Comunicaciones Orales (CO-01/CO-30)

Sesión I. Herramientas de terapia génica (CO-01/CO-06)

CO-01. Generación, amplificación y caracterización de Vectores virales para terapia génica en la unidad de producción de vectores del Centre de Biotecnología Animal i Teràpia Gènica (Universitat autónoma de Barcelona)

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La Unidad de Producción de Vectores es una plataforma tecnológica para el diseño, producción, purificación y caracterización de vectores virales. Se localiza en el Centre de Biotecnologia Animal i Teràpia Gènica (CBATEG), un centro de investigación de la Universitat Autònoma de Barcelona (UAB). Esta Unidad consta de un área de seguridad biológica de nivel 2, de 125 m^2, con cuatro laboratorios de cultivo celular y producción de vectores, y un área de seguridad biológica de nivel 3, de 25 m^2.

El objetivo de la Unidad de Producción de Vectores es producir vectores de calidad GLP para terapia génica en respuesta a la demanda por parte de laboratorios de investigación de instituciones públicas o privadas, nacionales o internacionales.

Actualmente, la Unidad de Producción de Vectores puede generar, amplificar y caracterizar vectores adenovirales de los serotipos humanos 2, 5 y 40, así como vectores AAV del serotipo 2. Además, a partir del 2005 se podrán generar también AAV de los serotipos 1 al 8, y adenovirus caninos CAV-2.

Una vez producido y titulado el vector, se seleccionan las fracciones con una elevada concentración, ya que ello permite la obtención de mejores resultados cuando se trabaja *in vivo* con modelos animales, y se someten a una bateria de tests de control de calidad, para descartar la presencia de micoplasma, bacterias, hongos y levaduras.

Como ejemplo, los valores medios de las preparaciones adenovirales obtenidos son: 3,30 x 1012 PP/ml y 1,25 x 1011 IU/ml. El tiempo medio de generación (clonación del gen de interés) es de 4-8 semanas, mientras que el tiempo medio de amplificación y caracterización es de 4-6 semanas.

CO-02. Reparación de mutaciones puntuales utilizando oligonucleótidos TFBOs

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Los oligonucleótidos formadores de triple hélices bifuncionales (TFBOs) contienen un dominio reparador y un dominio TFO que, a parte de dirigir el TFBO a la zona del genoma donde se encuentra la mutación, pueden actuar como inductores eficientes de la recombinación homóloga. Como modelo se ha escogido el gen dihidrofolato reductasa (*dhfr*), ya que disponemos de una colección de mutantes puntuales. La selección de las colonias revertantes (DHFR negativas a positivas) tras incubación con los TFBOs se realiza en medio selectivo para la actividad DHFR.

Para el presente estudio se han diseñado 5 TFBOs diferentes teniendo en cuenta las reglas de apareamiento de los TFOs al DNA. Utilizando uno de ellos se ha conseguido reparar la mutación puntual a nivel de proteína con una frecuencia de recombinación de $5x10^{-5}$. Utilizando hidroxiurea se ha conseguido incrementar la recombinación homóloga y la de revertantes producidos por los TFBOs hasta alcanzar una frecuencia de 10^{-3}.

Mediante la estrategia para reparar mutaciones puntuales con TFBOs se conseguiría reemplazar la secuencia mutada por la salvaje en su locus endógeno sin causar problemas insercionales. Esto sería de gran interés en terapia génica de enfermedades monogénicas, donde una sola mutación en un gen provoca la patología.

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CO-03. Epidermal growth factor mediated gene delivery into tumor cells

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The purpose of this study is to improve the transfection activity of cationic liposomes targeting the complexes to the epidermal growth factor receptor (EGFr), which is overexpressed in different tumors. For this, we prepared DOTAP/cholesterol liposomes labelled with EGF as follows: cationic liposomes for in vitro experiments were formulated at 1/2, 1/1, 2/1 and 5/1(+/-) lipid/DNA charge ratio by sequentially mixing the liposomes with the ligand and adding the reporter or the therapeutic plasmid gene, pCMVLuc (pVR1216) or pCMVIL12. HepG2, DHDK12 pro and SW620 cells were used for in vitro experiments, which were performed in the presence of 60% serum. The characterization of EGF-lipoplexes indicated a size close to 300 nm and a variable net surface charge as a function of the amount of EGF associated to the cationic liposomes. EGF-lipoplexes, which showed an increased transfection activity, were positively charged, not cytotoxic and highly effective in protecting DNA from DNAse I attack. Transfection activity *in vitro* resulted in an enhancement in the luciferase and IL12 expression by EGF-lipoplexes compared to those without ligand (plain-lipoplexes) and to naked DNA. Results observed in experiments performed with an excess of free EGF or with SW620 cells, which are deficient in EGFr, supported the hypothesis that targeted complexes are recognized by this receptor and that the complexes are taken into the cell via receptor-media-

ted endocytosis. These findings indicate that these complexes may be an adequate alternative to viral vectors for EGF-receptor expressing cells *in vivo*.

CO-04. Evaluación de SLN como sistema de administración de ADN

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El objetivo del estudio ha sido la elaboración y caracterización de nanopartículas lipídicas (SLN) como sistema de administración de ADN. Se elaboraron diferentes formulaciones mediante una técnica de emulsificación y evaporación del solvente. Todas las nanopartículas contenían Precirol® ATO 5, Tween® 80 y CTAB o DOTAP como lípido catiónico en diferentes proporciones. La caracterización de las partículas se realizó mediante la determinación del tamaño y la carga superficial, obteniéndose partículas de alrededor de 600 nm con una carga positiva de entre +49 mv y +59 mv. Una vez caracterizadas las SLN, se realizaron estudios de toxicidad utilizando la línea celular de mioblasto de ratón C2C12. Los ensayos de toxicidad mostraron una mayor viabilidad celular con las formulaciones que contenían DOTAP frente a las que contenían CTAB. La incorporación de Tween® 80 no influyó de manera importante en la toxicidad de las SLN. Una vez evaluada la toxicidad, se prepararon los complejos SLN:ADN. El plásmido utilizado fue el pCMS-EGFP, que codifica para la proteína verde fluorescente. Los complejos se caracterizaron midiendo el tamaño, la carga superficial y la eficiencia de unión mediante electroforesis en gel de agarosa. Por último, se comprobó la capacidad de transfección de las formulaciones elaboradas en células C2C12.

CO-05. DNA-Chitosan nanoparticles as gene delivery system

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The objective of this work was the evaluation of DNA-chitosan nanoparticles (Np) as non-viral gene delivery systems when administered by either oral or hydrodinamic routes. The hydrodinamic administration permited us to determine the kinetic of the luciferase expression. Using the CDD camera, the transgene (tg) expression was localised in the liver and quantified during at least 100 days. The determination of the parameters of the tg expression in the liver versus time, revealed that both the naked DNA and nanoparticles displayed the same AUC (area under the curve). On the contrary, the MRT (mean residence or expression time) of Np formulation revealed that the luciferase expression was delayed 3 to 5 times, with a decrease in the maximal levels of luciferase expression.

Finally, *in vivo* studies performed by single intraduodenal administration in mice showed

that nanoparticles were able to transduce enterocytes and the transgene expression was maintained for at least 3 days. All of these findings can be of interest to develop new strategies based on induction of DNA sustained releases.

Key words: chitosan, nanoparticles, gene delivery, enterocytes, liver.

CO-06. Role of the putative HSG-Binding site of the adenovirus fiber shaft on liver detargeting and knob-mediated retargeting

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Retargeting adenovirus from liver to tumors is a major milestone for cancer gene and virus therapies. We have studied the transduction properties of vectors unable to bind the adenovirus receptors CAR, integrin and HSG. RGD was inserted into the fiber to study the compatibility of these detargeting mutations with knob-mediated transduction.

In vitro, HSG contribution to infectivity becomes evident when interaction with CAR and integrins is missing. Conversely, in vivo, CAR and Integrin binding ablation did not reduce liver transduction and the shaft mutation that precludes HSG binding was necessary and sufficient to abrogate liver transduction. The RGD motif insertion into the HI loop of fiber knob results in a very efficient integrin-mediated infectivity. However, in the context of the shaft mutation the RGD insertion at the HI loop only partially rescued this integrin-mediated infectivity. The shaft mutation affects the structure of fiber impairing the knob-mediated entry. This hypothesis is compatible with a hepatocyte entry pathway in vivo that involves the interaction of the knob with serum factors. The insertion of ligands at the hexon or protein IX may be required to benefit from this fiber shaft mutation detargeting properties.

Sesión II. Herramientas de terapia génica (CO-07/CO-10)

CO-07. Nuevas herramientas para terapia génica: Estudio del posible papel de una relaxasa conjugativa bacteriana como integrasa específica de ADN en el genoma humano

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Las bacterias utilizan la conjugación para enviar moléculas de ADN a otras bacterias, y en ciertas condiciones también a células eucariotas. Cualquier ADN de cualquier longitud puede ser transferido *in vivo* por conjugación, convirtiéndose en un atractivo método de introducción de ADN en células humanas[1].

La proteína clave es la relaxasa, que inicia la transferencia y supuestamente conduce al

ADN hasta la célula receptora[2]. La relaxasa TrwC, además, cataliza una recombinación sitio-específica[3]. Su estructura tridimensional[4] es muy similar a la proteína Rep, que cataliza la integración del genoma de virus AAV en un sitio específico del cromosoma 19 humano[5]. Con vistas a su posible utilización en terapia génica, hemos analizado algunas de sus características funcionales. Hemos comprobado que:

1. TrwC entra en la célula receptora, y allí cataliza una recombinación sitio-específica.
2. TrwC entra al núcleo.
3. TrwC cataliza las reacciones de transferencia de cadenas utilizando como diana una secuencia del cromosoma X humano.

En teoría, podríamos adaptar un sistema bacteriano para introducir por conjugación cualquier ADN a células humanas, y la proteína TrwC lo conduciría hasta el núcleo. Finalmente, los resultados sugieren que TrwC podría integrar el ADN exógeno en un sitio específico del cromosoma X.

Referencias

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CO-08. Transferencia génica al hígado de un gen terapéutico mediante vectores no virales de estructura y tamaño semejante a virus

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Uno de los problemas más importantes de la terapia génica no viral es el desarrollo de vectores de tamaño pequeño, que sean capaces de atravesar la barrera capilar. La mayoría de vectores no virales, tanto lipídicos como poliméricos, producen grandes agregados ADN/vector muy heterogéneos en composición y tamaño, que asocian múltiples unidades de plásmido, resultando partículas excesivamente grandes.

