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Jrgang Cheng[1]

[1] National Cancer Institute at Frederick

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Abstract

Uterine receptivity needs to be synchronized with embryonic development, so the blastocyst stage of the embryo can implant. Leukemia Inhibitory Factor (LIF) is an essential factor for implantation, which is involved in the initiation of the window of implantation. However, the process by which the LIF signal pathway is transduced in the uterine luminal epithelium (LE) that leads to uterine receptivity is not completely elucidated. We tested the ability of cellular signaling inhibitors to disrupt uterine support of the embryo. Only Tyrphostin-AG490, an inhibitor of Jak2, can interfere with LIF signaling. Not only can AG490 reduce phosphorylated STAT3 levels in isolated LE, but it also ablated implantation when injected into uterine lumen. Furthermore, AG490 treatment in wild-type animals mimics the consequences of genetic ablation of LIF that results in free floating hatched embryos, which are unable to implant. Our results support the notion that Jak2 is the sole Janus kinase to mediate LIF activation in LE, and the signaling pathways of cytokines can serve as contraception targets.

Index terms— leukemia inhibitory factor (LIF), implantation, janus kinase 2 (Jak2), tyrphostin, AG490, DMSO, signal transduction, contraception.

I. Introduction mbryonic implantation is a complex and dynamic physiological interaction between embryo and uterine tissues 1 . Prior to implantation, the uterus shifts from a "refractory" phase to a "receptive" phase during which the embryos can attach and survive. This "window of implantation" can be characterized both hormonally and morphologically in the uterus 2 , which is primarily regulated by the ovarian steroid hormones estrogen (E2) and progesterone (P4) 3 . In rodents, a rise in E2 levels on the 4 th day of pregnancy (called nidatory estrogen) initiates the window of implantation and the onset of the receptive state 4 .

The effect of nidatory E2 is in fact mediated by LIF, as not only does E2 up-regulate LIF expression in the endometrial glands, but a single injection of LIF into hormone-primed and ovariectomized mice can replaces nidatory E2 efficiently, resulting in implantation 5 . Genetic ablation of LIF in the mice results in female infertility. Without LIF, female mice have normal mating and ovulation yet avoid both embryonic attachment and the initiation of decidulization resulting in implantation failure 6,7 .

To understand the signaling pathways employed by LIF that are necessary for uterine receptivity, different inhibitors were tested to block LIF function. Only AG490, a Jak2 kinase inhibitor, is capable of blocking the formation of implantation nodules and yielding similar phenotypes as that of LIF null females 6 . To initialize the window of implantation in mice, LIF binds to LIFR/gp130, activates Jak2, which in turn phosphorylates STAT3. These results also suggest JAK/STAT signaling pathways may serve as potential contraceptive targets.

1 II. Material and Methods

Mice. LIF-deficient mice were maintained in an existing colony. Six to eight week female mice (B6C3F1) were purchased from Charles River Laboratories. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, 1985). Surgical procedures were performed under tribromoethanol (Avertin) anesthesia according to institutional guidelines (NCI-Frederick ACUC Guidelines and Policies). All mice were naturally mated with the assumption that mating occurred around midnight, with day 1 of pregnancy being equivalent to the day with plug after mating(Day1 E Abstract- Uterine receptivity needs to be synchronized with embryonic development, so the blastocyst stage of the embryo can implant. Leukemia Inhibitory Factor (LIF) is an essential factor for implantation, which is involved in the

initiation of the window of implantation. However, the process by which the LIF signal pathway is transduced in the uterine luminal epithelium (LE) that leads to uterine receptivity is not completely elucidated. We tested the ability of cellular signaling inhibitors to disrupt Only Tyrphostin-AG490, an inhibitor of Jak2, can interfere with LIF signaling. Not only can AG490 reduce phosphorylated STAT3 levels in isolated LE, but it also ablated implantation when injected into uterine lumen. Furthermore, AG490 treatment in wild-type animals mimics the consequences of genetic ablation of LIF that results in free floating hatched embryos, which are unable to implant. Our results support the notion that Jak2 is the sole Janus kinase to mediate LIF activation in LE, and the signaling pathways of cytokines can serve as contraception targets.

