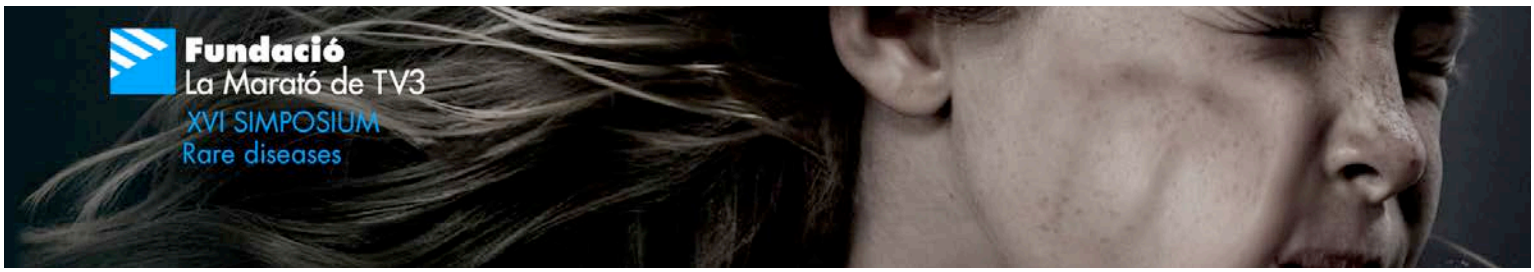

Notch pathway inhibition as therapeutic target in rhabdomyosarcoma

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1. Project Summary

Theoretical Framework: Rhabdomyosarcoma (RMS) is a malignant tumor derived from skeletal muscle precursors. It is a rare disease with an incidence of 5.3 cases/million/year, affecting mainly children under 15 years. Current 5-year overall survival is approximately 65%. Our in vitro studies suggest that Notch inhibition produces a significant decrease in the invasion of RMS cell lines. In addition, activation of the Notch pathway seems to play a crucial role in the maintenance of tumor initiating cells (TICs) in other neoplasms and there is evidence in the literature that RMS cells positive for fibroblast growth factor-3 (FGFR3 +) have characteristics of TICs.

Main objective: To analyze the effects of Notch inhibition using a murine model of xenotransplantation of RMS to identify new therapeutic targets in this neoplasia.

Specific objectives: 1 - To analyze the antioncogenic effect of Notch pathway downregulation by alpha- and gamma-secretase inhibitors in RMS cells *in vitro*. 2 - To test the effect of alpha- and gamma-secretase inhibitors in vivo in a rhabdomyosarcoma mouse model. 3 – To analyze the effects of the inhibition of the Notch pathway in rhabdomyosarcoma cancer stem cells (FGFR3+) as a potential therapeutic target to overcome chemoresistance and reduce or block their metastatic potential. 4 - Determining the proteomic profile in SCID mice xenografts treated or untreated, to identify and quantify proteins belonging or related to Notch pathway as a new therapeutic or diagnostic tool.

2. Results

Section 1 - The antioncogenic effect of Notch downregulation by alpha- and gamma-secretase inhibitors have been tested in rhabdomyosarcoma cells in vitro.

These results allowed us to select gamma-secretase inhibitors as the most effective, which were selected for subsequent phases of the project.

Section 2 - We conducted two Notch pathway inhibition studies in vivo in primary tumors arising in immunocompromised mice. Two different drugs, GSI-XXI and DAPT, were used. DAPT significantly reduced tumor growth but was not able to block it completely. During the second year we completed the development of our metastasis model and the treatment with gamma-secretase inhibitors was performed. The results showed a significant decrease in the appearance of metastases in the treated mice. At the same time, the importance of inhibiting the Hedgehog pathway was also studied, which has an important compensatory effect. **These results allowed us to reach the objective or even surpass it, because they were supplemented by the study of the Hedgehog pathway, closely related to Notch and with proven synergistic effects on tumorigenicity.**

Section 3 – The cytometric analysis of cancer stem cells in both cultured cells and RMS tumors was also performed. During the third year of the project the previously reported FGFR3 was discarded as a cancer stem cell marker because it proved not to be good for selecting cancer stem cells in terms of clonogenic potential and differentiation capacity. For this reason, new markers were incorporated since recent advances in the understanding of tumor stem cells have suggested other markers (such as CD133 and C-met). **These markers permitted more reliable progress towards the ultimate goal, which is to isolate and characterize tumor stem cells in the RMS.**

The development of this project also permitted the discovery of a new method for the detection of tumor cells which will make it possible to analyze their presence in peripheral blood and bone marrow (published in Cytometry A).