Recientemente, nuestro laboratorio ha desarrollado un nuevo tipo de vector capaz de inducir el colapso monomolecular de una única molécula de plásmido, resultando complejos muy pequeños, típicamente en el rango de 20-40 nm. Las partículas resultantes, esféricas y constituidas por un plásmido único, tienen una estructura y tamaño que recuerda al de los virus, y han sido caracterizadas por microscopía electrónica y electroforesis en cuanto a su tamaño y resistencia a la degradación en suero. Estas partículas "pseudovirales" presentan excelentes posibilidades potenciales de distribución y escape del torrente sanguíneo y son una promesa de una nueva generación de vectores similares a virus en cuanto a la eficacia de diseminación por los tejidos. Asociadas a un plásmido portador de un gen terapéutico hepático (alfa-1 antitripsina) pueden emplearse para la terapéutica de reemplazo, restituyendo al tejido hepático copias funcionales del gen defectuoso.

CO-09. Lyophilization of adenoviral vectors for gene therapy

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One of the key factors concerning the success of any multicentre gene therapy trial is the maintenance of the vector's viability during shipping and storage. The aim of this study was to develop a procedure for the lyophilization of this sensible material and, therefore, improve their facilitating its manipulation and use.

Adenoviral vectors expressing luciferase were produced and purified under GMP conditions. Then the vectors were dispersed in an aqueous solution containing a cryoprotector, dosed in uni-dose vials, and lyophilised. The influence of both pH and cryoprotector on the viability of adenoviral vector was studied after quantification of luciferase activity by bioluminescence. Sucrose and lactose offered good results with little lost of the original vector's activity. Concerning the pH, dispersion of the vectors in buffer Tris prior congelation offered the best conditions to maintain the vector viability. Thus, preparations with either sucrose or lactose in buffer. Tris enabled us to obtain survival rates of about 100%. When the freeze-dried vials were stored at –20 ºC, the vectors maintained their activity for at least 3 months. This study will be prolonged for 12 months.

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CO-10. Importancia de la caracterización de los modelos animales previa a la aplicación de terapia génica: esclerosis lateral amiotrófica

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La caracterización de los modelos animales para su uso en el estudio de la efectividad de los tratamientos de terapia génica es de gran importancia. Una buena elección en los animales elegidos para dicho estudio resulta imprescindible para conseguir una correcta interpretación de los resultados obtenidos. Nuestro equipo ha realizado la caracterización del modelo animal G93A, ratones transgénicos que sobreexpresan la superóxido dismutasa humana mutada y que son utilizados para el estudio de la esclerosis lateral amiotrófica. Con este fin, se sometió al animal a diferentes ensayos específicos (valoración de síntomas por un experto, evolución del peso, momento de inicio de los síntomas, supervivencia, test de fuerza, rotarod y electromiografía). También se ha observado la evolución (posible pérdida) de copias del transgen en nuestra colonia a lo largo de distintas generaciones. Tras la comparación de los distintos test se observó la influencia del sexo en la aparición de síntomas, influyendo también en el

momento óptimo para aplicar cada una de estas pruebas y su eficacia. Sin embargo, el número de copias de la enzima no resultó determinante sobre la supervivencia de los animales. Estos resultados se deben tener en cuenta en los tratamientos experimentales realizados en este modelo animal.

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Sesión III. Aplicaciones de terapia génica. Cáncer (CO-11/CO-17)

CO-11. Incremento en la generación de hepatocitos derivados de la médula ósea inducido por el tratamiento con factores movilizadores de células madre hematopoyéticas

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Se ha descrito que células de la médula ósea (MO), bajo determinadas condiciones, generan células funcionalmente activas de tejidos no hematopoyéticos. Esto permitiría la vehiculización de genes a diferentes tejidos mediante el trasplante de células madre hematopoyéticas (CMHs) manipuladas genéticamente. Determinados factores de crecimiento movilizan CMHs desde la MO al torrente sanguíneo. En nuestro laboratorio hemos establecido un modelo murino para investigar si la movilización *in vivo* de CMHs con el factor de crecimiento de granulocitos (G-CSF) puede incrementar la generación de hepatocitos derivados de MO (HdMO). Ratones trasplantados con MO expresando la proteína verde fluorescente fueron tratados con tetracloruro de carbono (CCl4) para inducir daño hepático. El tratamiento generó un marcado desbalance en los parámetros hepáticos plasmáticos y una estructura hepática alterada. La presencia de HdMO, evaluada mediante técnicas de inmunohistoquímica e inmunofluorescencia, fue de 1 por cada 250.000 hepatocitos totales. La movilización de progenitores hematopoyéticos con G-CSF provocó un aumento de 17 veces en el porcentaje de HdMO. Estos resultados demuestran que la movilización de CMHs a sangre periférica incrementa significativamente la presencia de HdMO en hígados dañados, sugiriendo la posibilidad de utilizar factores movilizadores de CMHs para facilitar su alojamiento y potencial transdiferenciación a diferentes tejidos del organismo.

CO-12. Análisis de sitios de integración de vectores lentivirales en el genoma de precursores hematopoyéticos humanos primitivos

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Observaciones recientes sobre mutagénesis insercional aconsejan profundizar en la investigación de regiones del genoma en las cuales se integran preferentemente los diferentes vectores de interés en el campo de la terapia génica. Para estudiar la integración de vectores lentivirales en células madre hematopoyéticas humanas, se ha utilizado el modelo de trasplante xenogénico de hematopoyesis humana en ratones inmunodeficientes. Se han utilizado vectores lentivirales autoinactivantes de tercera generación, que codifican EGFP bajo el promotor CMV. Las muestras humanas transducidas se trasplantaron en ratones NOD/SCID, analizándose la cinética del injerto en aspirados de médula ósea. En muestras con un alto porcentaje de células $EGFP^+$ se aplicó la técnica de LM-PCR para determinar los sitios de inserción lentiviral. En total, se detectaron 34 integraciones, de las cuales 28 se pudieron mapear. Un 71% de éstas se localizaron en genes RefSeq. Un análisis más detallado de los sitios de integración ha demostrado por primera vez que la integración de vectores lentivirales en el genoma de precursores hematopoyéticos humanos primitivos ocurre con preferencia en regiones codificantes. Tres de las integraciones lentivirales se localizaron en genes con papel en tumorigenesis, si bien el fenotipo hematopoyético de estos animales no mostró evidencias de malignización.

CO-13. Susceptibilidad de células madre hematopoyéticas murinas a vectores lentivirales

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Para estudiar la susceptibilidad de distintos tipos de células repobladoras a vectores lentivirales y la posible ventaja proliferativa que este tipo de construcciones podría conferir, se realizaron ensayos de repoblación competitiva de médula ósea transducida con médula ósea sin manipular genéticamente. En estos ensayos, dos poblaciones celulares, una (Ly5.1) transducida por vectores lentivirales y otra (Ly5.2) tratada bajo las mismas condiciones en ausencia del virus fueron trasplantadas en receptores irradiados letalmente. En estos experimentos se han utilizado vectores lentivirales que codifican EGFP bajo el control del promotor CMV. La médula ósea de los receptores primarios se trasplantó en receptores secundarios para ensayar la capacidad de reconstitución y automantenimiento de las células madre hematopoyéticas transducidas. Los resultados obtenidos mostraron cómo la población EGFP+ incrementaba dentro de la población Ly5.1, mientras que la proporción Ly5.1/Ly5.2 se mantenía estable en el tiempo. El mantenimiento o aumento de la población transducida en los sucesivos trasplantes confirmó la eficacia de los vectores lentivirales para transducir

células madre hematopoyéticas sin comprometer su potencial de reconstitución. Además, estos resultados nos permiten concluir que el aumento observado en la población transducida no es debido a una ventaja proliferativa conferida por el vector lentiviral.

CO-14. Corrección genética del fenotipo de progenitores hematopoyéticos de pacientes con anemia de Fanconi

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La terapia génica de pacientes con anemia de Fanconi surge como una prometedora alternativa para pacientes que carecen de un donante apropiado. Con objeto de investigar las posibilidades de corregir genéticamente células madre de estos pacientes, en primer lugar hemos estudiado la reserva hematopoyética de los mismos. Nuestros estudios muestran que estos pacientes presentan un contenido reducido de progenitores hematopoyéticos en su médula ósea; del orden del 30% de los valores normales de CD34+, y del 5% de Unidades Formadoras de Colonias. Como es característico en células de Fanconi, los progenitores de médula ósea de pacientes que pertenecen al grupo de complementación FA-A mostraron una muy elevada sensibilidad a agentes entrecruzantes del DNA, tales como la MMC. Experimentos de transducción de médula ósea de estos pacientes con vectores retrovirales que codifican el gen *FANCA* demostraron que la complementación génica revierte el fenotipo característico de sensibilidad a la MMC y mejora de forma significativa el número de progenitores con capacidad clonogénica. Las perspectivas de la terapia génica aplicada a esta enfermedad será discutida en base a los resultados experimentales obtenidos.

CO-15. Mobilization of stem cells by slug in heart development andancer

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The SNAIL-related zinc-finger transcription factor, SLUG (SNAI2), is critical for the normal development of neural crest-derived cells and loss of-function SLUG mutations have been proven to contribute to piebaldism and Waardenburg syndrome type 2 in a dose-dependent fashion. While aberrant induction of SLUG has been documented in cancer cells, relatively little is known about the consequences of SLUG overexpression either in normal development or in malignancy. We report SLUG duplication in a child with a *de novo* 8q11.2–> 8q13.3 duplication associated with tetralogy of Fallot, submucous cleft palate, renal anomalies, hypotonia and developmental delay. To investigate the potential role of SLUG overexpression in development and in cancer, we generated mice carrying a tetracycline-repressible Slug transgene. These mice were morphologically normal at birth, inferring that Slug overexpression is not sufficient to cause overt morphogenetic defects. In the adult mice, there was a 20% incidence of sudden death, cardiomegaly and cardiac failure associated with incipient mesenchymal tumourigenesis (leukemias and sarcomas). While the timing of the cardiomyopathy favours secondary causation, it remains possible that SLUG overexpression may directly contribute to this acquired cardiac phenotype. The mesenchymal cancers observed in the Slug-mice represent an *in vivo* demonstration that transformation depends upon genetic changes that allow undifferentiated cells to grow outside their normal environment. However, the survival conferred by Slug, while reversible *in vitro*, can escape such control *in vivo*. The implicactions of these results in human stem cell-based therapies will be discussed.