LIF binds to the heterodimeric LIF receptor/ gp130 complex, which is expressed in the LE and to a lesser extent the glandular epithelium, but not in the stroma in the uterus 8 . LIF receptors recruit Janus kinase, Jak1, Jak2, Jak3 and TYK2, to initialize the signaling cascade. LIF's action in the uterus and activation of STAT3 is primarily centered on the LE, which in turn plays an obligatory role in interacting with the embryonic trophoblast in attachment and in controlling decidualization. Two major pathways, the Jak/STAT and ras/MAP kinase, have been identified as being activated by LIF binding to the LIFR/gp130 receptor complex in the uterine LE, embryonic stem cells, and neurons 9,10,11 .

uterine support of the embryo. The primary antibodies were either polyclonal antibodies to P-Ty-STAT3, STAT3 (Cell Signaling Technology) or a monoclonal antibody to STAT3 (BD -Transduction Labs). Peroxidase conjugated anti-rabbit or anti-mouse IgG antibodies were used to detect binding. Specific bands were visualized with chemiluminescence (ECL plus, Amersham) by using a DCC camera (Stratagene) and exposure to film (Kodak). Signal quantification was performed by NIH-Image (v1.62).The responsiveness of LE to LIF was determined by the ratio between tyrosine phosphorylated STAT3 and total STAT3 signal, with respective antibodies on the same protein blot.

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Inhibitors of signaling pathways. Inhibitors used to block signaling as follow: A. EGF signaling inhibitor Tyrphostin, AG 1478 (4-(3-Chlotoanilino)-6,7-dimethoxyquinazoline; (IC50= 3 nM -EGFR).B. Jak2 kinase and EGF inhibitors, AG490 (Tyrphostin B42; ?-Cyano-(3,4dihydroxy)-N-Benzylcinnamide; (IC 50 =100 nM -EGFR; 10µM -Jak2)), and AG43 (Tyrphostin A64; ?-Cyano-(4hydroxy) dihyfrocinnamonitrile; as a negative control). C.Inhibitor of MEK1/2 U0126 (1,4-Diamino-2, 3-dicyano-1,4-bis(2-aminophenylthio) butadiene (IC 50 = ~65 nM)). MEK inhibitor PD98059 (2'-Amino-3'-methoxyflavone (IC 50 = 2 µM-MEK1)), and as a negative control U0124 (1,4-Diamino-2,3-dicyano-1,4-bis (methylthio)butadiene; negative control). D.Inhibitor of p38 kinase, SB 203580 (4-(4-Flurophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1H-imidazole (IC50=~0.4 µM-p38 MAPK; 4 µM-PKB) and as a negative control SB202474 (4-Ethyl-2-(pethoxyphenyl)-5-(4'-pyridyl)-1H-imidazole). All reagents were from Calbiochem or Sigma. Reagents were dissolved in DMSO at a concentration of 20mg/mL shortly before injection. With M.W. around 300 ~400 per mole, the molarity of each chemicals is between 50 ~67 mM.

Uterine injection. Mice were anaesthetized with 0.45mL of 1.2% Avertin. A surgical incision was made through the midline of the back, between the two ovaries with the mouse lying ventrally, and the right uterine horn was pulled from the peritoneal cavity via the fat-pad attached to the ovary. A solution of 20µL (0.4 mg) was injected into the uterine horn by either mouth pipette or capillary syringe near the oviduct on the morning of Day3 pc (or the time indicated). To reduce any "stress" the injection might cause, a limited amount of solution and only one uterine horn was injected; a procedure similar to embryo transfer. Even with the injection of 1 horn, solutions can have effect on the other horn by diffusion or circulation. After three days (or Day6 pc), the mouse was sacrificed and its uterus examined. The ovary from the unmanipulated uterine horn was removed to mark the injected side, and the uterus was isolated and stuck onto to a strip of 3mm filter paper (Whatman) to prevent it from contracting and curling. The straightened uterus was then measured with a ruler to determine the implantation sites distances from the cervix. If a uterus showed no signs of implantation, flushing was performed to confirm the presence or absence of hatched blastocysts. When blastocysts are present, animals were marked as blocking. Without a viable embryo mice are considered non-pregnant.

