Academic year 2010/2011

PhD in Biochemistry and Molecular Biology

Work-in-progress yearly reports

Elenco delle presentazioni dei dottorandi per l'idoneita' a sostenere la tesi finale di dottorato

- Lunedi' 24 Ottobre ore 9.30, Aula 2A2: 20 min+10 min discussion

3rd year students:
Bellomaria Alessia XXIV ciclo
Chadramouli Balasubramanian XXIV ciclo
Coletta Andrea XXIV ciclo
Fabrini Raffaele XXIV ciclo
Viticchiè Giuditta XXIV ciclo

ASSENTI GIUSTIFICATI

Relazioni attività di ricerca - III° Anno

Alessia Bellomaria, XXIV ciclo
Docente guida: Prof. Maurizio Paci

Advances in the molecular recognition mechanism of p63 by itch-E3 ligase

Itch E3–ligase mediates the degradation of several important proteins such as p63 and p73. Several signalling complexes, which are mediated by these domains, have been implicated in human diseases (Muscular Dystrophy, Alzheimer's Disease, Huntington Disease etc.). Itch contains four WW domains, which are essential for the target recognition process. These domains are highly compact protein-protein binding modules that interact with short proline-rich sequences and are considered belonging to the Group I of the domains binding polypeptides with a PY motif.
It is likely that the Itch-p63 interaction results from a direct binding of Itch-WW2 domain with the PY motif of p63. Indeed, we studied the in vitro interaction between Itch-WW2 domain and p63(534-551), an 18-mer peptide encompassing a fragment of the p63 protein including the PY motif, by fluorescence, CD and NMR spectroscopy. 3D heteronuclear NMR experiments with uniformly ^{13}C, ^{15}N-labelled Itch-WW2 were recorded to assign the backbone and side chains and the residues of Itch-WW2 involved in the interaction with p63-peptide were identified.
We have, also, evaluated the effects of a site specific mutation of p63, I549T, that has reported in both Hay–Wells syndrome and Rapp–Hodgkin syndrome, both on the conformation of pep63 and on the Itch-WW2-pep63 interaction and an extended PPxY motif for the Itch recognition was proposed.
Moreover, a cyclization of pep63 was performed and the binding with Itch-WW2 was analysed by fluorescence spectroscopy. The cyclization of the peptide leads an increase of both the resistance to proteases cleavage and to the ability of the peptide to bind the WW2 domain. Thus, the data here presented suggest the possibility to use the cyclic form of pep63 for an *in vivo* inhibition of the recognition mechanism of Itch E3-ligase.

Chandramouli Balasubramanian, XXIV ciclo
Docente guida: Prof. Alessandro Desideri

Structural investigation on HIV-1 envelope glycoprotein gp120 through molecular simulation technique

The entry of HIV-1 into the host cell initiates with the interaction of its envelope glycoprotein gp120 with a CD4 receptor and a co-receptor that can be either CCR5 or CXCR4. The initial interaction of gp120 with CD4 results in significant conformational changes that presents the previously hidden portion of gp120 for subsequent interaction with the co-receptor. A HIV strain, based on its co-receptor preference, is designated as CCR5 or CXCR4 tropic. CCR5 specific strains predominate the population at the initial stage of infection while CXCR4 strains tend to predominate with the disease progression and the ability of HIV to alter the co-receptor usage is known to be an important characteristic to escape from the immune response. The sequence and structural features of the third variable loop (usually referred as V3 loop) within gp120 is known to dictated the HIV's choice of co-receptor usage. In our work, we have examined by molecular simulation the structure-dynamics relationship of two gp120 proteins representing a CCR5 and CXCR4 specific strains in the CD4-free and CD4-bound states. The results confirms that, while the V3 loop have a similar dynamical behaviour in the absence of CD4, show significantly difference in the presence of CD4. Irrespective of the strain specificity, the CD4 contacts with the two gp120 is strongly conserved. These results let us to propose that the flexibility and net charge of the V3 loop are important criteria for the co-receptor recognition.

Andrea Coletta, XXIV ciclo
Docente guida: Alessandro Desideri

Studio di inibitori della topoisomerasi IB mediante tecniche di Chimica Teorica

La topoisomerasi è un enzima presente negli eucarioti, nei procarioti e in alcuni virus. La topoisomerasi IB umana (htopoIB) è il target specifico di una classe di farmaci chemioterapici di largo utilizzo, il cui capostipite è la camptotecina: un alcaloide ricavato dalla pianta *Camptotheca acuminata.* L'azione delle camptotecine avviene mediante la stabilizzazione del complesso binario htopoIB+DNA che si forma nella fasi di trascrizione e duplicazione, trasformando la htopoIB in un veleno e inducendo apoptosi nelle cellule in elevata proliferazione. La camptotecina non trova un uso clinico a causa della sua scarsa idro-solubilità, dei suoi gravi effetti collaterali e della presenza, in molte linee cellulari tumorali, di mutazioni della htopoIB che rendono quest'ultima resistente all'inibizione. Tuttavia sono in uso clinico o in fase di trial numerosi composti derivati della camptotecina (topotecano, irinotecano). Inoltre esistono numerosi studi sperimentali che suggeriscono la topoisomerasi quale target nel trattamento chemioterapico di infezioni parassitarie quali la Leishmaniosi (*Leishmania donovani*), sono infatti noti dei composti inibitori della ldtopoIB. Al fine di comprendere i meccanismi molecolari che influenzano l'azione inibitoria di questa classe di composti, si sono già rilevati di grande efficacia le tecniche della Dinamica Molecolare, il Molecular Docking e il Drug Design (e.g.: QSAR). Nel corso del mio dottorato mi sono occupato della caratterizzazione, mediante tecniche di chimica teorica (calcolo della struttura elettronica, potenziale elettrostatico, realizzazione di modelli di meccanica molecolare, etc.) di un farmaco antitumorale attualmente in uso clinico (topotecano)[1-3] di un composto che presenta attività inibitorie nei confronti della LdtopoIB [4], e di alcuni rappresentanti di una nuova classe di farmaci antitumorali di derivazione sintetica