CO-16. Transducción y transplante *in utero* de progenitores hematopoyéticos de hígado fetal (Lin-Aa.1+) como modelo para el tratamiento de enfermedades hereditarias

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El transplante *in utero* de células hematopoyéticas es una alternativa al transplante postnatal para el tratamiento de enfermedades hereditarias que se pueden diagnosticar en estadíos tempranos del desarrollo. Previamente hemos demostrado en un modelo murino la posibilidad de injertar vía trasplante intra-utero progenitores hematopoyéticos provenientes de médula ósea adulta transducidos con vectores retrovirales. En el presente trabajo se pretende evaluar la eficacia de transducción y la capacidad de injertar *in utero* células derivadas de hígado fetal, como modelo para la evaluación del potencial uso de células de origen embrionario o perinatal en este tipo de trasplante. Para ello, poblaciones celulares procedentes de hígado fetal de día 14.5 de desarrollo fueron enriquecidas en progenitores y células madre hematopoyéticas (células Lin-AA4.1$^+$). Estas células fueron transducidas con vectores retrovirales expresando la proteína verde fluorescente, bajo condiciones descritas por nuestro laboratorio. Más del 20% de los progenitores granulomacrofágicos presentes en la muestra expresaron la EGFP. Entre 2-3 x 10^4 células Lin$^-$AA4.1$^+$ transducidas fueron inyectadas en fetos de 14,5 días. El grado de quimerismo en sangre periférica y a diferentes tiempos después del nacimiento en los animales transplantados será discutido.

CO-17. Estudios genéticos preclínicos para la terapia génica de la anemia de Fanconi en España

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La anemia de Fanconi (AF) es una enfermedad heterogénea que presenta una alta complejidad genética con 11 grupos de complementación. En relación a la terapia génica en esta enfermedad, es de interés la presencia de pacientes con mosaicismo somático en los cuales se produce una reversión natural de la mutación patogénica, y pacientes adscritos a ciertos grupos de complementación, con características clínicas especiales. Esto hace necesario un estudio genético previo a cualquier consideración de terapia génica. El trabajo que se está desarrollando dentro de la Red Española para el Estudio de la AF, incluye la caracterización de los grupos de complementación

a que pertenece cada uno de los pacientes españoles con AF, el análisis de sus mutaciones y la determinación de los pacientes con mosaicismo somático. En la actualidad, se han analizado 65 pacientes. El 80% de los pacientes analizados pertenece al grupo de complementación FA-A, siendo significativa la ausencia de pacientes dentro del grupo FA-C, relativamente frecuente en otros países. Es de destacar la elevada incidencia de la enfermedad en pacientes de la etnia gitana. Todos estos pacientes pertenecieron al grupo FA-A y presentaron la misma mutación, debido a un efecto genético fundador. Es significativo también el hallazgo de un 17% de pacientes adscritos a grupos poco frecuentes, y un 15% de pacientes con mosaicismo somático.

Sesión IV. Aplicaciones de terapia génica. Cáncer (CO-18/CO-24)

CO-18. PET IMAGING ALLOWS MONITORING TRANSGENE EXPRESSION IN GENE THERAPY OF LIVER CANCER USING ADENOVIRAL VECTORS

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Goal: Clinical development of gene therapy is hindered by the lack of tools that may help evaluating the intensity and duration of transgene expression. In animal models, PET imaging using a fluorine-18 labeled penciclovir analog (^{18}FHBG) as a radiotracer can monitor in vivo the expression of thymidine kinase. We have explored this strategy in patients with liver cancer. **Methods:** Patients were enrolled in a clinical trial in which increasing doses of an adenoviral vector encoding thymidine kinase (Ad.tk) were given intratumorally followed 2 days later by oral valganciclovir. At day 2, transgene expression was evaluated after IV injection of 18FHBG both on the treated lesion and on a whole-body basis. In a few patients, PET study was repeated at day 9 and after a second dose of Ad.tk injected one month later. **Results:** Transgene expression within the tumor was detected in all patients that received ≥1012 viral particles. The surrounding liver was not transduced significantly, and transgene expression was not detected in distant organs. Expression had virtually vanished by day 9 and was not detected after readministration of Ad.tk. **Conclusions:** We have shown consistently for the first time that transgene expression can be monitored in cancer patients using PET imaging. This can prove to be a most valuable tool for the rational progress of clinical gene therapy.

CO-19. Improving the evaluation of new cancer treatments: challenges and opportunities

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Abstract: There are, at present, ten times more anticancer drugs being tested in clinical trials than there were 15 years ago. Many of the new classes of agents and gene therapy approaches, however, are predicted to work in only small subpopulations of patients, target unconventional aspects of tumour development and interact with other agents in an unpredictable manner. How can clinical trials be re-designed to accommodate the new features of targeted anticancer drugs and/or approaches?

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CO-20. Terapia génica antiangiogénica/ antitumoral. Acción de PEDF en el desarrollo de tumores primarios y metástasis de melanoma humano

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La inhibición de la angiogénesis ha demostrado ser una estrategia efectiva en la terapia antitumoral. El factor derivado del epitelio pigmentado, PEDF ha sido propuesto como uno de los inhibidores de la angiogénesis más potentes descritos en el ojo al inducir apoptosis en las células endoteliales activadas.

Como una primera aproximación a la terapia génica antiangiogénica, se determino el efecto terapéutico de PEDF en un modelo de melanoma cutáneo humano. Para ello se sobreexpresó este factor a través de transferencia génica, generando para ello, un vector retroviral bicistronico portador del cADN de PEDF humano y el cADN de la proteína verde fluorescente (GFP) como control. Tras la inyección subcutánea de estas células, se observó una importante inhibición del crecimiento tumoral y una inhibición total en el desarrollo de metástasis pulmonares inducidas en ratones inmunodeficientes cuando las células fueron inyectadas de forma intravenosa. Para evaluar el efecto antimetastásico de PEDF sistémico, se generó un vector adenoviral que se administró por via intravenosa a ratones inmunodeficientes (*targeting* hepático). En este caso, los resultados obtenidos, aunque preliminares, demostraron que, tras inocular las células de melanoma, 3 días post-inyección del vector, tanto el número como el tamaño de las metástasis encontradas en los pulmones de los ratones inyectados con el Ad-PEDF fueron menores que en el caso de los ratones control.

CO-21. TAT8-TK/GCV ELECTROTRANSFER INDUCED PANCREATIC TUMOR REGRESSION *IN VIVO*

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Suicide gene therapy using the thymidine kinase of the Herpes simplex virus (TK) in combination with ganciclovir (GCV) has been shown to produce therapeutic efficacy, although limited, due to the poor gene transfer efficiency and the reduced bystander effect. Here we report that fusion of TK to an 8-amino-acid peptide from the basic domain of the HIV-Tat protein significantly increases the cytotoxic efficacy of the TK/GCV system in pancreatic tumors. The application of intratumoral DNA electrotransfer of TK or Tat8-TK together with high doses of GCV administration led to overall statistically significant reduction in tumor growth (TK $p = 0,0008$, Tat8-TK $p < 0,0001$). Interestingly a reduction in the initial tumor volume was statistically significant in the Tat8-TK group (59,5% reduction $p = 0,0002$). Tumors from this group showed enhanced apoptosis as observed in the number of TUNEL positive cells. Moreover, in this group a 50% complete eradication of the tumors was achieved. Similarly, when low doses of GCV were administered overall reduction in the tumor growth reached statistically significance only in the Tat8-TK group ($p = 0,0079$)

Thereby, our results suggest that intratumoral electrogene transfer of a modified TK fused to the Tat8 peptide followed by GCV administration might be a feasible approach in the treatment of pancreatic tumors.

CO-22. MODULATION OF GEMCITABINE SENSITIVY THROUGH ADENOVIRUS-MEDIATED REINTRODUCTION OF NUCLEOSIDE TRANSPORTERS AND P53 IN HUMAN PANCREATIC CANCER

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Gemcitabine is the most effective drug for the treatment of pancreatic cancer. Different nucleoside transporters (concentrative, CNTs, or equilibrative, ENTs) are responsible for gemcitabine uptake. The observed alterations in their expression could determine the sensitivity of human tumours to this drug. In this work, we have used a panel of human pancreatic tumour cell lines to analyze the role of nucleoside transporter expression patterns on gemcitabine-induced cytotoxicity. Moreover, two new adenoviruses carrying hENT1 or hCNT1 nucleoside transporters have been generated to study the impact of their overexpression on gemcitabine cytotoxicity. Their combination with p53 reintroduction has also been assayed since p53 has a key role in controlling and regulating drug cytotoxic effects and it is also mutated in more than 50% of pancreatic tumours. We have observed that the equilibrative transporter hENT1 is the major component responsible for gemcitabine uptake in all cell lines. After

treatment with this drug a decrease in the basal transport activity was detected. Overexpression of both transporters (hENT1 or hCNT1) modulated gemcitabine cytotoxicity and p53 reintroduction provoked a significant increase in chemosensitivity. From all these results, we conclude that gene therapy approaches based on the modulation of nucleoside transport and combination with p53 function restoration may be of interest to potentiate gemcitabine cytotoxicity.

Modalidad: Comunicación oral breve.

CO-23. Optimization of the transcriptional control of E1A with E2F promoter to achieve tumor-selective replication of adenovirus

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E2F acts as a transcriptional repressor when bound to unphosphorylated Rb during G1 or Go phase. Upon Rb phosphorylation, E2F is released from the E2F-Rb complexes to activate transcription. In tumor cells Rb is absent or hyperphosphorylated and "free" E2F levels increase. One of the best-characterized E2F-responsive promoters is the promoter of the E2F1 gene itself. Several groups have used this promoter to control adenovirus E1a gene expression as a strategy to achieve tumor-selective replication of adenovirus.

This strategy can be combined with mutations in E1a that also target Rb-pathway defective cells. We have constructed a series of oncolytic adenoviruses that combine E1a mutations with E1a transcriptional control using E2F1 promoter. We demonstrate that insulators are necessary to maintain the proper regulation of E2F1 promoter in the viral genome. Tight control of E1a expression reduced in vivo hepatic toxicity. Reduced levels of E1a expression and virus replication that were found in some tumor cell lines was palliated by the introduction of a kozak sequence at E1a start codon.