3 III. Results

4 a) Interfering Implantation

To address which signal transduction pathway (Jak/STAT or MAP kinase) is necessary for LIF function, selected blocking chemicals were injected into the mouse uterus and then verified as lacking of implantation nodules, which is a sure sign of implantation failure. These blockers fall into one of the four different signal pathway categories: JAK2, EGF, MAP kinase (Mek1/2), and p38 MAP kinase. All chemicals are prepared in the same manner, with 20 mg/ml in DMSO and 20µl solution was applied (0.5 mg/ per animal). Based on the peak of LIF mRNA expression around Day4 pc (Shade area), injection was performed on Day3 (Figure 1A). For an easy and unambiguous way to determine whether embryos have implanted or not, Day6 uteri were examined instead of using Skyblue to mark sites of early decidualization. Uteri showing no sign of implantation were double-checked with flushing to verify the existence of embryos and rule out those animals without an embryo. Unmanipulated horns served as controls.

During the pilot experiment, it was noticed that the injected horn was more prone to be devoid of implantation than the control side without injection. As the common denominator is the physical injection and AG490 inhibition of LIF induced STAT3 phosphorylation. To confirm the inhibitory effect of AG490 on the Jak2/STAT3 pathway, levels of tyrosine phosphorylation of STAT3 were monitored. LE from late Day3 p.c. mice was purified and incubated with the indicated concentration of AG490 overnight at 37 0 C in serum-free Opti-MEM (Gibco-BRL/Invitrogen) 12 . LIF (100ng/mL, Chemicon) was then added to activate the Jak2/STAT3 pathway with or without AG490 treatment for 30 min. LE was also purified from LIF null females, due to its lower p-Ty-STAT3 background, was treated with 1mM AG490 for 3 hours before LIF treatment. Treated LEs were collected by centrifugation, solubilized in SDS PAGE protein lysis buffer, with trituration using a 1 ml syringe with a 27-gauge needle. Protein extracts were collected, aliquoted and stored at -80 0 C. Control samples were handled in parallel with those of the treated group. Duplicated samples were prepared, run on the gel, and proteins transferred to a PVDF membrane for immunoblotting. Protein blotting was performed using standard procedures. the use of DMSO as the solvent, it was decided to check whether the injection itself or the solvent played any role in blocking embryo implantation. The same volume of 20 µl common solvents, DMSO, DMF, Isopropanol and ethanol, was injected into one of the uterine horns of Day3 pc animals, and three days later, both horns were examined. All injected solvents had no effect on the unmanipulated (non-injected) horn (Figure 1B, shaded table), meaning that all the animals listed were pregnant and implantations occurred on that side. Thus, the results of various treatments are centered on the injected uterine horn. DMSO completely nullified the sign of any implantation nodule in the injected horn. When the concentration of DMSO was diluted with PBS, its effect was reduced as well. DMF (dimethyforamide) exhibited no effect on implantation, thus the punch wound generated by the injection itself had no apparent effect. Injection with ethanol and isopropanol also did not block the formation of implantation nodules. However, the implantation nodules of the injected horn were reduced in size when compared with the unmanipulated side in the same animal, indicating a reduction or delaying decidualization response. among chosen inhibitors, Jak2 inhibition by AG490 required higher concentration (10 µM). For the above reasons, the blocking chemicals were dissolved in DMSO, injected into one horn, and the unmanipulated horn of the same animal was subsequently examined for either pregnancy or implantation failure. The injected horn was used as a successful injection control, in that a sufficient volume of chemicals was delivered.

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The numbers of mice with various outcomes after being injected with different chemicals on the Day3 pc are shown (Figure 2A). Of the chemicals injected into the uterus, only tyrphostin AG490 achieved a blocking rate of nearly 50%. Both Mek1/2 blockers (U0126 and PB98059) showed no effects. Particularly, U0124, a negative control for U0126, showed a rare blocking effect as well as aberrant uterine morphology. Despite the small sample size (3), SB233580, a p38 MAP kinase blocker, also had no effect on forming implantation nodules. Tyrphostin AG490 inhibits both Jak2 (IC 50 = 10 µM; also Jak3, which is not expressed in LE) and EGF pathways (IC 50 =100 nM). However, another tyrphostin AG1478 (IC 50 =3 nM), which has a higher affinity and better specificity than AG490 with regard to the inhibition of EGF pathways, showed no effects on blocking implantation.