(indenoisochinoloni). L'uso di queste tecniche ha già permesso di correlare le bande di assorbimento UV-Vis del topotecano con il suo micro-ambiente di solvatazione [3]. L'obbiettivo è quello di applicare le medesime tecniche agli altri composti in esame e di estendere la ricerca allo studio dell'interazione di questi con il DNA e i loro target farmacologici, mediante tecniche di Chimica computazionale quali la Dinamica Molecolare non all'equilibrio e calcoli Quanto-Meccanici.

Raffaele Fabrini, XXIV ciclo
Docente guida: Prof. Giorgio Ricci

Erythrocyte glutathione transferase as a potential new biomarker to evaluate the uremic toxicity in chronic kidney diseases and the hemodialysis adequacy.

The erythrocyte glutathione S-transferase (e-GST) is a member of a superfamily of enzymes involved in cell detoxification that shows an increased expression in chronic kidney disease (CKD) patients. We propose a new automated analysis procedure for e-GST activity that has been validated in 72 CKD patients and 62 maintenance hemodialysis patients (MHD). An increased e-GST activity was confirmed in MHD patients (N = 62; 10.2 ± 0.4 U/g_{Hb}) compared with healthy subjects (N = 80; 5.8 ± 0.4 U/g_{Hb}), and as an original finding, a significant increase of e-GST activity was observed in pre-dialysis CKD patients with a positive correlation with disease severity weighted according to the four stages of K-DOQI classification (7.4 ± 0.5, 8 ± 1, 9.5 ± 0.6, 12 ± 1 U/g_{Hb}, respectively). No correlation was found between e-GST activity and usual markers of systemic inflammation and renal function, while a significant correlation was observed for the first time between plasma homocysteine (Hcy) in MHD patients. Hcy, however, was not identified as an inhibitor of e-GST enzyme. The results in this study suggest the potential for automated e-GST analysis as a valuable tool to further explore phase II-related uremic toxicity in CKD and MHD patients.

A further study a further study was focused on hemodialysis patients and hemodialytic techniques. The Kt/V_{urea} ratio only reflects the efficiency of a single dialytic session against small toxins, but it does not measure the depuration ability against middle (> 500 Da) and protein bound toxins. This limit may explain the controversial association between patient survival and Kt/V_{urea}. Our preliminary results reveal that patients under convective dialysis display e-GST activity (7.2 ± 0.4 U/g_{Hb}, N = 59) significantly lower than patients under diffusive treatment (10.2 ± 0.5 U/g_{Hb}, N = 44) confirming the superiority of the former procedure. No correlation has been found with Kt/V_{urea} parameter. e-GST, whose level cannot be varied during the erythrocyte life-span, provides a long-time mediated information on the blood toxin content and could be proposed as a new biosensor for dialysis adequacy.

Giuditta Viticchiè, XXIV ciclo
Docente guida: Prof. Gerry Melino

Analysis of miR-203 in different biological processes: skin re-epithelialization during wound healing, and cellular invasion and migration in prostate cancer cells.

Keratinocyte proliferation and migration are crucial steps for the rapid closure of the epidermis during wound healing, but the molecular mechanisms involved in this cellular response remain to be completely elucidated. Here, by *in situ* hybridization, we characterize the expression pattern of miR-203 after wound induction in mouse epidermis, showing that its expression is down regulated in the highly proliferating keratinocytes of the "migrating tongue" while is strongly expressed in the differentiating cells of the non lesional skin surrounding the wound. Our data suggest that miR-203 exerts a specific role in wound re-epithelialization and epidermal homeostasis reestablishment of injured skin by controlling the expression of target proteins that are responsible for both keratinocytes proliferation and migration.

Prostate cancers show a slow progression from a local lesion (primary tumor) to a metastatic and hormone-resistant phenotype. After an initial step of hyperplasia, in a high percentage of cases a neoplastic transformation event occurs that, less frequently, is followed by epithelial to mesenchymal transition and invasion of healthy tissues (usually bones). MicroRNA-203 (miR-203) is a tumor suppressor microRNA often silenced in different malignancies. Here, we show that miR-203 is downregulated in clinical primary prostatic tumors compared to normal prostate tissue, and in metastatic prostate cancer cell lines compared to normal epithelial prostatic cells. Overexpression of miR-203 in brain or bone metastatic prostate cell lines (DU145 and PC3) is sufficient to induce a mesenchymal to epithelial transition with inhibition of cell proliferation, migration and invasiveness. We have identified CKAP2, LASP1, BIRC5, WASF1, ASAP1 and RUNX2 as new miR-203 direct target mRNAs involved in these events. Therefore, miR-203 could be a potentially new prognostic marker and therapeutic target in metastatic prostate cancer.