CO-24. Semliki forest virus vectors engineered to express higher IL-12 levels induce efficient elimination of murine colon adenocarcinomas

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Vectors based on alphaviruses can transiently express recombinant proteins at high levels in many cell types, offering great potential for vaccination and gene therapy. To evaluate the use of these vectors for tumor treatment two SFV vectors expressing different levels of IL-12 were constructed. One vector expresses both IL-12 subunits from a single subgenomic promoter (SFV IL-12), while in the second vector each IL-12 subunit is expressed from an independent subgenomic promoter fused to the SFV capsid translation enhancer (SFV enhIL-12). This latter strategy provided an 8-fold increase in

the level of IL-12 expression. We evaluated the therapeutic potential of SFV vectors using the poorly immunogenic MC38 colon adenocarcinoma model. A single intratumoral injection of 108 viral particles of any of the two vectors induced ≥ 80% complete tumor regressions with long-term tumor-free survival. However, lower doses of SFV-enhIL-12 were more efficient than SFV-IL-12 in inducing antitumoral responses, indicating a positive correlation between the level of IL-12 and the therapeutical effect. Moreover, repeated intratumoral injections of suboptimal doses of SFV-enhIL-12 increased significantly the antitumoral response. In all cases SFV vectors were more efficient in eliminating tumors than a first-generation adenovirus vector expressing IL-12. In addition, the antitumoral effect of SFV vectors was only moderately affected by previous immunizations of the animals with high doses of the same type of vector. This antitumoral effect was produced, at least in part, by a potent CTL-mediated immune response.

Sesión V. Aplicaciones de terapia génica. Enfermedades hereditarias e infecciosas. (CO-25/CO-30)

CO-25. Specific inhibition of foot-and-mouth disease virus infection by transcripts targeted to non-coding RNA regions

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Foot-and-mouth disease virus (FMDV) RNA translation and replication is significantly blocked by highly specific antisense oligodeoxynucleotides targeted to crucial sites within the 5' and 3' non-coding regions (NCRs), as we have demonstrated previously. In this report we have studied the antiviral effects when these transcripts are expressed in susceptible cells. Transient expression of antisense transcripts corresponding to the 5' and 3' NCR and their combination confer specific inhibition of homologous (serotype C) viral infection in BHK-21 cells. To further explore the antiviral potential of the FMDV interfering transcripts, BHK-21 cell clones stably expressing these transcripts were selected. The constitutive expression of antisense 5' NCR transcripts (5´AS) exerted the higher levels of inhibition to homologous and heterologous FMDV infection. These inhibitions were also observed, albeit to a lesser extent, in clones stably expressing antisense 3'NCR transcripts. The antiviral response was specific for FMDV. In all cases, a correlation was found among the levels of transcript expression found in transfected clones and the extent of viral inhibitions observed. This results open the possibility to develop genetic strategy for to reduce the susceptibility of animals to FMDV.

CO-26. RNAs antisense catalíticos. Aplicación a la inhibición de la replicación del HIV-1

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Una de las herramientas de terapia génica que más interés ha despertado en los últimos años es la utilización de RNAs inhibidores. Resultados previos demuestran la utilidad de combinar diferentes dominios inhibidores en una misma molécula de RNA. En este trabajo ensayamos la capacidad de un nuevo tipo de RNAs inhibidores que llamamos RNAs antisense catalíticos, de inhibir la replicación del HIV-1 en células U87-CD4-CXCR4. Estos RNAs inhibidores portan un dominio catalítico (ribozima hairpin o ribozima hammerhead) y un dominio de RNA antisense. El dominio antisense se diseña atendiendo a las características de estos elementos en sistemas naturales de control de expresión génica. Hemos ensayado una colección de RNAs inhibidores en la que ambos dominios se dirigen frente a la región LTR del HIV, obteniendo inhibiciones próximas al 90% medido por niveles de antígeno p24. Sorprendentemente RNAs inhibidores con la actividad catalítica abolida conducen igualmente a una inhibición significativa de la replicación viral, mientras que no se observa inhibición alguna cuando se ensayan cada uno de los dominios inhibidores utilizados independientemente. Ensayos in vitro demuestran una buena correlación entre eficiencia de unión del RNA inhibidor al substrato viral y su capacidad de inhibir la replicación del virus en células.

CO-27. Inhibición de la traducción dependiente de IRES del HCV mediada por RNAs aislados a partir de poblaciones combinatoriales

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El virus C de la hepatitis (HCV) es el agente etiológico causante de la hepatitis C, uno de los mayores problemas de salud mundial. La identificación de inhibidores eficientes de su replicación constituye por tanto un reto importante. A partir de una población aleatoria de más de 105 variantes y utilizando una novedosa estrategia de selección molecular *in vitro* hemos identificado una colección de RNAs que se muestran como eficientes inhibidores del HCV. El método de selección desarrollado consta de dos pasos consecutivos de selección que permite el aislamiento de moléculas híbridas de RNA seleccionadas por su capacidad de unión y corte del IRES de HCV (formadas por un dominio catalítico o ribozima y un aptámero). El análisis de la población de RNAs después de seis ciclos de selección permitió la identificación de 7 familias de variantes definidas por la existencia de un dominio consenso de secuencia para cada una de ellas. Estos dominios son perfectamente complementarios a regiones distintas del IRES del HCV. La actividad inhibidora de un representante de cada familia se ensayó en extractos celulares. Todos los RNAs seleccionados inhiben significativamente el IRES del HCV llegando en algunos casos hasta el 95%.

CO-28. Alterations in the podia formation capacity of tymphoblastoid B cells from Wiskott-Aldrich syndrome patients can be rescued after WASP-retroviral gene transfer

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Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disorder caused by mutations in the Wiskott-Aldrich Syndrome Protein (WASP) gene. WASP-deficient cells have been reported to present alterations in the cytoskeleton architecture. The ability of the cytoskeleton to induce extension of podia play an important role in the migratory process and in cell-cell communication. Recently, new morphologies of pseudopod, magnupodia, tenupodia and osmopodia, have been described to be present in human hematopoietic cells.

We have investigated the ability of B and T cells from WAS patients to induce this particular types of membrane projections. We have found that Epstein-Barr virus (EBV)-lymphoblastoid B cells but not primary T cells from control individuals form nice long podia. However EBV-lymphoblastoid B cells from WAS patients with mutations R86H and E133K were not able to form it. Interestingly, when these cell lines were transduced with recombinant retroviruses expressing the WASP gene the capacity of the cells to form pseudopodia was rescued. On the other hand we have found that EBV-lymphoblastoid cell lines from WAS patients were more sensitive than control cells to podia formation observed under osmotic stress conditions. Importantly, the reintroduction of WASP in patient cells resulted in a decrease in the number of osmopodia.

CO-29. Construction of episoma vectors carrying the intact human factor VIII gene for gene therapy

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Haemophilia A is a disorder caused by deficiency in the activity of factor VIII. Two aspects make this disease attractive for gene therapy; it is caused by mutations in one gene and low levels of expression are expected to have a therapeutic effect. So far, all gene therapy approaches have been based on expression of FVIII from cDNA minigenes due to the large size of the gene (180 kb).

In order to make a BAC carrying the intact factor VIII gene, we have used homologous recombination (the Red system) in *E. coli* to recombine together two overlapping BACs, each containing part of the factor VIII gene. We now have a 230-kb BAC containing not only all the exons of the gene, but also about 50 kb upstream of the first exon and 30 kb downstream of the last exon, hopefully including all the regulatory elements needed for correct specific expression of the gene.

In a second step we have introduced onto this BAC either oriP/EBNA-1 which con-

fers multicopy episomal maintenance, or alphoid DNA which is able to form mammalian artificial chromosomes (MACs). These constructs will be introduced into different human and mouse cell lines and expression of factor VIII will be analysed.

C0-30. Terapia génica para la deficiencia de la piruvato kinasa eritrocitaria

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La deficiencia en Piruvato Kinasa Eritrocitaria humana (hRPK), un desorden autosómico recesivo producido por mutaciones en el gen PKLR, es una de las causas más comunes de anemia hemolítica crónica no esferocítica. La expresión transgénica de la RPK en precursores eritropoyéticos deficientes puede resultar una terapia alternativa en aquellos pacientes con síntomas severos. En este trabajo hemos construido vectores retrovirales que expresan el cDNA de la hRPK y la EGFP en un único RNA mensajero, separados por un IRES eucariótico (SF11RPKXEG). Líneas celulares murinas 3T3 y MEL (células de eritroleucemia murina) y humanas HeLa y 293T fueron transducidas con sobrenadantes retrovirales infectivos. Análisis de western-blot y de FACS demostraron la expresión estable de EGFP y hRPK, respectivamente, con expresión estable del transgén durante más de 20 semanas en cultivo. La expresión de los trasngenes no varió significativamente durante el tiempo del cultivo. Además, la inducción a eritrodiferenciación de células MEL no modificó la expresión de hRPK ni de eGFP. Posteriormente, progenitores hematopoyéticos Lin-Sca-1+ fueron transducidos *in vitro* con SF11RPKXEG y transplantados en receptores normales letalmente irradiados. Tres meses post-transplante, una elevada expresión de hRPK y eGFP fue observada en leucocitos, plaquetas y eritrocitos maduros. Estos resultados indican que los vectores retrovirales pueden ser un sistema efectivo para abordar la corrección fenotípica de los defectos eritropoyéticos asociados a la deficiencia de Piruvato Kinasa Eritrocitaria humana mediante de protocolos de terapia génica.

