

MIFEPRISTONE INHIBITS MPA- AND FGF2-INDUCED MAMMARY TUMOR GROWTH BUT NOT FGF2-INDUCED MAMMARY HYPERPLASIA

JUAN P. CERLIANI¹, SEBASTIAN GIULIANELLI¹, ANA SAHORES¹, VICTORIA WARGON, ADRIAN GONGORA¹, ALBERTO BALDI¹, ALFREDO MOLINOLO², CAROLINE A. LAMB¹, CLAUDIA LANARI¹

¹Laboratorio de Carcinogénesis Hormonal, Instituto de Biología y Medicina Experimental (IBYME)- Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina,

²Oral and Pharyngeal Cancer Branch, NIDCR, NIH, Bethesda

Abstract We have previously demonstrated a crosstalk between fibroblast growth factor 2 (FGF2) and progestins inducing experimental breast cancer growth. The aim of the present study was to compare the effects of FGF2 and of medroxyprogesterone acetate (MPA) on the mouse mammary glands and to investigate whether the antiprogestin RU486 was able to reverse the MPA- or FGF2-induced effects on both, mammary gland and tumor growth. We demonstrate that FGF2 administered locally induced an intraductal hyperplasia that was not reverted by RU486, suggesting that FGF2-induced effects are progesterone receptor (PR)-independent. However, MPA-induced paraductal hyperplasia was reverted by RU486 and a partial agonistic effect was observed in RU486-treated glands. Using C4-HD tumors which only grow in the presence of MPA, we showed that FGF2 administered intratumorally was able to stimulate tumor growth as MPA. The histology of FGF2-treated tumors showed different degrees of gland differentiation. RU486 inhibited both, MPA or FGF2 induced tumor growth. However, only complete regression was observed in MPA-treated tumors. Our results support the hypothesis that stromal FGF2 activates PR inducing hormone independent tumor growth.

Key words: breast cancer, mammary gland, mammary carcinomas, FGF2, progestins, RU486

Resumen *La mifepristona inhibe el crecimiento de carcinomas mamarios inducidos por MPA o por FGF2 pero no las hiperplasias mamarias inducidas por FGF2.* Hemos demostrado previamente

que la vía de señalización del factor de crecimiento fibroblástico 2 (FGF2) interactúa con la vía de los receptores de progesterona (RP) induciendo el crecimiento del cáncer de mama experimental, y hemos postulado que el FGF2 estromal activaría los RP en los tumores hormono independientes. El objetivo de este trabajo es comparar los efectos del FGF2 y del acetato de medroxiprogesterona (MPA) en la glándula mamaria de ratón e investigar si el antiprogestágeno RU486 induce la regresión del tumor hormono dependiente C4-HD que crece con MPA o con la administración intratumoral de FGF2. Demostramos que la administración diaria local de FGF2 induce una hiperplasia intraductal mamaria que no es revertida por el tratamiento con RU486. Por otra parte, el RU486 revierte la hiperplasia paraductal inducida por MPA y sólo induce un efecto agonista parcial. Estos datos sugieren que el efecto del FGF2 en la glándula mamaria es RP independiente. Demostramos que el tumor C4-HD crece *in vivo* con la administración intratumoral de FGF2. En este caso, la histología revela un mayor grado de diferenciación, similar al observado en el tumor C4-HI que crece sin el aporte exógeno de hormonas. El RU-486 inhibió tanto la estimulación inducida por MPA como por FGF2. Los resultados apoyan la hipótesis de que el FGF2 estromal activa al RP induciendo el crecimiento hormono independiente de tumores mamarios.

Palabras clave: cáncer de mama, glándula mamaria, carcinomas mamarios, FGF2, progestágenos, RU486

Most breast cancers express estrogen receptors alpha (ER α) and progesterone receptors (PR) at the time of diagnosis and are thus, susceptible to an endocrine treatment, that currently aims at either blocking the ER or to significantly decrease the levels of circulating estrogens¹. However, there is increasing evidence indicating that

progesterone receptors (PR) are also involved in breast cancer growth², suggesting that PRs may also be valid targets for breast cancer treatment.