Interestingly, some unmanipulated uteri had implantation nodules close to the oviduct, which is always spaced out evenly along a whole horn, indicating the effect of the chemicals declines along the uterine horn away from the injection site. In addition, a single intraperitoneal injection with DMSO/AG490 showed no similar effect. The mouse uterus is a tube-like structure and solution can diffuse to another horn more easily than thoughed circulation. Thus, the effectiveness of a specific chemical can be exhibited as the "range of action" with the injection site as the point of origin. Subtracting the effect of DMSO, the effective distance (E.D.) of chemicals can then be defined as the distance between the cervix (where DMSO lost its efficacy) to the first implantation site (designated as the center of the implantation nodule) divided by the length of the uterine horn (Fig. 2B). With such assumptions, and assigning a complete blockage of implantation with the score of 1, quantitative measurements for each chemical's effectiveness can be computed. The measurement results elucidate that AG490 not only yield better than 50% of complete blocking, but also showed longer range of efficiency than that of other chemicals (Fig. 2C). The wide range of E.D. from individual animals after treatments prohibits drawing a conclusive result with any other specific chemical. There is also no change of appearance or size of implantation nodules. Nevertheless, as the data indicates, the Jak2 pathway is necessary for the continuation of pregnancy. The general morphology of an AG490 treated uterus deprived of embryonic implantation shows a Day3-like appearance without any signs of edema (Fig. 2B). When performing uterine flushing at Day6 p.c., hatched embryos can be collected similar to those from LIF null females 6 (Fig. 2D).

6 b) AG490 inhibits LIF Activation of STAT3

As chemicals delivered outside of the uterus showed neither DMSO nor AG490 effect, injection into the lumen of uterus is necessary for their actions. To confirm whether DMSO or AG490 can block the activation of LIF signaling pathways in the luminal epithelium, the purified LE was pre-treated with AG490, and then with LIF (100 ng/ml) for 30 min. Activation of p-STAT3 is normalized by the total STAT3 signals in the immunoblot (Fig. 3). Without AG490 (but with DMSO) pretreatment, LIF can increase the ratio between p-STAT3 / STAT3 around 3.7 fold, which is consistent with previous findings of using LE 8 . It also indicated that DMSO didn't

seem to have any effect on LIF activation. With pretreatment of 0.1 mM AG490, the LIF effect was more than 50% reduced to 1.6 fold. With 1 mM AG490 pretreatment, the LIF activation of STAT3 was blocked completely in both WT and LIF null animals after incubation with AG490. Surprisingly, the basal level of p-STAT3 was reduced dramatically after AG490 treatments. Since AG490 can block pregnancy completely with only 50% efficiency as demonstrated in the previous injections (Figure 2), the subsequent question was whether or not injection of AG490 at different points in time during early pregnancy could alter its efficacy. Using similar approaches, AG490 was injected with two different concentrations into uterine horns at three different time points: the morning of Day2, the morning of Day3, and the morning of Day4. The results were summarized in Figure 4A. There are two major conclusions that can be drawn. First, injection on Day2 has better efficacy of stop pregnancy than injection on Day3, and there is no blocking effect when injection was done on Day4. Second, similar to the effect of AG490, which diminished abruptly on Day4, the effect of DMSO on the injected horn also disappeared on Day4. In fact, not only does Day4 injection of chemicals have no effect on inhibiting the formation of implantation nodules in the unmanipulated horns, but also only high concentrations of AG490 in DMSO can inhibit implantation in the injected horns, indicating a synergistic effect of both components. Using the values of Average Effective Distance (Fig. 2B), the change in AG490 effects on different days of injection can be more appreciated (Fig. 4B). The effects of DMSO are limited within injected horn. There is also a dramatic decrease in efficacy of low doses of AG490 (20 µl of 2mg/ml) from Day2 to Day3 and again from Day3 to Day4. At high dosages of AG490 (20 µl of 20 mg/ml), the change from Day2 to Day3 is not very significant. However, there is a dramatic reduction of efficacy when compared to AG490 effect on Day3 with Day4.

7 Dose and Time Effects on AG490 Administration c)

Volume XVI Issue II Version I utilizes Jak2, and activates STAT3 to initiate the uterine receptivity.