PÓSTERS
(P-01/P-31)

P-01. CATONIC PLGA MICROPARTICLES FOR GENE DELIVERY TO CANCER CELLS

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The aim of this study is the preparation of biodegradable microparticles with a cationic surface to improve the delivery of DNA into cancer cells. For this, we prepared PLGA microparticles with an optimised water-in-oil-in-water double emulsion process, by using several type of polymers with different molecular weight (RG502, RG503, RG504, RG502H, RG752). Microparticles were characterized in terms of particle size, encapsulation efficiency (EE %) and release rate. Cell culture in vitro studies were performed with HepG2 cells (human hepatocellular carcinoma). Our results confirmed that, by increasing the molecular weight of the polymer, particle size and EE (%) of DNA was also increased. On the other hand, copolimerization 50:50 (lactic:glycolic) in the polymer lead to a better EE (%) than copolymer with 75:25 (lactic:glycolic). Besides, the presence of acidic radicals in the polymer (RG502H), lead to a better EE (%) and a higher release rate. Plasmid DNA extracted from PLGA microparticles preserved both, structural and functional integrity, as evidenced in experiments by agarose gel electrophoresis and in vitro transfection studies. These particles protected the plasmid DNA from digestion by deoxyribonuclease I (DNAse I) in vitro and maintained it in the supercoiled form. Finally, cationic microparticles prepared with PLGA in the presence of the cationic lipid DOTAP were able to transfect efficiently liver tumor cells, which indicate that these vectors could be used, after further studies, for cancer gene delivery.

P-02. NEW LIPOPOLYPLEXES FOR GENE THERAPY OF HEPATOCELLULAR CARCINOMA

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The main objective of this study is the development, optimization and characterization of a novel vector composed of polyethylenimine (PEI), cationic liposomes and DNA (lipopolyplexes), that can deliver genetic material into liver tumour cells. Lipopolyplexes were preparared by first mixing a solution of PEI with DNA at N/P ratio of 4 and after 15 minutes of incubation different amounts of cationic liposomes were added in order to prepare complexes at different molar ratios (lipid/DNA).

In vitro transfections were performed with HepG2 cells in the presence of 60% FBS. Different protocols were used to prepare the complexes, not showing diferences in the particle size or zeta potential. On the other hand, as expected, complexes with a higher lipid to DNA molar ratio showed increased values in the surface charge. Condensation assays, revealed that PEI was effective in condensing DNA at N/P ratio of 4. These non-viral systems were superior to PEI/DNA (polyplexes) and lipoplexes in transfecting HepG2 cells in the presence of 60% serum. Transfection activity of DNA increases by increasing the molar ratio lipid/DNA in the lipopolyplexes, showing branched PEI of low molecular weight better efficacy compared to high molecular weight PEI. At the same time, lipopolyplexes formed with linear PEI were more effective than branched PEI in transfecting liver cancer cells. These vectors were not cytotoxic and highly effective in protecting DNA from attack by DNAse I. Further studies measuring *in vivo* gene expression, to investigate its suitability for this application, are in progress.

P-03. Mecanismos implicados en la transferencia génica hidrodinámica al hígado

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El hígado es una importante diana en terapia génica por sus importantes y numerosas funciones metabólicas, fuente de muchas enfermedades monogénicas susceptibles de tratamiento por dicha vía. Entre los mecanismos posibles, la inyección hidrodinámica ha resultado muy efectiva particularmente en este órgano, por lo que resulta crucial elucidar los mecanismos subyacentes a tan alta eficacia de transfección, inyectando el DNA de la forma más sencilla y segura posible: desnudo. Utilizando el plásmido pTG7101, que contiene el gen humano de la α1-antitripsina controlado por su promotor natural, hemos podido comprobar que las dosis múltiples acumuladas del transgén son bien toleradas por los animales de experimentación y conducen a niveles terapéuticos de la proteína en plasma. La medida simultánea de las presiones en las venas porta y cava muestra una inversión de las mismas durante la inyección hidrodinámica, lo cual implica una inversión del flujo sanguíneo hepático. Esta inversión ha sido observada mediante microscopía intravital, comprobando que se produce un estasis circulatorio intrahepático durante algunos minutos, principalmente en los sinusoides pericentrales. Además en los hepatocitos de dicha zona, la microscopía electrónica de transmisión muestra una presencia masiva de vesículas endocíticas megafluídicas. Estas observaciones nos ofrecen nuevas perspectivas acerca del proceso de transfección hidrodinámico en el hígado, cuya comprensión es de vital importancia para desarrollar nuevos procedimientos de terapia génica seguros y eficaces con aplicación clínica.

P-04. Biodistribution of semliki forest virus vectors in mice when administered by different routes

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Semliki Forest virus (SFV) based vectors have a very broad tropism, being able to infect cells from many different origins, including tumor cells. These vectors have been used to express cytokines and other antitumoral molecules in different tumor models, leading to the induction of efficient antitumoral immune responses. It was of great interest to study if SFV vectors were able to target tumor nodules in vivo when given by different routes of inoculation, as well as to analyze the biodistribution of these vectors in the organism. In this study we studied the *in vivo* distribution of an SFV vector expressing firefly luciferase (SFV-luc) after intravenous, intraperitoneal or intratumoral administration in mice bearing MC38 tumors or in mice without tumors. Specific infection of the tumoral tissue was only found when the virus was inoculated intratumorally. Analysis of CCD whole body images and of luciferase activity in tissue homogenates from mice systemically inoculated with SFV-luc showed a high infectivity in the lung and a medium infectivity in kidney and spleen. Preimmunization of animals with SFV vectors was able to significantly inhibit expression of luciferase in animals inoculated systemically with SFV-luc, but not in those inoculated intratumorally, indicating that although SFV vectors are immunogenic, the generated antivector immune response is not able to prevent tumor infectivity.

P-05. Improvement of liver gene transfer with first and third generation adenoviral vectors

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Recombinant adenoviruses are the most extensively used vectors in gene therapy. To optimize adenovirus-mediated liver transduction we studied the effect of the route of administration using first generation adenovirus vectors expressing human IL12 and luciferase and a helper-dependent adenovirus vector expressing hIL12 using direct intrahepatic (ih) injection, and the systemic administration via tail vein (iv). Intrahepatic injection of first generation adenovirus resulted in higher transgene expression and the kinetic and localization of the expressed protein differs substantially to the pattern showed after iv injection. Intrahepatic injection of third generation adenovirus expressing hIL-12 under the control of an inducible promoter also resulted in an increase of transgene expression. Furthermore macrophage depletion showed that ih injection partially circumvents macrophages' phagocitic activity and revealed that the number of transduced hepatocytes is lower. Interestingly, the administration of empty liposomes increases transgene expression without macrophage depletion. Thus, intrahepatic injection allows us to reduce the dose of recombinant virus to obtain the same therapeutical effect. In fact, the ih injection of a helper-dependent adenovirus carrying murine IL12 resulted in an improved antitumoral efficacy in a

mice tumor model. Intrahepatic injection of adenoviruses increases the transgene expression and reduces the escape being useful for liver gene transfer.

P-06. Comparison between *in vitro* and *in vivo* produced rSV40 vectors

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Recombinant Simian Virus 40 (rSV40) vectors are good candidates for gene therapy approaches as they infect most cell lines, they have long-term expression and they are poorly immunogenic. We have set up a protocol to produce high titters of good quality rSV40 vectors using COS-1 cells as a packaging cell line. The therapeutic rSV40 vectors produced were efficient in the treatment of experimental models of liver cirrhosis and colon cancer. However, two problems are derived from this method of production: (i) our *in vivo* produced rSV40 vectors can only pack up to 2.5 Kb of foreign genome (ii) low amount of the therapeutic protein is produced form rSV40 vectors. Both problems could be solved by using an alternative method of production in which the rSV40 vectors are produced in vitro after mixing the viral major structural protein (VP1) with the vector DNA. rSV40 vectors driving the expression of luciferase under the control of several mammalian and viral promoters were produced *in vitro* or in COS-1 cells. Comparison of the expression and the titer of the vectors produced using both methods will be shown.

P-07. Feasibility of retroviral vector-mediated in utero gene transfer to the fetal rabbit

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Background: Successful treatment or prevention of severe hereditary diseases could conceivably be achieved by genetic intervention early in development. Viral vector-mediated fetal gene transfer is proving a valuable tool to test the above concept in relevant animal models. Although the pregnant rabbit is a well-recognized model for fetal therapy, few pre-clinical assays have used it to validate fetal gene transfer approaches. In this preliminary study we assessed for the first time the feasibility of retroviral vector-mediated *in utero* gene transfer in the fetal rabbit.

Methods: Different amounts of the VSV-G pseudotyped MFG(nls)lacZ retroviral vector, expressing a nuclear-localised ß-galactosidase reporter protein were injected intraperitoneally and intrahepatically into 20-22 day old fetuses. At 8-9 days post-treatment, the pups were sacrificed and the tissues harvested for

analysis. Evidence of gene transfer was obtained by PCR amplification of proviral sequences within genomic DNA isolated from the treated samples. Transgenic ß-galactosidase expression was assessed by X-gal histochemical staining.

Results: By intraperitoneal injection 43% of the viable fetuses treated (3/7) showed evidence of successful *lacZ* gene transfer and low-level ß-galactosidase expression into liver and heart, whereas by intrahepatic injection roughly 38% (3/8) of the livers were positive for *lacZ* gene transfer and expression. The success rate for the viable fetuses rose to 67% positive livers (4/6) when a near double amount of recombinant virus was injected using a 10-fold concentrated virus stock. In terms of short-term safety, fetal and maternal survival rates approached 80% of treated fetuses, and 100% of treated does.

Conclusions: The pregnant rabbit is a useful and reliable model allowing the design of further studies to optimize the conditions for effective, safer, and persistent retroviral vector-mediated fetal gene transfer.

P-08. Development of new noncytopathic vectors derived from Semliki Forest Virus

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Alphavirus vectors can express high levels of recombinant proteins in many different cells types. However, this expression is transient, due to the cytopathic nature of viral replication. Several noncytopathic mutants have been obtained from Sindbis virus (SIN) and Semliki Forest virus (SFV) which could be used when sustained transgene expression is desired. We have constructed new SFV vectors by introducing the mutations described to turn SIN noncytopathic in conserved positions in SFV. These new vectors (SFVnc) have been compared with previosly described SFV noncytopathic vectors. We found that two SFVnc mutations produced an interesting phenotype in transfected cells. One of these mutants, G1E in nsp2, was still cytopathic, but gave rise with low frequence to noncytopathic variants. The second mutant, P718T in nsp2, was apparently not able to replicate in most cells, but gave rise with a relative high frequency to noncytopathic variants that formed colonies without selection. We rationalized that the noncytopathic effect was due to a second mutation that appeared naturally in the SFVnc vectors. To isolate these noncytopathic variants we inserted the pac gene into each of these mutants and puromycine resistant cell clones were selected in BHK cells. The noncytopathic replicons were then cloned by RT-PCR. As hypothesized, one noncytopathic replicon was rescued in this way containing in addition to P718T a second mutation, R649H, in the nuclear localization signal of nsp2. Characterization of this double mutant will be discussed in detail.