Even though these steroid hormone pathways require the presence of the hormone to activate their cognate receptors, neoplastic deregulation of other growth signals may contribute to the aberrant activation of the steroid receptors in the absence of such ligand. This mechanism might explain why hormone-dependent cells expressing steroid receptors start to grow in the absence of hormones³. Our results indicate that one such pathway may be that of Fibroblast growth factor 2 (FGF2), a survival fac-

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Postal address: Dra. Claudia Lanari, IBYME, Vuelta de Obligado 2490, 1428 Buenos Aires, Argentina
Fax: (54-11) 4786-2564 e-mail: clanari@dna.uba.ar

tor involved in neoangiogenesis that induces mitogenic and chemotactic responses in different cell types (reviewed in⁴). Its activities and those of other FGFs are mediated by their binding to a family of four receptor tyrosin kinases (RTK) designated FGF receptors (FGFRs) 1-4, which have an extracellular portion containing three immunoglobulin-like domains and intracellular tyrosin kinase domains. Heparin and heparin sulfate proteoglycans play a critical role facilitating FGF binding to FGFR (reviewed in⁴).

Even though the c-erbB family and steroid receptor signaling remain the most studied proliferation pathways in breast cancer, there is compelling evidence suggesting that FGFRs are also among the key regulators of breast cancer growth⁵. In this regard, a single nucleotide polymorphism (SNP) in FGFR-2 was recently related to increased ER+ breast cancer risk^{6, 7}.

Using a hormonal carcinogenesis murine breast cancer model⁸, we have shown that mifepristone (a progestin antagonist, RU486) inhibited the growth of mammary carcinomas that express high levels of ER and PR isoform A and are able to grow without exogenous hormone supply. We proposed that these tumors (C4-HI), recruit carcinoma associated fibroblasts that provide growth factors such as FGF2 that, by binding to their cognate receptors, are able in turn, to activate PR regardless of the presence of the natural hormone. As a proof of principle, we investigated whether FGF2 was able to stimulate the growth of a hormone dependent tumor, C4-HD, in the absence of hormones, and to evaluate if the FGFR inhibitor PD 173074 decreased the growth of the hormone independent variant, C4-HI. Interestingly, FGF2 stimulated C4-HD, and PD 173074 decreased C4-HI tumor growth⁹.

The aim of the present study was to compare the effects of FGF2 and MPA on adult mouse mammary glands and to investigate whether the antiprogestin was able to reverse the FGF2-induced effects on the mammary gland and on C4-HD tumor growth.

Two-month-old BALB/c or NOD/SCID female virgin mice were used in the experiments. The animals were fed *ad libitum* and kept in air-conditioned rooms at 20 ± 2 °C with a 12 h light-dark period. Animal care and manipulation was in agreement with institutional guidelines, which are in accordance with the Guide for the Care and Use of Laboratory Animals. Two experiments were performed using 3 mice in each group. To study the mammary glands, BALB/c mice were treated for one week either with 0.1 ml of medroxyprogesterone acetate (MPA) depot (20 mg; Medrosterona, Laboratorio Craveri, Buenos Aires) in the contralateral flank, daily injections in the 4th mammary gland of 5 µg FGF2 or saline. All groups were simultaneously treated subcutaneously (s.c.) with vehicle or RU486 (12 mg/kg body weight; Sigma-Aldrich, St Louis, MO) in the back. FGF2 was synthesized as described previously⁹. Animals were euthanized and the 4th mammary glands were excised, fixed in buffered formalin and embedded

in paraffin. Vehicle-treated glands showed duct structures surrounded by adipocytes (Fig. 1A). MPA induced branching or paraductal hyperplasia as previously published¹⁰ (Fig. 1C). RU486 inhibited most of the MPA-induced effects (Fig. 1D), although it had a partial growth agonist effect when administered alone (Fig. 1B). FGF2-treated mammary glands showed solid intraductal hyperplasia without branching, associated with a dense inflammatory infiltrate composed of poly and mononuclear cells in the surrounding adipose tissue (Fig. 1E). The combined treatment of FGF2 together with RU486 did not inhibit the intraductal hyperplasias or diminished the inflammatory response (Fig. 1F). The fact that RU486 inhibited only the effects induced by MPA suggests that the intraductal hyperplasia induced by FGF2 is PR independent.