3. Relevance and possible implications

Rhabdomyosarcoma is the most common soft tissue sarcoma in children. Overall survival from this neoplasia is around 65%; however, patients with metastasis or disease progression during or after therapy have particularly poor prognosis. For these patients, the search for new therapies that directly affect molecular targets would result in more effective means of overcoming resistance to treatment, thereby improving their chances of cure.

Before the beginning of this project the possible biological role of the Notch pathway in rhabdomyosarcoma was absolutely unknown. This project has shown an important role for this pathway that is conducive to the mobility and invasiveness of rhabdomyosarcoma cells in vitro, which could result in increased metastatic potential in vivo. This project has clarified the role of the pathway in tumor progression and metastatic capacity in immunocompromised animal models.

This project has shown the antioncogenic effect of Notch pathway inhibitors in a mouse model, thereby verifying their potential as anti-cancer therapy in vivo. This will afford not only better understanding of the role of this signaling pathway in RMS, but also the possibility of using these inhibitors in the near future as targeted therapy for high-risk, recurrent or refractory RMS.

The project has first described several very innovative concepts and great clinical implication for RMS

1. Notch as a potential therapeutic target. It also suggests cotreatment with Hedgehog pathway inhibitors. **Cotreatment with both inhibitors could be very powerful in the treatment of patients who do not currently respond to therapy.**

2. The presence of tumor stem cells in the two main types of rhabdomyosarcoma and the promotion of their differentiation when treated with Notch and Hedgehog inhibitors suggested these pathways as potential therapeutic targets that may also be effective against this specific subpopulation of cells. **These results could open the door to new treatments based on Notch and Hedgehog inhibitors, which could contribute to improved survival rates.**

3. We have developed a new method based on flow cytometry to determine the presence of tumor cells in the blood of rhabdomyosarcoma patients. This determination is not well resolved to date and can help to early detection thereby improving survival rates.

In addition, the project has allowed agreement signature with two biotechnological companies to develop new compounds with potential clinical applicability (ITGA9 and inhibitors of Hedgehog ligands) and to study the applicability of existing compounds such as Notch inhibitors (DAPT and GSI-XXI). Two co-operation contracts have been signed with two companies (BCNpeptides and Iproteos) to develop these compounds towards a possible patent license and product development in the short term. Both contracts are deposited in the Vall d'Hebron Research Institute and available to anyone who wishes to consult them. The fact that private companies have shown interest in these findings is illustrative of the potential that can be derived from the findings of this project. This encourages us to continue this line of research with new funding that we have already obtained and we expect to increase in the future, largely thanks to the aid we received from the TV3 Marathon Foundation just 3 years ago.

4. Publications

Publications directly related

AUTHORS: Roma J, Almazán-Moga A, Sánchez de Toledo J, Gallego S. **TITLE:** Notch, Wnt and Hedgehog pathways in rhabdomyosarcoma: from single pathways to an integrated network. **JOURNAL:** Sarcoma. 2012:695603.

AUTHORS: Masià A, Almazán-Moga A, Velasco P, Reventós J, Torán N, Sánchez de Toledo J, Roma J, Gallego S. **TITLE:** Notch-mediated induction of N-cadherin and α9-integrin confers higher invasive phenotype on rhabdomyosarcoma cells. **JOURNAL:** Br J Cancer. 2012 Oct 9;107(8):1374-83.

AUTHORS: Almazán-Moga A, Roma J, Molist C, Vidal I, Jubierre L, Soriano A, Segura MF, de Toledo JS,Gallego S. **TITLE:** Optimization of rhabdomyosarcoma disseminated disease assessment by flow cytometry. **JOURNAL:** Cytometry Part A. 2014 Dec;85(12):1020-9.

[MANUSCRIT EN PREPARACIÓ: Ligand-dependent Hedgehog pathway activation in RMS: the oncogenic role of the ligands. Almazán-Moga A, Nitzki F, Velasco P, Molist C, Giralt I, Vidal I, Segura MF, Soriano A, Jubierre L, Navarro S, Martinez-Tirado O, Ferreres JC, Santamaría A, Rota R, Hahn H, Sánchez de Toledo J, Roma J, Gallego S. To be submitted to Dev Cell.]