P-09. Generación de adenovirus gutless *helper free* mediante el uso del doble sistema de recombinasas C31/CRE

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Para evitar los problemas de inmunogenicidad del adenovirus (Ad) se han desarrollado los Ad-*"gutless"*, que no contienen ninguna región viral codificante y permiten insertos de hasta 36 Kb. Su sistema de producción está compuesto por tres elementos: a) Ad-*"gutless"* con los genes terapéuticos, b) Ad-*helper* que proporciona *"in trans"* las proteínas necesarias para el empaquetamiento del genoma y c) línea celular que exprese una recombinasa. Para evitar la contaminación del Ad-helper, se han desarrollado sistemas que utilizan las recombinasas Cre y FLPe. Sin embargo, los niveles de contaminación son del 0,1-1% y por ello proponemos utilizar la recombinasa unidireccional ΦC31 en un sistema de doble recombinasa ΦC31/Cre que permita la producción del Ad-*"gutless"* de calidad GMP que puedan ser utilizados en ensayos clínicos humanos. En la actualidad hemos creado una familia de Ad-helpers cuya señal de empaquetamiento Ψ está flanqueada por diferentes combinaciones de secuencias attB/attP (ΦC31) y loxP (Cre). Asimismo, se ha generado una línea celular productora 293 que expresa conjuntamente las dos recombinasas (293Cre/ΦC31). Finalmente, hemos amplificado 27 Kb del locus Xq28 humano que actúa como ADN *"stuffer"* en los Ad "gutless". Nuestro objetivo final es analizar su potencial como vectores de terapia y para ello se analizará tanto su capacidad de no inducción de la respuesta inmune celular, como la duración y nivel de expresión de los genes utilizados, en diferentes modelos animales de enfermedades humanas.

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P-10. Optimizing stable retroviral transduction of primary human an murine synovial fibroblast

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Fibroblast like synoviocytes (FLS) are the main resident cells in the normal joints and play a major role in the development of the rheumatoid arthritis (RA). Efficient gene targeting of FLS are in the basis of current therapy approaches as well as experimental studies on RA. Retrovirus-mediated gene transfer is a developing methodology for constitutive or inducible expression from integrated transgenes copies, leading to the desired long lasting delivery of biological products. However, the ex vivo

infection with replication-defective retrovirus require complex protocols restricting both the success and the reproducibility of the method.

We have investigated the effect of experimental conditions as well as of intrinsic properties of the cells in the efficiency of retroviral transduction of human FLSs. Optimising factors affecting the early interaction of the virus with the cell membrane (method and rounds of inoculation, polycations, Retronectin®) as well as those related to cellular and virus properties (cell density and passage number, viral titre), led us to establish a procedure that render consistent and reproducible efficiency of transduction close to 70%. Importantly, eventual differences due to unique features of each FLSs line assayed are negligible in the efficiency of transduction, thus making affordable high rate of gene target into synoviocytes.

P-11. Increased efficacy and safety in the treatment of liver cancer with a novel adenovirus-alphavirus hybrid vector

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An improved viral vector for cancer gene therapy should be capable of infecting the tumor with high efficiency, inducing specific and high level expression of the transgene in the tumor and destroying selectively the tumor cells. In the design of such a vector to treat hepatocellular carcinoma (HCC) we took advantage of 1) the high infectivity of adenoviruses for hepatic cells, 2) the high level of protein expression and the pro-apoptotic properties that characterize the Semliki Forest Virus (SFV) replicon and 3) the tumor selectivity provided by the alphafetoprotein (AFP) promoter. Thus we constructed a hybrid viral vector composed of a helper-dependent adenovirus containing a SFV replicon under the transcriptional control of AFP promoter. The transgene was inserted in the SFV replicon and was placed under the control of the SFV subgenomic promoter. Hybrid vectors containing murine interleukin 12 (mIL-12) genes or the reporter gene LacZ showed very specific and high level expression of transgenes in AFP-expressing HCC cells, both *in vitro* and *in vivo* in an HCC animal model. Infected HCC cells were selectively eliminated due to the induction of apoptosis by SFV replication. In a rat ortothopic liver tumor model, treatment of established tumors by the hybrid vector carrying mIL-12 gene resulted in a strong antitumoral activity without accompanying toxicity. This new type of vectors may provide a potent and safe tool for cancer gene therapy.

P-12. Rechazo preventivo del desarrollo del melanoma mediante vacunas genéticas celulares: efecto de la combinación de genes de citocinas

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Algunos de los mayores éxitos en terapia génica antitumoral se han obtenido empleando vacunas con células modificadas genéticamente. Por su parte, citoquinas como GM-CSF e IL-12 han mostrado sobradamente su potencial antitumoral. Por ello elaboramos un experimento de vacunación antitumoral basado en la línea de melanoma murino B16 transfectada, mediante métodos no virales, con diferentes plásmidos basados en el vector p2F, para producir las citadas citoquinas y también B7.2, molécula coestimuladora que interviene en la presentación de antígenos al sistema inmune y por tanto debe potenciar su respuesta. Con tres dosis de vacunación (días -21, -7 y +7; implantación tumor día 0), y garantizando una producción ≥ 35 ng/ml/10^6céls/24h de m-GMCSF, medida por ELISA, se logró una supervivencia del 100% de los animales vacunados con 2x10^5céls. B16-p2F-mGMCSF hasta más de 5 meses después de la implantación del tumor; pero además se logró un 60% de supervivencia con p2F-mGMCSF+mB7.2, con la mitad de producción de la citoquina, resaltando el importante papel conjunto de ambas moléculas.

IL-12 y sus combinaciones con GMCSF alcanzaron también el 60% de supervivencia aunque con muertes más prematuras. No obstante, el incremento en la vida media fue claramente superior en las combinaciones con GMCSF lo que apoya la idea de que esta citoquina, que desencadena inicialmente la respuesta, desempeña como gen único el mejor papel, mientras que otras citoquinas, que pueden tener interés independientemente, reducen la eficacia que él solo presenta.

P-13. Dendritic Cells Delivered inside Human Carcinomas are Sequestered by Interleukin-8

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In the course of a clinical trial consisting of intratumoral injections of dendritic cells (DC) transfected to produce Interleukin-12, the use of ^{111}In-labeled tracing doses of DC showed that most DC remained inside tumor tissue, instead of migrating out. In a search for factors that could explain this retention, it was found that tumors from patients suffering hepatocellular carcinoma, colorectal or pancreatic cancer were producing IL-8 and that this chemokine attracted monocyte-derived dendritic cells that uniformly express both IL-8 receptors CXCR1 and CXCR2. Accordingly, neutraliz-

ing anti-human IL-8 monoclonal antibodies blocked the chemotactic attraction of DC by recombinant IL-8, as well as by the serum of the patients or culture supernatants of human colorectal carcinomas. In addition, human DC injected inside human colon cancer xenografted in nude mice were retained inside tumor tissue in an IL-8-dependent fashion. IL-8 production in malignant tissue and the responsiveness of DC to IL-8 are a likely explanation of the clinical images, which suggest retention of DC inside human malignant lesions. Impairment of DC migration towards lymphoid tissue could be involved in cancer immune-evasion and, therefore, interference with IL-8 functions should be considered therapeutically.

P-14. Intratumoral Injection of Dendritic Cells Engineered to Secrete Interleukin-12 by Recombinant Adenovirus in Patients with Metastatic Gastrointestinal Carcinomas

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Purpose: To evaluate the feasibility and safety of intratumoral injection of autologous dendritic cells (DC) transfected with an adenovirus encoding interleukin-12 genes (AFIL-12) for patients with metastatic gastrointestinal carcinomas. Secondarily, we have evaluated biological effects and antitumoral activity.

Patients and Methods: Seventeen patients with metastatic pancreatic (n=3), colorectal (n=5) or primary liver (n=9) malignancies entered the study. DC were generated from $CD14^+$ monocytes from leukapheresis, cultured and transfected with AFIL-12 before administration. Doses from $10x10^6$ to $50x10^6$ cells were escalated in 3 cohorts of patients. Patients received up to three doses at 21-day intervals.

Results: Fifteen (88 %) and eleven of seventeen (65%) patients were assessable for toxicity and response, respectively. Intratumoral DC injections were mainly guided by ultrasound. Treatment was well tolerated. The most common side effects were lymphopenia, fever and malaise. IFNγ and IL-6 serum concentrations were increased in 15 patients after each treatment, as well as peripheral blood NK activity in five of them. DC transfected with AFIL-12 stimulated a potent antibody response against adenoviral capsides. DC treatment induced a marked increase of infiltrating CD8+ T lymphocytes in 3 out of 11 tumor biopsies analyzed. A partial response was observed in one patient with pancreatic carcinoma. Stable disease was observed in two patients and progression in eight cases, two of them fast progressing during treatment.

Conclusion: Intratumoral injection of DC transfected with an adenovirus encoding IL-12 to patients with metastatic gastrointestinal malignancies is feasible and well tolerated. Further studies are necessary to define and increase clinical efficacy.

P-15. An Oncolytic Adenovirus controlled by a modified Telomerase promoter is attenuated in Telomerase-negative cells, but shows reduced activity in cancer cells

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The promoter for human telomerase reverse transcriptase (TERTp) is preferentially active in malignant cells. Recently, it has been used to control de expression of the viral E1a gene for the development of oncolytic adenoviruses. In order to ensure maximal repression in normal cells, the inclusion of additional binding sites for Myc/Mad transcription factors (E-boxes) in the proximal region of the core promoter has been described.

We found that the transcriptional activity of this artificial sequence (T-255-4DEB) is minimal in normal cells, but it is also reduced in all the cancer cell lines tested. The cancer specificity of a new oncolytic adenovirus based on this promoter (AdTE1) was evaluated by direct comparison with wild type adenovirus type 5 (AdWT) in a panel of normal and transformed human cells. In all the parameters studied, AdTE1 was attenuated in normal cells, but the efficacy in cancer cells showed a parallel reduction, suggesting a lack of specificity. However, when we compared the cytotoxicity of AdTE1 and AdWT in senescent fibroblasts, the attenuation of AdTE1 was more intense.

We conclude that AdTE1 is preferentially attenuated in cells that are permanently devoid of telomerase expression, but it shows reduced activity in cancer cells compared with AdWT. Further modifications in the telomerase-based promoters should be introduced to combine maximal attenuation in normal tissues and enhanced activity in tumors.