When evaluating the requirements of signal pathways in embryo implantation with specific blockers, no blocking effects are observed with MAP kinase P44/42, MAPK p38 and the EGFR. Since this experiment was designed to interfere with the function of LE during uterine preparation with specific timing and action sites (in lumen), it cannot be ruled out that the requirements of those pathways in earlier (proliferation) or later (LE apoptosis or decidualization) stages of implantation in LE are additionally contributory. Indeed, some aberrations have been observed after treatments, such as a sausage-like swelling that showed no spacing between implantation sites (DMF (1); U0124 (1); AG490 Day4-20 (2) animals). The implantation nodules also varied in size within the same horn (U0124 (3); PB98059 (1) animal). Furthermore, there was an implantation nodule-like swelling located in the cervix (U0126 (1) animal) (diagram shown in Fig. 2B). All these interesting observations indicate that those chemicals might interfere with different aspects of uterine-embryo interaction, such as implantation sites spacing, the progress of decidualization or embryo viabilities. However, as there is no consistent correlation between phenotype and a specific chemical but AG490, no further assay were employed to understand the mechanism of those abnormalities.

When injected earlier, even the lower concentrations of AG490 showed implantation blocking (2 mg/ml in Fig. 4), which is prior to naditory estrogen, likely indicating that AG490 has different yet unknown targets during Day2, there is an early requirement of Jak2, the effect of AG490 last, or has better efficacy before signal was activated. Based on the Day4 injection result that AG490 has no effect in blocking implantation, which is supposed to take place on Day4 evening, this finding supports that once LIF pathway is activated, it could not be reversed. Alternatively, a recent study linked Jak2 with Angiotension II-induced smooth muscle construct, thus changing blood pressure **??**7 . The uterus does experience edema and becomes rich in blood circulation prior to the implantation. However, the direct correlation between the blood flow and the implantation is not well established. It would be of interest to elucidate the unknown target of AG490 or Jak2 activator(s).

While using chemical blockers to dissect the essential signaling pathways for implantation, a surprising finding was that DMSO exhibited a reproducible effect in the inhibition of implantation despite a limited effective range. Time course studies indicated a narrower effective period than that of AG490. The gross feature of the uterine horn with injection is similar to that of Day2/3 pc uteri. However, unlike uterine horns treated with AG490, uterine flushing yielded zero or rarely hatched embryos. It is possible that DMSO is Discussion IV.

Our results suggest that Jak2 has a unique and essential role in LIF signaling pathways during implantation, despite the fact that Jak1, and to a lesser extent Trk2, are also expressed in the LE (unpublished results) 13,14 . It is surprising that blocking Jak2 with AG490 not only blocks STAT3 activation by LIF but also lowers the p-STAT3 basal levels. This indicates that not only Jak2 is the sole signal mediator of LIF in activating STAT3 but also suggests the presence of a strong counter-effect, likely from tyrosine phosphatases, against Jak2 by de-phosphorylating STAT3 in LE. A prior study has demonstrated that the nucleus translocation of STAT3 is associated with LIF null phenotype in the uterus 9 . When endogenous gp130 was replaced with mutated gp130 containing c-terminal truncation that had lost the STAT3 docking site, the homozygote female showed identical implantation deficiencies as that of a LIF null 15 . Using STAT3 membrane permeable oligo to sequester STAT3 binding in the uterus lumen may also lower implantation rates **??**6 toxic to the embryo, but its effect was attenuated along the uterus with dilution from the uterine fluid or infused into uterine tissue as implantation can occurred in unmanipulated horn **??**8 . However on Day3 embryos still resided in the oviduct, so the DMSO did not have direct contact with the embryo. In addition, such explanation contradicts the observation that the blastocytes were spared, since similar to AG490, the effect of DMSO was completely gone on Day4 p.c. If

DMSO is toxic to the embryo, it is likely before the forming of blastocysts. It is also possible that the effect of DMSO in reducing inflammation may also be a reason for blocking implantation, as the implantation process mimics an inflammation response ??9 . However, the exact mechanism of interfering with either inflammation or implantation by DMSO is still unknown.