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Review Article

Notch, Wnt, and *Hedgehog* Pathways in Rhabdomyosarcoma: From Single Pathways to an Integrated Network

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British Journal of Cancer (2012), 1–10

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Full Paper

Notch-mediated induction of N-cadherin and α9-integrin confers higher invasive phenotype on rhabdomyosarcoma cells

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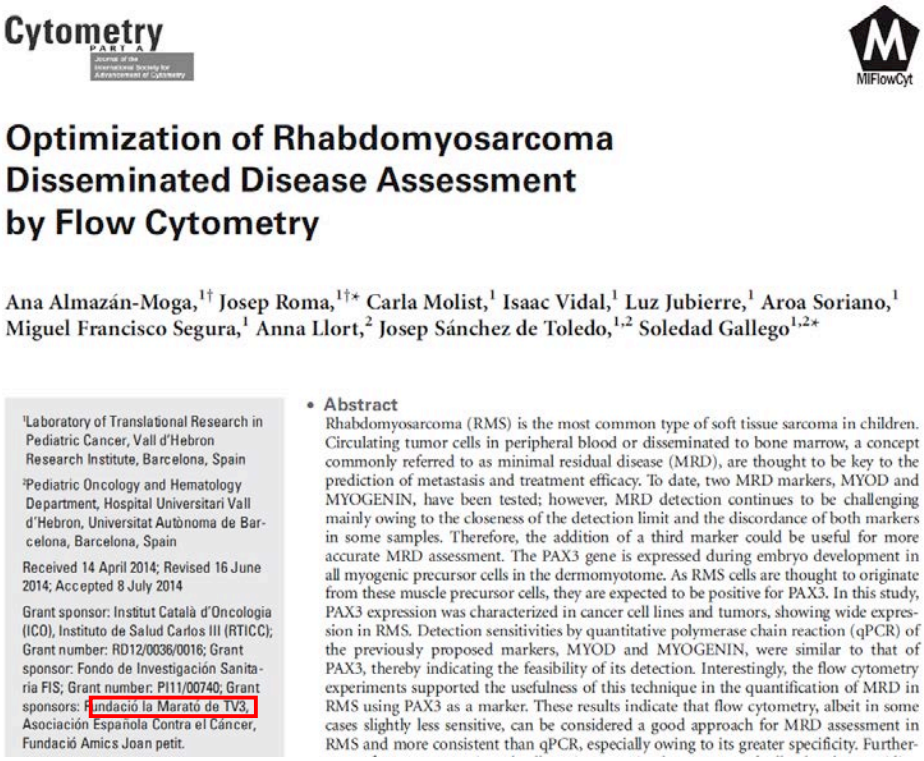

Cytometry PART A

MIFlowCyt

Optimization of Rhabdomyosarcoma Disseminated Disease Assessment by Flow Cytometry

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• Abstract

Rhabdomyosarcoma (RMS) is the most common type of soft tissue sarcoma in children. Circulating tumor cells in peripheral blood or disseminated to bone marrow, a concept commonly referred to as minimal residual disease (MRD), are thought to be key to the prediction of metastasis and treatment efficacy. To date, two MRD markers, MYOD and MYOGENIN, have been tested; however, MRD detection continues to be challenging mainly owing to the closeness of the detection limit and the discordance of both markers in some samples. Therefore, the addition of a third marker could be useful for more accurate MRD assessment. The PAX3 gene is expressed during embryo development in all myogenic precursor cells in the dermomyotome. As RMS cells are thought to originate from these muscle precursor cells, they are expected to be positive for PAX3. In this study, PAX3 expression was characterized in cancer cell lines and tumors, showing wide expression in RMS. Detection sensitivities by quantitative polymerase chain reaction (qPCR) of the previously proposed markers, MYOD and MYOGENIN, were similar to that of PAX3, thereby indicating the feasibility of its detection. Interestingly, the flow cytometry experiments supported the usefulness of this technique in the quantification of MRD in RMS using PAX3 as a marker. These results indicate that flow cytometry, albeit in some cases slightly less sensitive, can be considered a good approach for MRD assessment in RMS and more consistent than qPCR, especially owing to its greater specificity. Further-

Other related publications

Gallego S, Roma J, Sánchez de Toledo J. Molecular genetics of rhabdomyosarcoma. Encyclopedia of Life Sciences (ELS). John Wiley & Sons, Ltd: Chichester. (February 2012).

Soriano A, Jubierre L, Almazán-Moga A, Molist C, Roma J, de Toledo JS, Gallego S, Segura MF. microRNAs as pharmacological targets in cancer. Pharmacological Research.

Huertas-Martínez J, Rello-Varona S, Barrau I, Herrero- Martín D, García-Monclús S, Sáinz-Jaspeado M, Lagares-Tena L, Núñez-Álvarez Y, Mateo-Lozano S, Mora J, Roma J, Toran N, Moran S, López-Alemany R,Gallego S, Esteller M, Peinado MA, Garca del Muro X, Tirado OM. Caveolin-1 is down-regulated in alveolar rhabdomyosarcomas and negatively regulates tumor growth. Oncotarget. 2014 Oct 30;5(20):9744-55.