. On the contrary, only a little up-regulation was observed for some neuronal marker genes, indicating that for the neural differentiation the FGF treatment is dispensable. This data confirmed our previous observations made by immunofluorescence technique.