Next, we evaluated the effect of RU486 on MPA- or FGF2-induced tumor growth. Although we had already demonstrated that RU486 induces tumor regression in hormone-independent tumors of our model⁸, we had not

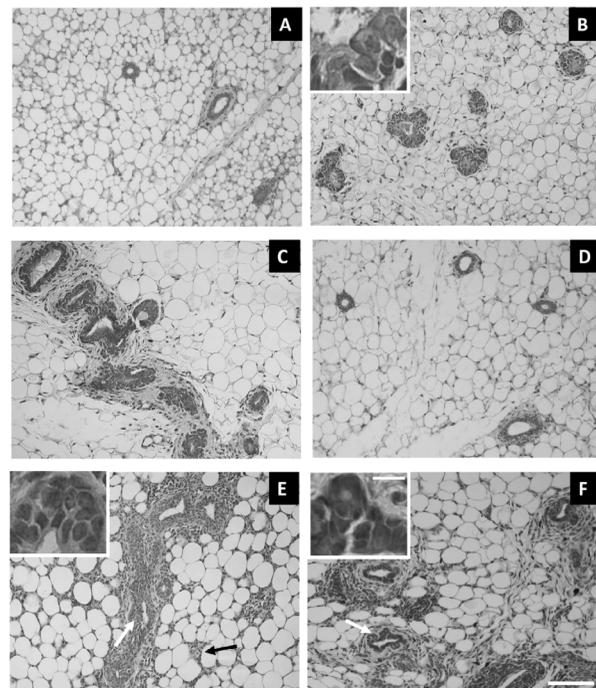


Fig. 1.— Mammary glands (4th) from virgin BALB/c mice treated for one week with saline (A), RU486 (B), MPA (C), MPA + RU486 (D), FGF2 (E) or FGF2 + RU486. MPA induced branching, RU486 inhibited MPA-induced effects but when administered alone induced a slight progestin agonistic effect. FGF2 induced an intraductal hyperplasia. The white arrow shows the multilayered ducts and the black arrow shows the inflammatory cells intermingled with the adipocytes. In FGF2-treated glands, RU486 induced glandular differentiation (white arrow) within the multilayered ducts and did not reduce the inflammatory response; bar 20X: 100 µm, bar 40X: 10 µm.

yet tested whether RU486 reverted MPA-induced tumor growth *in vivo*. As shown in Fig. 2A⁸, C4-HD only grows in MPA-treated mice and RU486 induced complete tumor regression in spite of the presence of MPA. To evaluate the effect of FGF2 the experiments were carried out in BALB/c and in NOD/SCID mice to rule out possible effects of FGF2 acting as an immunogen. Animals were transplanted s.c. with C4-HD tumors, and treated with MPA (20 mg pellets) as described previously⁸. When tumors reached a size of about 25 mm², the pellets were removed and the mice were administered with intratumor injections of FGF2, saline, or they were re inoculated with MPA. After one week, half of the FGF2-treated mice were simultaneously treated with daily doses of RU486 as described above for another week. Tumors in untreated mice regressed almost completely. As reported previously⁹, tumors treated only with FGF2 continued growing, while a significant decrease in growth was observed with the combined treatment with RU486 (Fig. 2B; $p < 0.001$ Student *t* test comparing the tumor sizes at the end of treatment). MPA- and FGF2-treated tumors showed a similar mitotic [MPA: 3.5 ± 0.27 ; FGF2: 2.96 ± 0.48 mitotic figures/high power field ($x \pm SE$)] and apoptotic index [MPA: 1.26 ± 0.21 ; FGF2: 1.231 ± 0.26 apoptotic figures/high power field ($x \pm SE$)]. A significant decrease in mitosis ($p < 0.01$) and an increase in apoptosis ($p < 0.01$) was observed in (FGF2 + RU486)-treated mice compared to FGF2-treated tumors. The outcome was similar in either BALB/c or NOD/SCID mice. These results confirm the existence of a cross talk between the FGF2 and PR signaling pathways.

Tumors growing in the presence of MPA are composed of nests of epithelial cells with low degree of gland differentiation surrounded by stromal cells (Fig. 2C, left). FGF2-treated, as well as (FGF2 + RU486)-treated tumors showed glandular differentiation (Fig. 2C, middle and right respectively). A high reactive stroma was observed in the latter.