P-16. Optimization of the protocols for the treatment of liver metastases by inducible expression of interleukin-12 in a murine model

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Gene therapy of liver diseases using "gutless" or high capacity adenoviral vectors with inducible and tissue-specific transgene expression systems shows promising results in liver cancer treatment but stills needs some improvements.

In this study, we have used a new murine model of hepatic metastases to determine the therapeutic window of murine interleukin-12 (IL-12), and the most suitable protocol to achieve the maximum therapeutic effect with minimal toxicity. Colon cancer metastases were induced in syngenic C57BL/6 by intra-hepatic injection of MC38 cells stably transduced with the luciferase reporter gene. A high-capacity adenoviral

vector expressing IL-12 under the control of a liver-specific inducible system was injected in the liver, and the expression of IL-12 was induced by exogenous administration of mifepristone. The anti-tumor effect was evaluated by in vivo bioluminescence analysis and direct tumor measurement.

Our result show that the intra-hepatic route of administration is effective, but the variability in the levels of IL-12 achieved requires frequent monitorization and careful adjustment of the inducer drug to avoid toxicity. Increasing the dose of the vector (up to 5 x 10^8 iu) with moderate mifepristone levels (125 microg/kg) was more effective than increasing the dose of mifepristone (1000 microg/kg) with lower viral load (1 x 10^8 iu). Optimized protocols for using gutless adenoviral vectors expressing inducible and liver-specific IL-12 will allow appropriate regulation of transgene expression for future application in clinical trials.

P-17. FGF2-Redirected adenoviruses enhance trasduction efficicency and increase the therapeutic efficacy of the suicide system ADCYP2B1/CPA in pancreatic cancer models

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Cancer cells are often resistant to adenoviral infection particularly due to the low expression of the CAR receptor. The redirection of adenovirus to receptors that are highly expressed in cancer cells may allow the vector to efficiently deliver the therapeutic gene in the tumour mass. In this study, we have evaluated the ability of adenovirus redirected to fibroblast growth factor receptors (FGFRs) to target pancreatic cancer cells and to increase the therapeutic outcome of the suicide CYP2B1/CPA system. Expression analysis of FGFRs showed elevated levels of the receptors in biopsy samples of human pancreatic ductal adenocarcinoma (PDAC) and in cells and xenografts derived from pancreatic tumours. Transduction efficiency of the retargeted adenovirus FGF2-Ad-luc demonstrated enhanced luciferase activity in both pancreatic cancer cells and mouse xenografts. Therapeutic efficacy of this retargeting strategy was evaluated by using an FGF2-redirected adenovirus carrying the CYP2B1 suicide gene. An enhanced cytotoxic effect (higher than 5–fold) was observed in pancreatic cancer cells that exhibit elevated levels of FGFRs. In vivo administration of FGF2-Ad-CYP2B1 lead to an increase in CYP2B1 expression in FGFR positive tumours. Moreover, preliminary results suggest that the retargeted virus have enhanced cytotoxicity *in vivo*.

P-18. Evaluation of antitumor effect of intrahepatic interleukin-12 expression from a plasmid vector in mice models of primary and metastatic liver cancer

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Interleukin-12 (IL-12) is a potent antitumoral cytokine, but it can be toxic at high doses. In order minimize side effects, we constructed a plasmid able to drive expression of murine IL-12 in a liver-specific and doxycycline (Dox)-inducible manner. The plasmid was transferred to mice by the hydrodynamics-based procedure and the antitumor effect of IL-12 was evaluated on two different animal models. One of them is a hepatic colon cancer metastasis model in syngenic mice induced by intra-hepatic implantation of CT-26 cells. When the plasmid was injected before cell implantation, a complete eradication of liver metastasis was observed in all animals receiving Dox for 10 days. In contrast, if cells were implanted before plasmid administration, only 20% of the treated animals showed complete tumor regression, although their survival was longer than untreated mice.

The second is a spontaneous primary hepatocellular carcinoma (HCC) model. Transgenic mice that over-express the c-myc gene in the liver under the control of the rat L-type pyruvate kinase (PK) promoter develop tumors in 7-8 months that resemble human primary HCC in terms of biology and gene expression profile. After treatment, induced IL-12 reached high levels, but none of these mice showed tumor remission, even after 20 days of Dox administration. The effect of IL-12 was also evaluated on histological tumor samples, by quantification of VEGF, IFN-gamma and TNF-alpha and by analysis of specific immune responses against tumor antigens. Our data indicate that antitumor activity of IL-12 can be strongly limited by the therapeutic procedure and more importantly by the type/origin of liver tumor assayed.

P-19. The kinetics of gene regulation in the liver using drug-inducible systems can be affected by cyotokine transgene expression

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Interleukin 12 (IL-12) is a potent cytokine endowed with strong antitumor properties but also considerable toxicity. Therefore, tight regulation of its expression is required when using IL-12 for cancer gene therapy. Pharmacological control of gene expression can be achieved by using the so-called "on/off" regulatory systems. Most of

them are composed of two expression units, one bearing the gene of interest under the control of an inducible promoter, and the other carrying a constitutively expressed chimeric trans-activator protein (TA) able to bind a specific drug and to mediate the activation or the repression of the inducible promoter activity. We have analyzed the kinetics of human (h) and murine (m) IL-12 expression in mice controlled by the doxycycline (Dox)- or the RU486-dependent gene switch systems using two different vectors directed to the liver: plasmids administered by hydrodinamic injection and a high capacity adenoviral vector. Whereas the levels of hIL-12 (that does not have biological effect in mouse) were quite stable, the amount of mIL-12 was drastically reduced in serum after daily drug administration. The data indicate that loss of DNA was not the cause for reduction in mIL-12 synthesis and that it was associated to a drastic decrease in TA expression. In order to analyze the kinetics of the TA promoter activity in the presence of IL-12, plasmids carrying the hAAT reporter gene driven by the same liver-specific promoter/enhancer sequences used in the Dox- or RU486-inducible systems were transferred to mice together with a Dox-inducible IL-12 plasmid vector. Our data showed that hAAT expression has a similar kinetics as IL-12 after several rounds of Dox-mediated gene induction. In summary, we conclude that the levels of TA directly determine the levels of mIL-12 (and vice-versa), indicating that the function of a drug-inducible system can be affected depending on the gene to be regulated.

P-20. Induction of gp120-specific protective immune responses by genetic vaccination with linear polyethylenimine-plasmid complex

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The induction of IFN-γ secreting CD8+ T cells and neutralizing antibodies to HIV-1 are both key requirements for prevention of viral transmission and clearance of pathogenic HIV. Although DNA vaccination has been shown to induce both humoral and cellular immune responses against HIV antigens; the magnitude of the immune responses has always been disappointing. In this report, we analyzed the ability of Polyethylenimine-DNA complex expressing an HIV-gp120 antigen (PEI-pgp120) to induce systemic CD8+ T cell and humoral responses against the gp120 antigen. The administration of PEI-plasmid complex resulted in rapid elevation of the serum levels of IL-12 and IFN-γ. Furthermore, a single administration of PEI-pgp120 complex elicits a 20 times higher the number of gp120-specific CD8+ T cells compared to that elicited by three intramuscular injections of naked DNA. Interestingly, we found that systemic vaccination with PEI-pgp120 induced protective immune responses against both systemic and mucosal challenge with a recombinant *vaccinia* virus expressing a gp120 antigen. The data also demonstrated that the depletion of macrophages with liposome-encapsulated clodronate completely abolished gp120-specific cellular response. Overall our results showed that a single administration of PEI-pgp120 complexes, eliciting strong immune responses, is an effective vaccination approach to generate protection against systemic and mucosal viral infections.

P-21. Diseño, producción y evaluación de un virus adenoasociado recombinante que expresa interferón alfa para el tratamiento de la hepatitis crónica B

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El interferón alfa (INFα) es una citoquina clave en la defensa frente a infecciones virales. Para aumentar la eficacia y minimizar los efectos adversos del tratamiento de la hepatitis crónica B con INFα, se ha estudiado en este trabajo la posibilidad, la eficacia antiviral y la toxicidad de la producción de interferón alfa en el músculo y en el hígado de marmotas crónicamente infectadas por el virus de la hepatitis de la marmota, un modelo de infección crónica por el VHB.

En primer lugar se compararon tres vías de administración de los virus adenoasociados (VAA) dirigidas al hígado en ratones Balb/C. Se escogió la inyección intraportal para la liberación hepática de INFα ya que combina altos niveles, gran distribución en el hígado y pocas diferencias entre sexos.

La expresión de INFα mediante un VAA tras inyección intraportal es eliminada en la mayoría de las marmotas a los dos meses de la inyección obteniéndose una eficacia antiviral limitada. En cambio, la expresión de luciferasa mediante inyección intraportal de un VAA se detectó durante más de seis meses. La inyección intramuscular provocó una expresión sostenida pero fluctuante de INFα que se asoció a un efecto terapéutico tardío en tres de cinco marmotas.

P-22. Inhibición del crecimiento bacteriano mediante transferencia adenoviral de péptidos antimicrobianos en queratinocitos

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La infección representa uno de los principales problemas asociados con el rechazo de los injertos de piel en pacientes quemados. El tratamiento tópico y sistémico con antibióticos convencionales hace cada vez más frecuente la aparición de cepas bacterianas multiresistentes. Los péptidos antimicrobianos (AMPs) forman parte del mecanismo de defensa innato de muchos organismos y son activos frente a un amplio espectro de bacterias. Algunos de estos péptidos se expresan en la piel humana en condiciones de inflamación. Mediante técnicas de transferencia génica utilizando vectores adenovirales, hemos expresado alguno de estos AMPs (HBD-2, HBD-3, LL-37) en queratinocitos humanos en cultivo. Los medios condicionados de estas células

mostraron actividad antibacteriana frente a diversas cepas frecuentemente asociadas a quemaduras. La coexpresión de algunos péptidos (HBD-2 y LL-37) produjo una actividad sinérgica frente a ciertas cepas bacterianas. Además, el crecimiento bacteriano sobre la epidermis de equivalentes cutáneos generados a partir de una matriz de fibrina también se ve significativamente reducido cuando se sobreexpresan estos péptidos por transferencia adenoviral. La función de estos péptidos también se ha relacionado con la reparación tisular y la inmunidad adaptativa. Así, el uso de una terapia génica antimicrobiana transitoria podría ser una solución terapeútica alternativa al uso de antibióticos sintéticos en pacientes quemados.