Elenco delle presentazioni dei dottorandi per il passaggio di annualita'

- Martedi' 25 Ottobre ore 9.30 Aula 2A2: 10 min+5 min

1st year students, XXVI ciclo:
Chimenti Maria Sole
Mancini Mara
Arno' Barbara
Evangelista Daniela
Latina Alessia
El Said Dalya
Iorio Egidio
Shantaram katkar Prafulla
Pinetti Valentina
Schillaci Carlo
Loria Rossella
Talamonti Emanuela

2nd year students, XXV ciclo:
Italiano Dafne
Cozzoli Eliana
Mastrangelo Nicolina
Memmi Elisa Maria
Lenti Elisa

ASSENTI GIUSTIFICATI

Relazioni attività di ricerca - I° Anno

Maria Sole Chimenti, XXVI ciclo
Docente guida: Prof. Gerry Melino

Multiplatform metabolomic study of CD4+ T-Lymphocytes after Methotrexate and Infliximab treatment: focus on rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease characterized by infiltration of mononuclear and polymorphonuclear cells into the joints with the development of inflammation that results in destruction of articular cartilage and adjacent bone. The mechanisms that give rise to RA are only partly understood. Activated TCD4+ cells comprise a large proportion of the inflammatory cells that invade the synovial tissue and may therefore be a cell type of pathogenic importance. A number of inflammatory mediators have also been implicated in the establishment and progression of inflammatory joint destruction, including proinflammatory cytokines such as tumor necrosis factor (TNFα), interleukin-6 (IL-6), and IL-17.
Conventional treatments include synthetical DMARDs, such as methotrexate (MTX), that lead to clinical improvement but are characterized by several side effects. Recently, Inhibitors of TNFα have demonstrated significant efficacy and safety profile in inflammatory disease such as RA.
Metabolomics is the rapidly evolving field of the comprehensive measurement of ideally all endogenous metabolites. The purpose of our study was the evaluation in CD4+ Tcells, from a multiplatform metabolomic study, of treatment with methotrexate and infliximab in order to investigate the metabolomic features that can be obtained.
Our results will be correlated with complementary set of clinical findings in order to associate metabolomic results to drugs efficacy and tolerability.

Mara Mancini, XXVI ciclo

Docente guida: Dr.ssa Eleonora Candi

Ruolo dei microRNA nella senescenza replicativa dei fibroblasti dermici umani

Il processo di senescenza cellulare è stato scoperto quaranta anni fa come fenomeno che previene la crescita di fibroblasti umani in coltura in maniera indefinita. L'accumulo di cellule senescenti, in un tessuto come la pelle, ne compromette la funzione fisiologica, il rinnovo, la capacità di ripararsi in seguito a qualsiasi tipo di insulto e può contribuire al suo invecchiamento. In questi ultimi anni, un numero sempre più grande di evidenze sperimentali ha correlato l'espressione dei microRNA al fenomeno della senescenza cellulare definendo loro possibili ruoli nell'induzione e nel mantenimento del fenotipo senescente in quanto coinvolti in differenti vie cellulari che regolano il ciclo cellulare. La nostra attenzione si è rivolta alla ricerca di microRNA coinvolti nel processo di senescenza replicativa nei fibroblasti dermici umani. A tal proposito è stato riprodotto in vitro un modello sperimentale di senescenza replicativa di fibroblasti dermici umani mantenuti costantemente in uno stato di sub-confluenza per 16 passaggi. In tali condizioni sperimentali l'induzione e il mantenimento del fenotipo senescente è accompagnato da un aumento dei livelli d'espressione della proteina p16, da una diminuzione della percentuale di cellule in fase S del ciclo cellulare, da un aumento della percentuale di cellule in fase G0/G1 e infine del cambiamento morfologico e dalla positività al saggio per la SA-β-Gal, tipiche di una coltura cellulare senescente. Nel modello sperimentale descritto, dal passaggio 1 al passaggio 15, è stato dimostrato l'aumento di espressione dei miR-138, 181a e 181b, 191, 134, 152 e 432 mediante *PCR-Real Time* quantitativa. L'analisi di predizione bioinformatica condotta per identificare possibili bersagli molecolari dei miR-138, 181a, 181b, 191, 134, 152 e 432 ha portato a considerare come bersagli putativi, il 3' UTR dei messaggeri di proteine strutturali della matrice extracellulare del derma. Durante l'invecchiamento il derma si indebolisce, le fibre di collagene si degradano, il loro rinnovamento naturale diventa più difficoltoso e l'elastina perde elasticità. A tal proposito quello che si vuole dimostrare in questo progetto è il coinvolgimento dei microRNA identificati, nel processo di senescenza dei fibroblasti dermici umani e la loro azione nel modulare l'espressione di proteine strutturali della matrice extracellulare.