The postnatal development of the mammary gland involves a series of sequential changes under the control of several steroid hormones and signal transduction pathways, among them the EGF and IGF family members¹¹. Less information is available regarding the role of FGFs, although it has been suggested that they may act activating FGFR of the epithelial cells¹². In this study we show that FGF2 induces ductal cell proliferation, in the normal mammary gland, whereas MPA actions are associated with branching and paraductal hyperplasia, a type of abortive branching of the ductal structures, suggesting that both effects are regulated by different mechanisms. The hormone effects associated with changes in the organ histoarchitecture, such as those induced by MPA, are probably complex phenomena involving the orchestration of many growth factor pathways. The exogenous administration of FGF2 induced a pro inflammatory milieu that may be participating in the induction of the intraductal hyperplasia. The antiglyucocorticoid effects described for

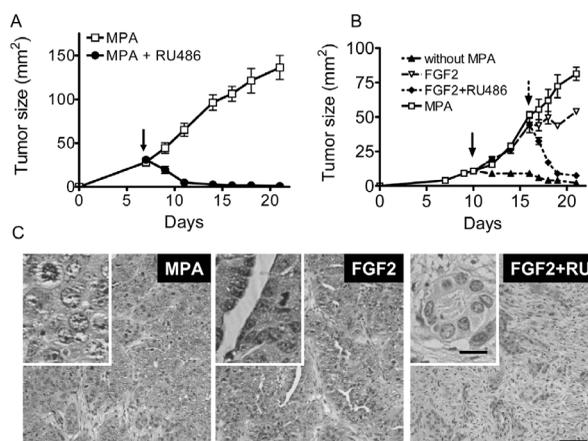


Fig. 2.— Effects of RU486 on MPA- or FGF2-induced C4-HD tumor growth. A: Animals were treated with MPA depot sc in the contralateral flank of tumor inoculum the same day of tumor transplantation. RU486 treatment started when tumors reached a size of 25 mm². Tumors regressed completely after one week of treatment. B: Mice were treated with 20 mg pellets of MPA to allow tumor growth. When tumors reached a size of 25-50 mm², MPA pellets were removed and tumors were treated with intratumor daily injections of FGF2, saline or re inoculated with MPA. After confirming that FGF2 stimulated tumor growth, half of the animals received RU-486 treatment for one week. A significant decrease in tumor size was observed with RU-486 treatment ($p < 0.001$, *t* test). C. H&E images of representative C4-HD tumors growing in MPA-treated, FGF2- treated or (FGF2 + RU486)-treated mice. Differentiated structures were observed in FGF2-treated tumors as well as vessels of different calipers. Differentiated glands and small nests of epithelial cells intermingled in a reactive stroma were observed in RU-486 plus FGF2-treated tumors. Insets show details of mitotic figures (left), differentiated structures (middle and right: FGF2 or FGF2+RU486-treated tumors). No tumor growth was observed in untreated animals; Bar: 70 μ m; Bar inset: 10 μ m.

RU486¹³ might be responsible for maintaining the inflammatory environment in the mammary glands treated with FGF2 combined with RU486. An interesting finding was that RU-486-treatment had a slight agonistic effect on the mammary glands. As it was suggested that antiprogesterins prevent BRCA1 induced hyperplasia¹⁴, these findings were unexpected.

In tumors, both FGF2 and MPA induced tumor growth. As shown in our recent array study¹⁵, FGF2 is upregulated in MPA-treated tumors, suggesting that FGF2 may be a mediator of the hormone effect. In this case, no inhibition of FGF2-induced tumor growth would have been expected with the antiprogesterin treatment. The data showed herein, point to either an interaction of FGF2 with the PR pathway¹⁶, or an involvement of PR in cell survival. In addition, they support the hypothesis that stromal FGF2 may be participating in hormone independent tumor growth. FGF2 activates STAT5 in our model (unpublished data) and activated STAT5 has been observed in differentiated

tumors, suggesting that the activation of STAT5 may have a pivotal role in hormone independent tumor growth.

In summary, in this study we provide evidence that the effects induced by FGF2 or MPA in the mammary glands are different, suggesting that the crosstalk observed between MPA and FGF2 in mammary carcinomas might have been acquired during the process of carcinogenesis. Our results support the idea that FGFR inhibitors and antiprogestins may be used in selected breast cancer patients.

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Conflicts of interest: The author(s) declare that they have no competing interests.

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No tu verdad: la verdad.
Y ven conmigo a buscarla.
La tuya, guárdatela.

Antonio Machado (1875-1839)