Jak2 is a prominent cancer target for leukemia treatment. Consequently, new generations of Jak2 inhibitors with better specificity and efficiency than AG490 will likely become readily available 20 . Although the effect of AG490 blocking implantation was performed with the mouse, it may have general application for contraception in other animals. The surge of LIF around the implantation period has been seen in many other mammalian species, including humans 9, ??1 . Thus, LIF signaling components can serve as good targets to block or enhance uterine receptivity for embryo implantation. Compared with inhibitory peptide and antibody blocking approaches ??7, ??2 , small chemicals can also provide advantages of both affordability and efficacy. In addition, both AG490 and DMSO treatments are reversible, as mice that went through the experiment without being sacrificed can have a normal pregnancy. With low toxicity (Acute oral toxicity (LD 50)= 14500mg/ kg) and acute dermal toxicity (LD50)= 40000 mg/kg (Calbiochem Safety Data sheet)), inexpensive cost, and a concentrated point of action (uterine lumen), DMSO, in conjunction with Jak2 inhibitors, which increase specificity and enhance range of action, could be a better alternative to hormone agonists and antagonists in achieving an effective and safe contraception.

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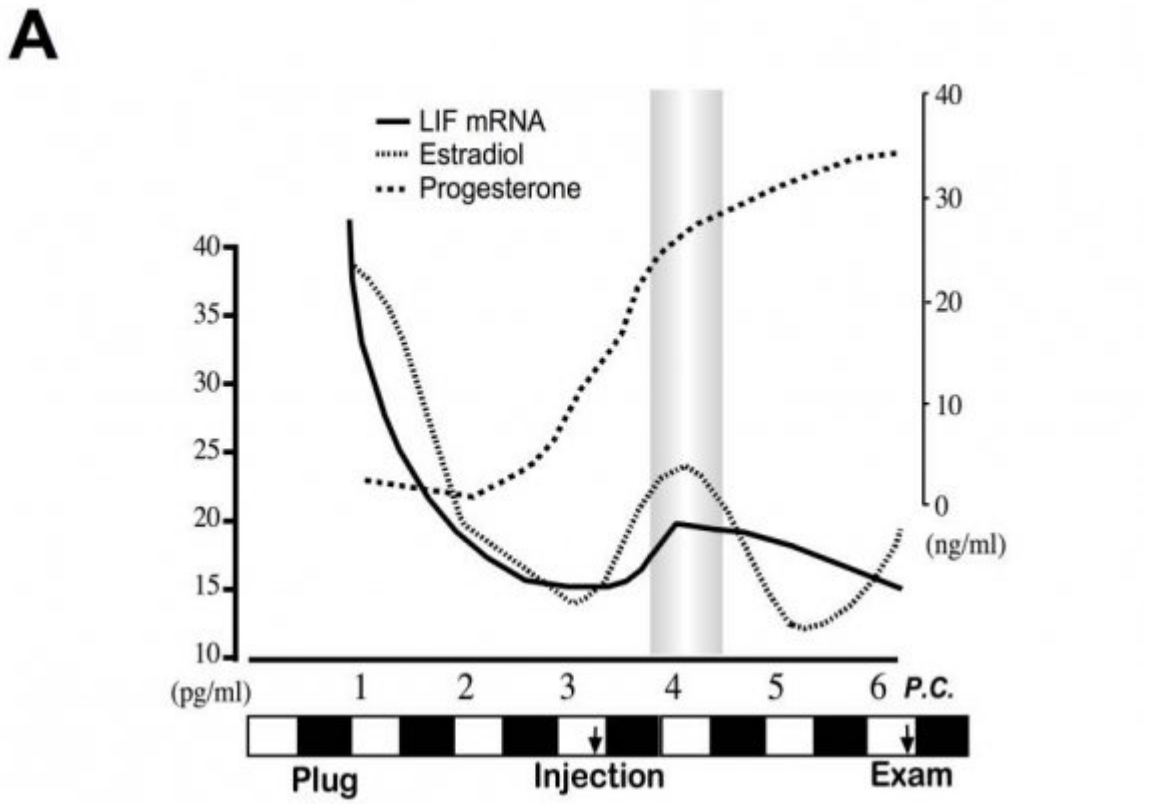
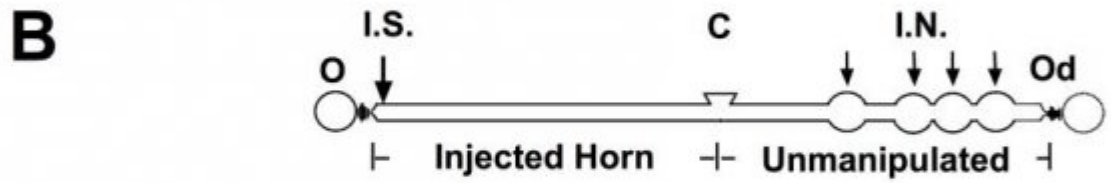
Figure 1:

[1]

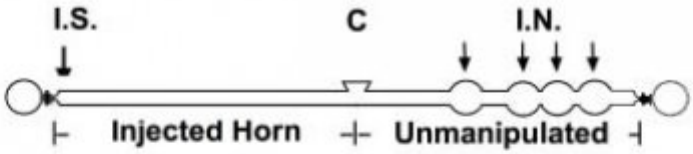




	Implanted	Blocked	Implanted	Blocked
DMSO:PBS (2:3)	1	1	2	0
DMSO:PBS (1:1)	2	2	4	0
DMSO	0	6	6	0
DMF	2	0	2	0
Isopropanol	2*	0	2	0
Ethanol	2*	0	2	0

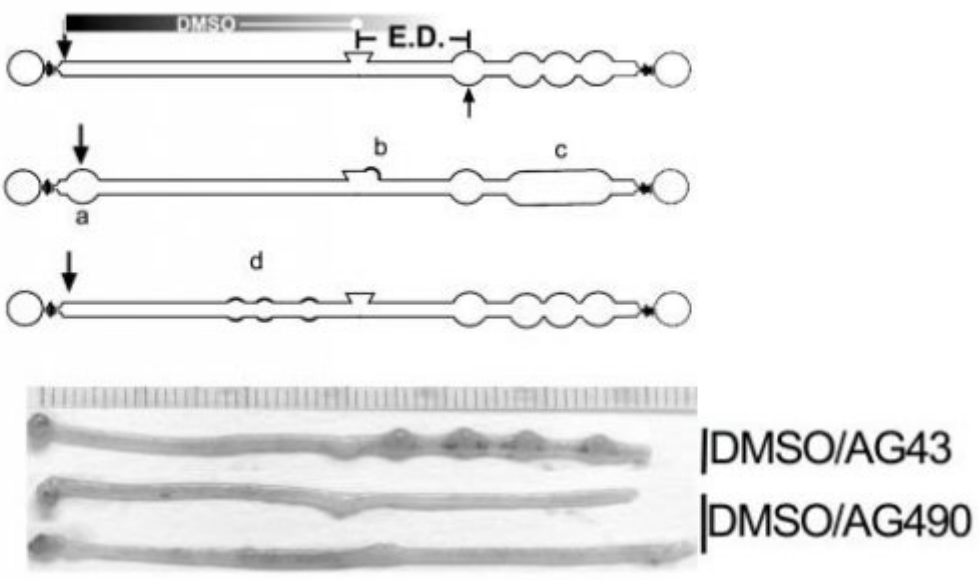
Figure 2:

A



	Implanted	Blocked	Implanted	Blocked	No Embryo
DMSO	0	7	6	0	1
AG43	0	20	19	0	1
AG490	0	23	9	10	4
AG1478	0	15	10	0	5
U0126	0	14	13	0	0
U0124	0	10	8	1	2
PB98059	0	5	3	0	2
SB233580	0	4	2	0	2
SB202474	0	5	1	0	4