Barbara Arnò, XXVI ciclo
Docente guida: Prof. Alessandro Desideri

Plasmodium falciparum topoisomerase I: a basic characterization

Malaria is an important human zoonotic disease transmitted by arthropod vectors and caused by parasites of the genus Plasmodium. Plasmodium falciparum infection is one of the most frequent acquired red blood cell disorders worldwide. These parasites undergo asexual multiplication within erythrocytes, causing anemia, fever, chills, nausea and, in several cases, coma and death. The spread of multi-drug-resistant strains has increased the need to identify new molecular targets for antimalarial chemotherapy. DNA topoisomerases may be considered possible candidates, due to their important role in cellular activities, including DNA replication. DNA topoisomerases alter the biological state of DNA by catalysing the breaking and rejoining of DNA strands and they are classified as type I and type II. Both are targets for antibacterial and anticancer agents. Type I topoisomerases are specifically inhibited by a natural plant alkaloid, camptothecin (CPT) and its derivatives, which are known anticancer compounds actually used in clinical therapy. The Plasmodium falciparum topoisomerase I (PftopoI) is a 839 amino acids monomer enzyme encoded by a single copy gene localized in chromosome 5. The protein

sequence shows a 42% identity with the human homologue and structural analysis reveals that PftopoI contains three insertion regions when compared to human topoisomerase I (htopoI): two in the core and one in the linker domain. In this work I have studied the effect of CPT on PftopoI, to investigate how the different structure could influence the response to the drug and to propose topoisomerase I as a critical target against malaria. Since previous studies have suggested that in the human enzyme the linker domain modulates the CPT effect, I have produced a chimera where the linker domain of htopoI has been swapped with the corresponding domain of PftopoI.

Daniela Evangelista, XXVI ciclo
Docente guida: Prof.ssa Luciana Avigliano

Interazione endotelio-linfociti: potenziali modulatori della cascata di adesione leucocitaria.

La migrazione cellulare è fondamentale in molti processi fisio-patologici, come la risposta immunitaria, l'infiammazione, l'angiogenesi, la formazione di metastasi tumorali. Il nostro studio si è focalizzato sulla modulazione della risposta infiammatoria, in particolare delle fasi precoci della cascata di adesione leucocitaria. E' stato innanzitutto messo a punto un modello sperimentale di infiammazione, utilizzando le cellule endoteliali ECV304 e le cellule linfocitarie Jurkat trattate con lipopolisaccaride (LPS), un componente della parete cellulare dei Gram negativi e potente induttore della risposta infiammatoria. Mediante saggi ELISA, abbiamo dimostrato che l'LPS induce il rilascio di molecole pro-infiammatorie (quali il TNF-α e la L-selectina) nel mezzo di coltura, con un effetto massimo a 0,1 μg/ml, che quindi è stata la concentrazione utilizzata negli esperimenti successivi. Una volta messo a punto il modello sperimentale, è stato valutato l'effetto degli endocannabinoidi sul processo infiammatotrio, dal momento che è stato suggerito un ruolo immuno-modulatore per questi mediatori lipidici, anche se i meccanismi d'azione rimangono ancora largamente oscuri. Cellule ECV304 trattate con concentrazioni crescenti di 2-AG (0,1-10 μM) mostrano un aumentato rilascio di TNF-α, in maniera analoga a quanto osservato con l'LPS. Inoltre, il 2-AG e l'LPS regolano, in maniera precoce e transitoria, l'esposizione sulla membrana plasmatica della P-selectina ed E-selectina nelle cellule ECV304; queste due proteine sono le prime molecole di adesione che sono attivate durante la cascata infiammatoria e mediano l'iniziale adesione dei leucociti ed il loro successivo rotolomento sull'endotelio. L'effetto del 2-AG sembra coinvolgere il recettore cannabico di tipo 1, dal momento che un antagonista selettivo è in grado di contrastare l'azione pro-infiammatoria del 2-AG.

Alessia Latina, XXVI ciclo
Docente guida: Prof. Gerry Melino

Correlazione tra ΔNp63α e i livelli di espressione dei microRNA nell'epidermide.

ΔNp63α è un fattore di trascrizione importante per lo sviluppo dell'epidermide. Il suo livello di espressione varia nei diversi strati dell'epidermide, è abbondante a livello dello strato basale e decresce negli strati superiori. Avvalendoci della tecnica del microarray abbiamo potuto constatare una variazione del profilo di espressione di diversi microRNA (miRNA) nei

cheratinociti epiteliali umani (HEK) in cui ΔNp63α era stato silenziato. Tale variazione è stata riscontrata anche in un secondo array in cui sono stati confrontati i profili di espressione dei miRNA in HEK proliferanti e HEK differenziate. Dall'analisi incrociata di questi due array sono stati selezionati 8 miRNA il cui livello di espressione si riduceva in entrambi i casi. Per validare questi dati in vitro abbiamo effettuato Real-Time PCR relativa su estratti di RNA di HEK indotte a differenziare o trasfettate per silenziare ΔNp63α. I risultati ottenuti dimostrano che in vitro solo tre miRNA (miR-150, miR-576-5p e miR-548c-5p) hanno un andamento in linea con i livelli di espressione di ΔNp63α in HEK. Per studiare se i tre miRNA sono regolati direttamente da p63, abbiamo analizzato, con il contributo di alcuni collaboratori, le regioni genomiche in prossimità di questi miRNA. Attraverso un esperimento di ChipSeq (immunoprecipitazione di cromatina seguita da sequenziamento genomico) abbiamo riscontrato un sito di legame per p63 a monte di miR-150.
Altri esperimenti saranno quindi necessari per confermare che p63 è responsabile dell'espressione diretta di questo miRNA. Inoltre cercheremo di identificare i suoi mRNA bersaglio attraverso esperimenti di trasfezione con pre-miR-150 e anti-miR-150 e di investigare il ruolo di questo miRNA nella proliferazione delle cellule epiteliali.