P-23. Gene delivery of a CD3δγ chimera into human CD3γ deficient T lymphocytes gives clues about TCR dynamics

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The CD3 subunits of the antigen-specific T-cell receptor (TCR) play a central role in regulation of surface TCR expression levels. Humans who lack CD3γ have a potentially lethal immunodeficiency, reduced surface TCR expression levels and abolished PMA-induced TCR down-regulation. We are investigating delivery of CD3 chains to treat this type of immunodeficiency and to understand TCR dynamics *in vivo.* The response to PMA has been clearly mapped to the di-leucine-based motif in the intracellular domain of CD3γ; however, the molecular cause of the reduced TCR surface expression in CD3γ negative patients is not known. We are using CD3 chimeras to approach this question. In the present work we report that retroviral transduction of a chimera containing the extra-cellular domain of CD3δ and the transmembrane and intra-cellular domain of CD3γ into CD3γ-deficient peripheral blood T lymphocytes restored TCR expression and down-regulation. Subtle differences in surface TCR staining could be observed with certain monoclonal antibodies when cells transduced with the chimera were compared with cells transduced with normal CD3γ. Since the CD3γ-deficient T lymphocytes contain CD3δ, the results suggest that the molecular basis for the reduced surface TCR expression level can be mapped to the transmembrane and intra-cellular domain of CD3γ. These results could be relevant for protocols involving gene transfer of TCR or CD3 chimeras to human T lymphocytes to treat cancer or immunodeficiency syndromes.

P-24. Gene therapy of haemophilia B through epidermal grafts genetically modified to secrete hFIX

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Haemophilia B is caused by a deficiciency in coagulation factor IX. It is due to mutations in the factor IX (FIX) gene. The gene has been mapped to the distal end of the long arm of the X chromosome, band Xq.27.1, leading to the classic X-linked inheritance. The FIX gene is 34 Kb in length and the essential genetic information is present in eight exons which encode 1.6Kb mRNA.

The FIX is synthesized by hepatocytes as a 461 amino acid precursor protein, and is dependent on post-translational processing for full functional activity.

Patients suffer from severe bleeding disorders that may cause chronic tissue inflamation and degeneration. The olny treatments availables nowdays are paliative ones.

Haemophilia is a good candidate for a gene therapy aproach since biologically active FIX can be synthesized by other cell types than hepatocytes , such as keratinocytes, and factor levels as low as 1.5% of normal (3-5 μg/ml) will amylorate symptoms.

We are investigating the use of human keratinocytes (epidermal stem cells) , as potencial target cells for gene therapy for haemophilia B. We have developed several retro and lentiviral bicistronic vectors containing the FIX gene. A human epidermal/dermal equivalent composed of a hFIX secretory epidermis will be grafted onto a new Hemophilic B/scid strain.

P-25. Molecular chimerism: towards gene therapy for autoinmune disease

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Transgene expression (molecular chimerism) in the hematopoietic system is usually associated with immune tolerance to the transgene products. We hypothesized that stably expressing the encephalitogenic peptide MOG_{40-55} in the marrow cells in a murine model of experimental autoimmune encephalomyelitis -EAE- will induce specific immune tolerance to the antigen, which will prevent or reduce susceptibility to the disease. To this end we designed and constructed a retroviral vector containing the coding sequence of the MOG_{40-55} peptide. We generated stable, high titer ecotropic retroviral vector producing cell lines which allow highly efficient gene transfer rates into murine marrow cells. We initially found that mice receiving a sublethal dose of radiation (4 Gy) developed a more severe disease than control non-irradiated animals. In order to circumvent the use of radiation as a conditioning regimen to allow long-term engraftment of the gene modified marrow cells with little or no effect on the course of the disease, we have investigated alternative treatments such as busulfan and treosulfan at non-myeloablative doses. These results and other preliminary data on EAE susceptibility and severity in mice transplanted with gene modified cells will be presented and discussed.

P-26. Nuevas estrategias de terapia génica para tratamiento de la enfermedad inflamatoria intestinal

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La enfermedad de Crohn se caracteriza por infiltración leucocitaria y producción elevada de citoquinas proinflamatorias en mucosa intestinal, lo que desestabiliza irreversiblemente las monocapas intestinales y obliga a la extirpación total del colon. Los tratamientos actuales consisten en la administración de factores antiinflamatorios pero tienen numerosos efectos secundarios. Por ello proponemos el uso de virus entéricos como vectores de terapia génica (adenovirus del subgrupo F: Ad40, Ad41) para incrementar la especificidad y la eficiencia de transfección al epitelio intestinal. Sin embargo, la dificultad para amplificar estos virus y el limitado conocimiento de su genoma ha potenciado la construcción de virus quiméricos. En colaboración con el Dr. Hamada, estamos amplificando y caracterizando adenovirus quimeras 5/40 (Ad40S, Ad40SL, Ad5.40, Ad40.5), que posteriormente se administrarán *in vivo* para analizar su biodistribución en ratón en función de la ruta de administración (oral, rectal, sistémica). También se analizará su tropismo en cultivos primarios y organotípicos de intestino de ratón y humano. Una vez seleccionado el vector quimérico con mejor especificidad y eficiencia de transfección en colon, se clonarán en su genoma genes y secuencias inmunomoduladoras (IL10, siRNA-TNF-alpha, etc.) y se determinará su efecto terapéutico en modelos murinos para la enfermedad.

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P-27. Neurotrophic factors and C fragment tetanus toxin fusion like treatment by gene therapy to ALS

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Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease currently without an effective treatment. Neurotrophic factors have been studied like molecules in the treatment of the disease. But their unspecific affinity to motor neurons population has made not successful the clinical trials.

C Fragment tetanus toxin has the ability to deliver enzymatic activities to motor neurons when it is fused with another protein. We have made the genetic fusion

between two neurotrophic factors, BDNF (Brain Derived Neurotrophic Factors) and GDNF (Glial Cell Line Derived Neurotrophic Factor) with TTC (C Fragment Tetanus Toxin) in order to study their possibilities for use in the treatment of ALS. *In vitro,* we have observed the survival abilities of BDNF and GDNF when they are fused to TTC. Both fusion proteins reduce the apoptosis rates in the cells. We proposed that these fusion proteins could be applied in the future like gene therapy in ALS treatment.

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P-28. Terapia génica para la cicatrización: efecto del factor de crecimiento de queratinocitos (KGF) en el tratamiento de heridas cutáneas

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Diversos grupos de investigación han demostrado que el factor de crecimiento de queratinocitos (KGF) es un modulador paracrino de los procesos de morfogénesis epitelial y de reepitelización cutánea. Estos resultados derivan de estudios realizados en modelos *in vitro* o en modelos animales murinos y porcinos. Todos ellos concluyen que el KGF es un candidato muy atractivo para mejorar la reparación cutánea. Recientemente en nuestro laboratorio hemos generado un modelo *in vivo* de cicatrización humana basado en la regeneración de piel humana en el dorso de ratones inmunodeficientes (ratón humanizado en piel). Utilizando este modelo hemos evaluado la eficacia del KGF (recombinante o producido por transducción con un vector adenoviral) como como posible factor terapéutico para el tratamiento de heridas en cutáneas en humano. Para ello se han analizado diversos parámetros que afectan tanto al proceso de reepitelización como al de remodelación dérmica en heridas cutáneas. Nuestros resultados sugieren que nuestro sistema podría utilizarse como modelo preclínico de cicatrización para evaluar tanto la potencialidad de distintos factores como la forma de administración (proteína recombinante o terapia génica *in vivo*).

P-29. Adenovirus VA RNA is processed to functional interfering RNAs

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Post-transcriptional gene silencing allows sequence-specific control of gene expression. Specificity is guaranteed by small antisense RNAs such as microRNAs (miRNAs) or small interefering RNAs (siRNAs). Functional miRNAs derive from longer double-stranded RNA (dsRNA) molecules that are cleaved to pre-miRNAs in the nucleus and are transported by Exportin 5 (Exp 5) to the cytoplasm. Adenovirus-infected cells express virus-associated (VA) RNAs, which are dsRNA molecules similar in structure to pre-miRNAs. VA RNAs are also transported by Exp 5 to the cytoplasm where they accumulate. Here, we show that small VAI-derived RNAs (sVAIRNAs) can be found in adenovirus-infected cells. sVAIRNAs are detected at 18 hours post-infection and accumulate at higher concentrations during the late phase of infection. VA RNA processing to sVAIRNAs requires neither viral replication nor viral protein expression, as sVAIRNAs accumulation can be detected in cells transfected with VA sequences. Finally, sVAIRNAs could behave as functional siRNAs, as they inhibit the expression of reporter genes with complementary sequences. Thus, sVAIRNA-mediated silencing could represent a novel mechanism used by adenoviruses to control cellular or viral gene expression.

P-30. Inhibiting expression of specific genes in mammalian cells with 5′ end-mutated U1 small nuclear mRNA targeted to terminal exons of pre-mRNA

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Reducing or eliminating expression of a given gene is likely to require multiple methods to ensure coverage of all of the genes in a given mammalian cell. We and others have previously shown that U1 small nuclear (sn) RNA, both natural or with 5′ end mutations, can specifically inhibit reporter gene expression in mammalian cells. This inhibition occurs when the U1 snRNA 5′ end base pairs near the polyadenylation signal of the reporter gene's pre-mRNA. This base pairing inhibits poly(A) tail addition, a key, nearly universal step in mRNA biosynthesis, resulting in degradation of the mRNA. Here we demonstrate

that expression of endogenous mammalian genes can be efficiently inhibited by transiently or stably expressed 5′_ end-

mutated U1 snRNA. Also, we determine the inhibitory mechanism and establish a set of rules to use this technique and to improve the efficiency of inhibition. Two U1 snRNAs base paired to a single pre-mRNA act synergistically, resulting in up to 700-fold inhibition of the expression of specific reporter genes and 25-fold inhibition of endogenous genes. Surprisingly, distance from the U1 snRNA binding site to the poly(A) signal is not critical for inhibition, instead the U1 snRNA must be targeted to the terminal exon of the pre-mRNA. This could reflect a disruption by the 5' end-mutated U1 snRNA of the definition of the terminal exon as described by the exon definition model.