Dalya Elsaid, XXVI ciclo
Docente guida: prof. Alessandro Finazzi-Agro'

Blood derived stem cells (BDSCS): Neural differentiation protocols for human therapy

Tissue regeneration is a process by which a tissue regenerates itself when apart has been removed or is necrotic. It should be distinguished from tissue repair, with which anew connective tissue replacing the lost tissue, such as scar tissue and repair of bone fractures .the nerve cells of a mammal such as do not regenerate, although the peripheral nervous tissue can regenerate if neurillematic sheath allow the orientation of the fibre. The importance of obtaining functional nerve cells is vital in neurodegenerative diseases. Neurodegenerative diseases are by definition progressive chronic diseases, characterized by a selective loss of neurons in areas and symmetric motor, sensory, cognitive and membership of the CNS, or loss\dysfunction of myelinated and non myelinated fibres in the PNS. Two degenerative neurological diseases but with clinical picture, evolution, prognosis and therapy are very different multiple sclerosis (ms) and amyotrophic lateral sclerosis (ASL). They are both diseases that affect the nervous system, are chronic, degenerative diseases with multifactorial etiology. Using a" Neuron Differentiation Kit" has allowed me to achieve the desired results: getting the nerve cells from stem cells. However, the differentiation protocols in the kit can not be applied in vivo due to toxicity of substances used. Knowing this, I aimed my work in the development of protocols for the differentiation of adult stem cells into nerve cells, with non-toxic and commonly used in therapy. The aim of my work is to:
1-devolop a protocol for the differentiation of stem cells into nerve cells and optimizes conditions for their differentiation (media, growth factors, culture media, stimulation for differentiation)
2-ensure that at the end of the cultured cells obtained were actually nerve cells (using PCR, monoclonal antibodies, Western blotting).
3-to study the type of nerve cell selectively grown in culture, trying to differentiate between the protocols in order to obtain from time to time , the desired type of nerve cell.

Egidio Iorio, XXVI ciclo
Docente guida: Prof. Maurizio Paci

Effects of downmodulation of choline Kinase and phosphatidylcholine-specific phospholipase C on the Magnetic Resonance Spectroscopy choline profile of epithelial ovarian cancer

Detection of an abnormal phosphatidylcholine (PtdCho) metabolism in epithelial ovarian cancer (EOC) by magnetic resonance spectroscopy (MRS) profile analysis, showed a significant increase in phosphocholine (PCho) content in EOC cells compared with non tumoral counterparts (Iorio E et al, Cancer Res 2005), associated with an altered activity profile of some PtdCho-cycle enzymes, including 12-to 25-fold activation of choline kinase (ChoK), and 5- to 17-fold increase in the activity of PtdCho-specific phospholipase C (PtdCho-plc), responsible for direct PCho production (Iorio E et al, Cancer Res 2010). Aims of the present study are to evaluate the biological relevance of ChoK and PtdCho-plc expression and activities in EOC and to define the possible role of MRS profiles in providing non invasive biomarkers to monitor the effectiveness of agents selectively targeted against ChoK and PtdCho-plc activities.
Inhibition of ChoK-alpha mRNA expression by transient RNA interference was associated with a significant reduction of overall ChoK protein expression and an about 70% drop in PCho content. We observed a partial inhibition of cell growth associated with a consistent increase in cells blocked in the G1-phase fraction. Pharmacological inhibition of PtdCho-plc induced a 30-to-40% reduction of PCho content and blocked cell proliferation. Our observations, confirming a main role for these two enzymes in deregulated choline metabolism in EOC tumors, warrant further investigations on the upstream and downstream signaling and metabolic alterations associated with ChoK and PtdCho-plc activation and suggest these enzymes as a promising targets for alternative therapeutic approaches.

Shantaram katkar Prafulla, XXVI ciclo
Docente guida: Prof. Alessandro Desideri

Characterization of effects of Gold (III) compound on the human topoisomerase IB catalytic cycle

A numbers of anticancer agents have topoisomerase as their target and they can act through several mechanism such as preventing DNA topoisomerase binding, DNA cleavage inhibition or stabilization of DNA-topoisomerase cleavable complex. The Gold (III) compound [Au(C^N^C)(IMe)] has been shown to have anticancer properties and it has been suggested that this is due to a perturbation of the human topoisomerase 1 B activity.
In this year, i have investigated the mechanism of inhibition of human topoisomerase IB activity, by analyzing the various steps of enzymatic catalytic cycle. Gold(III) compound inhibits the DNA super coiled relaxation and inhibit the catalytic cleavage of DNA substrate. Gold(III) compound does not permit the stabilization of cleavable complex by camptothecin when it is preincubated with enzyme and has no effect on the religation. Preincubation of enzyme with the compound before the addition of substrate increases the inhibitory effect. Analysis of DNA-

topoisomerase binding reaction indicates that the Gold(III) compound acts as a topoisomerase inhibitor preventing the enzyme-DNA interaction.

Valentina Pinetti, XXVI ciclo
Docente guida: Prof. Antonio Costanzo

Developmental factor IRF6 exhibits tumor suppressor activity in squamous cell carcinomas.

The transcription factor IRF6 regulates craniofacial development and epidermal proliferation. We have recently demonstrated that IRF6 is a component of a regulatory feedback loop that controls the proliferative potential of epidermal cells, being transcriptionally activated by ΔNp63 and inducing its proteasome-mediated downregulation, thereby limiting keratinocyte proliferative potential. We have hypothesized that IRF6 could be also involved in skin carcinogenesis. Therefore, we analyzed IRF6 expression in a large series of Squamo Cellular Carcinomas finding strong downregulation of IRF6 that correlated with their invasive and differentiation status. IRF6 downregulation in SCC cell lines and primary tumors correlates with methylation on a CpG island located in its promoter region. To identify the molecular mechanisms regulating IRF6 potential tumor suppressive activity, we performed genome wide analysis by combining ChIP-seq for IRF6 binding sites and gene expression profiling in primary human keratinocytes after siRNA-mediated IRF6 depletion. We observed dysregulation of cell cycle related genes and of genes involved in differentiation, cell adhesion and cell-cell contact. Many of these genes were direct IRF6 targets. We also performed *in vitro* invasion assays showing that IRF6 downregulation promotes tumor cell invasivity. Reintroduction of IRF6 in SCC cells strongly inhibits their growth, as shown by colony forming assays. Our data highlight a novel function for the developmental regulator IRF6 that exhibits tumor suppressor activity within carcinogenesis of stratified epithelia. Moreover, IRF6 binds regions near miRNAs which are involved in regulating the pluripotency reprogramming of stem cells. Some of these are also differentially expressed in cancer stem cells. Therefore, our future plan is to study the role of IRF6 as regulator of stemness and pluripotency.

Carlo Schillaci, XXVI ciclo
Docente guida: Prof. Maurizio Paci

The role of iron in generating ROS as inflammatory cause of Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) also known as cryptogenic fibrosing alveolitis is an interstitial lung disease characterized by progressive parenchymal fibrosis and ventilatory restriction [1]. IPF is a rare disease which affects approximately 5 million persons worldwide. The prevalence is estimated to be slightly greater in men (20.2/100,000) than in women (13.2/100,000) [2]. While pathogenetic mechanisms are incompletely understood, the currently accepted paradigm proposes that injury to the alveolar epithelium is followed by a burst of pro-inflammatory and fibroproliferative mediators that invoke responses associated with normal tissue repair. For unclear reasons, these repair processes never resolve and progressive fibrosis ensues. This theory is aligned with the most recent scientific data and has been summarized elsewhere [3-4]. Early theories on the pathogenesis of IPF focused on the role of chronic inflammation triggered by unknown stimuli that subsequently leads to lung injury and pulmonary

fibrosis[5]. Prognosis is poor; reported median survival is 3 years. Our attention is focused on the characterization and quantitation of the redox active chelatable iron that is presents in bronchoalveolar fluids (BAL) extracted from lungs[6]. In fact redox active iron is a powerful pro-oxidant in body fluids through its ability to convert poorly reactive oxygen species into highly reactive and damaging species such as the hydroxyl radical. Reactive oxygen species(ROS) are important in the pathophysiology of several diseases of the lung because their ability to oxidatively damage DNA, lipids and protein normally presents in BAL and lung tissue. For these reasons this work is aimed to quantify and make a speciation of the iron present in BAL from patients, to evaluate if the amount of iron is able to catalyze the production of ROS potentially dangerous for the inflammation of the alveolar tissue and to relate the chemical forms to different redox activity. The project will develop eventually through the screening of several iron chelators already used as drugs for other diseases , which inhibit oxidant activity of free iron in solution. In fact some type of chelating agent are able to inhibit the production of ROS but on the contrary others ligands increase this production depending on chemical structure and bioactivity. The comparison of anti oxidant activities and the pharmacophoric analysis of these drugs will help to design new chelators with new potential pharmaceutical effects.

Rossella Loria, XXVI ciclo
Docente guida: Dott.ssa Rita Falcioni

Silencing of PI3K pathway in human melanoma: a strategy to overcome the resistance to BRAF and NRAS-targeted therapies of new generation

Melanoma is a cancer that arises from melanocytes, specialized pigmented cells that are found predominantly in the skin. Recent discoveries in cell signaling cascade have provided greater understanding of the biology that underline melanoma, and these advances are being exploited to provide targeted drugs and new therapeutics approaches. A signaling pathway that is emerging as important in melanoma is the PI3K pathway whose activity is amplified in several melanomas suggesting a role of this kinase in the mechanisms of pharmacological resistance to anti-cancer drugs. The major activator of PI3K in nature is the receptor heterodimer HER2/HER3, with HER3 possessing six binding sites for p85, the regulatory subunit of PI3K. HER3 is frequently expressed in BRAF melanoma and metastases at elevated level (unpublished results) and is considered a determinant for poor prognosis. These observations support the involvement of HER3 growth factor receptor in the mechanism of resistance to new-targeted therapies. We propose to investigate the following aspects:

1. To analyze gene profile and microRNA between BRAF and N-RAS melanoma derived from human cell lines and primary tumors.
2. To analyze the role of HER3 in the mechanism of resistance to new-targeted therapies directed to BRAF and NRAS activity, such as PLX4720 or the Farnesyl transferase inhibitor, respectively.
3. To use SCID mice as model to analyze in vivo the capability to interfere with HER3 expression to improve new target therapies.

Emanuela Talamonti, XXVI ciclo
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Role of endocannabinoids in the modulation of inflammasomes pathway in macrophages

Endocannabinoids are a class of lipid mediators, which include amides and esters of long chain polyunsaturated fatty acids. These bioactive lipids modulate a variety of neuroinflammatory and neurodegenerative diseases, mainly through the activation of type-1 and type-2 cannabinoid receptors (CB_1 and CB_2). Immune cells express both receptors and possess also the whole machinery responsible for endocannabinoid metabolism. Not surprisingly, evidence has been accumulated showing manifold roles of endocannabinoids in the modulation of the immune system. Recently, studies have demonstrated immunomodulatory effects of AEA on macrophages and dendritic cells and their involvement in innate immunity. The activity of innate immunity is regulated by Toll-like receptors (TLR) and new signaling pathways collectively called "inflammasomes". The latter are involved in the activation of inflammatory caspases, mainly caspase-1, whose main substrates are interleukin (IL)-1β and IL-18, that are crucial inflammatory mediators. Based on this background, we will investigate the involvement of CB receptors in the possible effects mediated by endocannabinoids on cytokine release and expression of inflammasome members. As a first step, we will estimate the release of IL-1β and IL-18 from human macrophages and differentiated THP1 cells upon pretreatment with agonists and antagonists of CB receptors.

Relazioni attività di ricerca - II° Anno

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Identificazione di NCF2 come nuovo target di p53

L'analisi di due microarray realizzati in cloni stabili di cellule SAOS ad espressione inducibile per p53, TAp63α e ΔNp63α ha permesso di notare l'aumento dell'espressione di NCF2, subunità citosolica dell'enzima NADPH ossidasi, in risposta all'induzione di p53. Il reclutamento in membrana di NCF2 provoca l'attivazione della NADPH ossidasi e la conseguente generazione di ione superossido a partire dall'ossigeno molecolare. Sono stati quindi effettuati saggi di attività del promotore di NCF2, confermando l'attivazione da parte di p53 e, in misura minore, da parte di TAp63α. In seguito alla predizione bioinformatica dell'esistenza di tre siti presunti di legame di p53 al promotore di NCF2, attraverso immunoprecipitazione della cromatina (ChIP) è stato confermato il legame del fattore in almeno uno dei tre siti, rafforzando l'ipotesi. La regolazione è stata confermata attraverso Real Time PCR in diverse linee cellulari, anche in seguito a stimolazione di p53 attraverso danno al DNA, effetto sicuramente dovuto a p53 poiché non si verifica in assenza del fattore di trascrizione. Sarà quindi verificato l'effetto dell'aumento di NCF2 in termini di stato ossidativo della cellula e di apoptosi in cellule HCT116 sia in presenza che in assenza di p53, poiché in un modello cellulare con p53 mutato (HaCaT) in seguito al silenziamento genico di NCF2 si ha un aumento della presenza di specie reattive dell'ossigeno ed un considerevole aumento della percentuale di apoptosi.

Eliana Cozzoli, XXV ciclo
Docente guida: Prof. Nicola Rosato

Differenziamento in vitro delle BDSC in epatociti

Il trapianto di fegato è il trattamento di scelta per pazienti con patologia epatica cronica, cirrotica e/o neoplastica. Il limitato numero di donatori, nonché il rigetto e l'uso prolungato di immunosoppressori, ne limitano l'applicazione clinica. Inoltre, la possibilità di ottenere epatociti trapiantabili è ostacolato dal basso potenziale replicativo delle cellule epatiche, dalla loro concomitante perdita di funzionalità in coltura e dal ridotto numero di cellule vitali e funzionali che si ottengono dopo criopreservazione. Le cellule staminali emopoietiche pluripotenti (Blood Derived Stem Cells) sembrano essere una valida speranzosa alternativa. Queste tipologie di cellule CD34+/CD90+/CD117+ hanno capacità differenziativa "plastica", poiché in grado di dare origine non solo a cellule mature emopoietiche ma anche di altri tessuti, quali quello epatico. In particolare, le Blood Derived Stem Cells possiedono self-renewal e, se coltivate in vitro e sotto opportuno condizionamento, possono differenziare in osteoblasti, adipociti, condrociti, miociti, rappresentando un potenziale trapiantologico. Scopo del mio studio è valutare il grado di differenziamento in vitro delle BDSCs in epatociti ed il loro stato funzionale e comparare questi dati con quelli ottenuti in vivo.

Nicolina Mastrangelo, XXIV ciclo
Docente guida: Prof. Alessandro Finazzi-Agrò

The endocannabinoid system characterization in a mouse model of Huntington's disease

In my second year of formation I have analyzed the main components of the "endocannabinoid system" (ECS) in R6/2 mice, a widely used model of Huntington's disease (HD).
The HD is a degenerative genetic disease, caused by a cytosine–adenine–guanine (CAG) expansion in the gene coding for the " huntingtin" (htt). The R6 mice are the most extensively characterized animal models of HD and in particular R6/2 mice have a 150 CAG repeat and show severe motor and cognitive defects at earlier ages. The ECS which includes the endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG), their metabolic enzymes and the eCBs-binding receptors, is often involved in neurodegenerative disease. The endocannabinoids activate type-1 (CB_1) and -2 (CB_2) cannabinoid receptors or transient receptor potential cation channel (TRPV1). Thus, I measured eCBs binding activity at target receptors (CB_1, CB_2 and TRPV1) in the brain of wild-type and R6/2 mice of different ages. Moreover, I measured the endogenous content of AEA and 2-AG, of their biosynthetic (N-acyl-phosphatidylethanolamines-hydrolyzing phospholipase D, NAPE-PLD and diacylglycerol lipase, DAGL, respectively) and hydrolytic enzymes (fatty acid amide hydrolase, FAAH and monoacylglycerol lipase, MAGL, respectively) in the brain of wild-type and R6/2 mice of different ages. I also measured FAAH activity in lymphocytes of R6/2 mice, in order to evaluate whether central ECS alterations were mirrored by peripheral cells. In 12-week-old R6/2 mice I found a reduction of NAPE-PLD and DAGL activity, and of CB binding, as well as an increase in 2-AG content when compared to wild-type littermates, without any other change in ECS elements. The analysis of the ECS activities in different brain areas, striatum and cortex showed that striatum is the area mainly responsible of the ECS changes observed in the 12-week-old R6/2 brain.

Elisa Maria Memmi, XXV ciclo
Docente guida: Dr.ssa Bernassola

Role of p63 in regulating the self-renewal properties of mammary epithelial cancer stem cells.

p63 is a structural and functional homologue of p53, involved in the morphogenesis and maintenance of all stratified epithelia. The isoform ΔNp63 is a key lineage-specific determinant of the proliferative properties of stem cells (SCs) in mammalian epithelia, including epidermis, thymus, prostate and breast. In human normal mammary epithelial SCs the expression ΔNp63 isoforms is strictly confined to the basal/myoepithelial compartment, where SCs are expected to reside.Basically we're investigating if p63 confers increased self-replicative properties to mammary epithelial cancer stem/progenitor cells. ΔNp63 proteins act in a dominant negative fashion to functionally counteract p53 tumor suppressive properties. Importantly, p53 controls polarity of cell division in normal mammary SCs, while attenuation of p53 activation favors symmetric divisions in cSCs. These findings imply that ΔNp63 may have a role in regulating the self-renewal properties of cSCs via inhibition of p53.
Our mouse model is MMTV-ERBb2 transgenic mouse (Val^{664} to Glu^{664}), a well-characterized SC model of mammary carcinogenesis. We're studying the self-renewal properties of SCs in vitro through mammary sphere forming assay and in vivo through mammary transplantation assays and isolating stem cells utilizing the strategy of PKH26. We're creating a p63 know down system by lentiviral mediated RNAi delivery in mammospheres, and these p63 deficient mammospheres will be used to perform serial replating experiment in colture, limiting dilution transplantation assays, serial transplantation assays and finally time-lapse microscopy of PKH^{high} cells and assessment of p53 activation.Up to now our results strongly suggest that p63 over-expressing cells may identify a mammary stem/progenitor tumor-initiating cell sub-population. We hypothesize that this subpopulation of sSCs possesses increased replicative potential, which is critical for tumor formation and progression.

Elisa Lenti, XXV ciclo
Docente guida: Prof. Gerry Melino

Identification of target genes of oncogenic transcription factor Hox11 in organogenesis

It is now clear that many of the genes that orchestrate developmental processes such as organogenesis, are often reactivated in cancer. Among these, the oncogenic transcription factor Hox11 was shown to promote cellular proliferation during organogenesis and its loss of function mutation in mice causes spleen agenesis. Although few Hox11 target genes have been identified, the growth-related molecular network controlled by Hox11 during organogenesis is mostly unknown. To this end, we performed microarray analysis on wild-type and mutant embryonic spleen and found that loss of Hox11 causes deregulation of several pathways associated with patterning, organogenesis and cancer. In addition, we found that mutant splenic mesenchyme undergo premature differention and reduced vascularization. To uncovered direct target genes and pathways, we performed chromatin immunoprecipitation coupled with deep-sequencing (ChIP-seq) and identified several novel Hox11 target genes/pathways controlled by Hox11 during organ development. Among those targets, we found genes belonging to Wnt, Retinoic Acid, TGFb and Notch pathways. We focused on the Retinoic Acid pathway and found that several genes including RALDH, Cyp26b1, RARa and RXRa are deregulated in the absence of Hox11. Interesingly, ChIP assays showed that Hox11 binds the promoters of those genes

possibly regulating their expression during development. Using bioinformatic analysis of ChIP-seq data, we identified a common motif present in the promoter of Hox11 targets. We are currently validating this motif by performing transcriptional and electrophoretic mobility shift assays. We foresee that identification of physiological Hox11 target genes/pathways will shed light on the mechanisms by which ectopic activation of this oncogene induces cellular transformation and leukemogenesis.