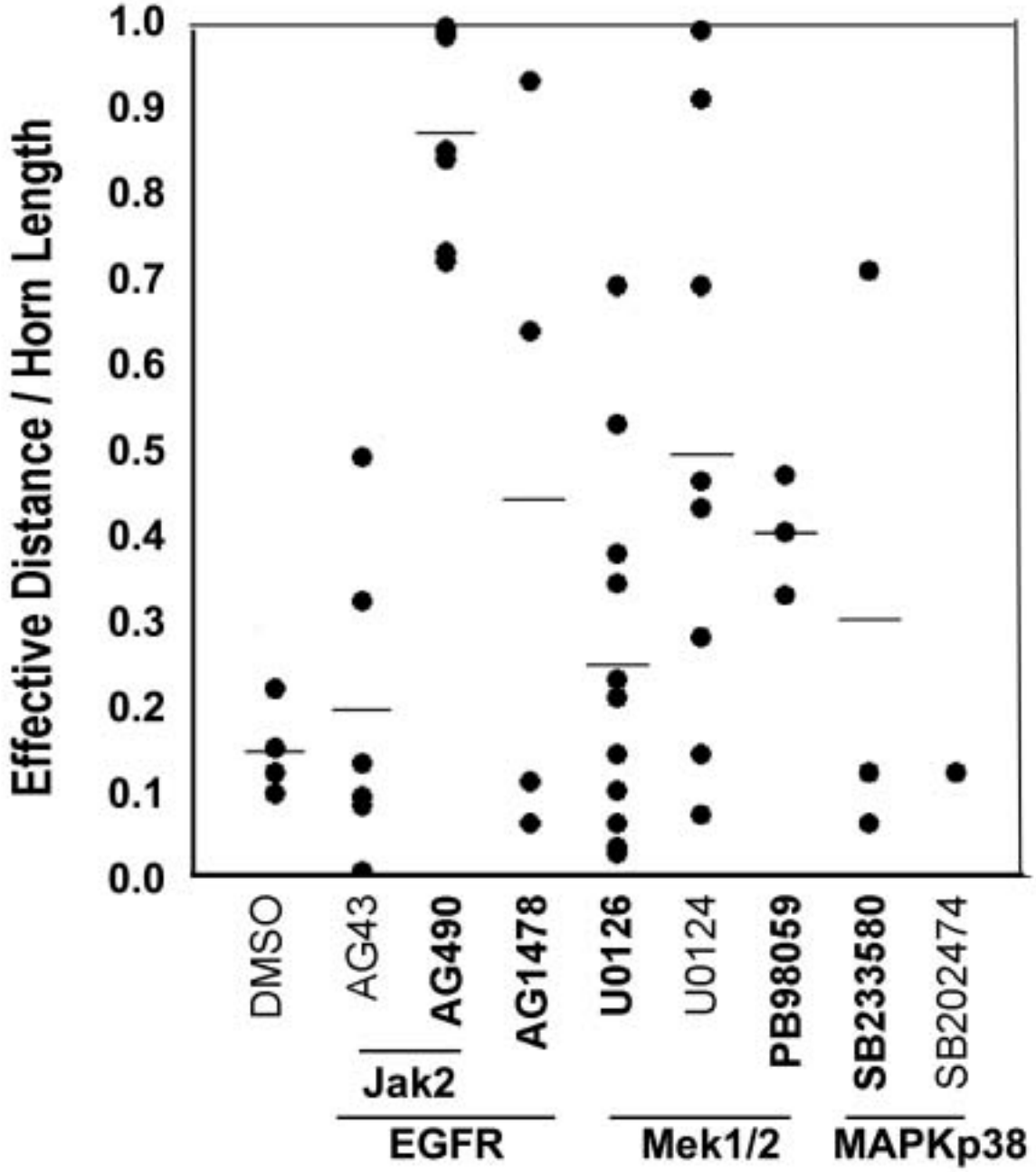
B



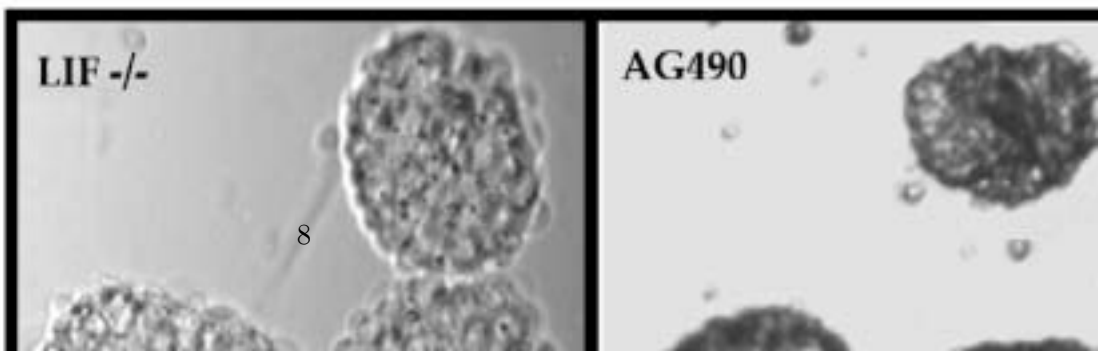
1

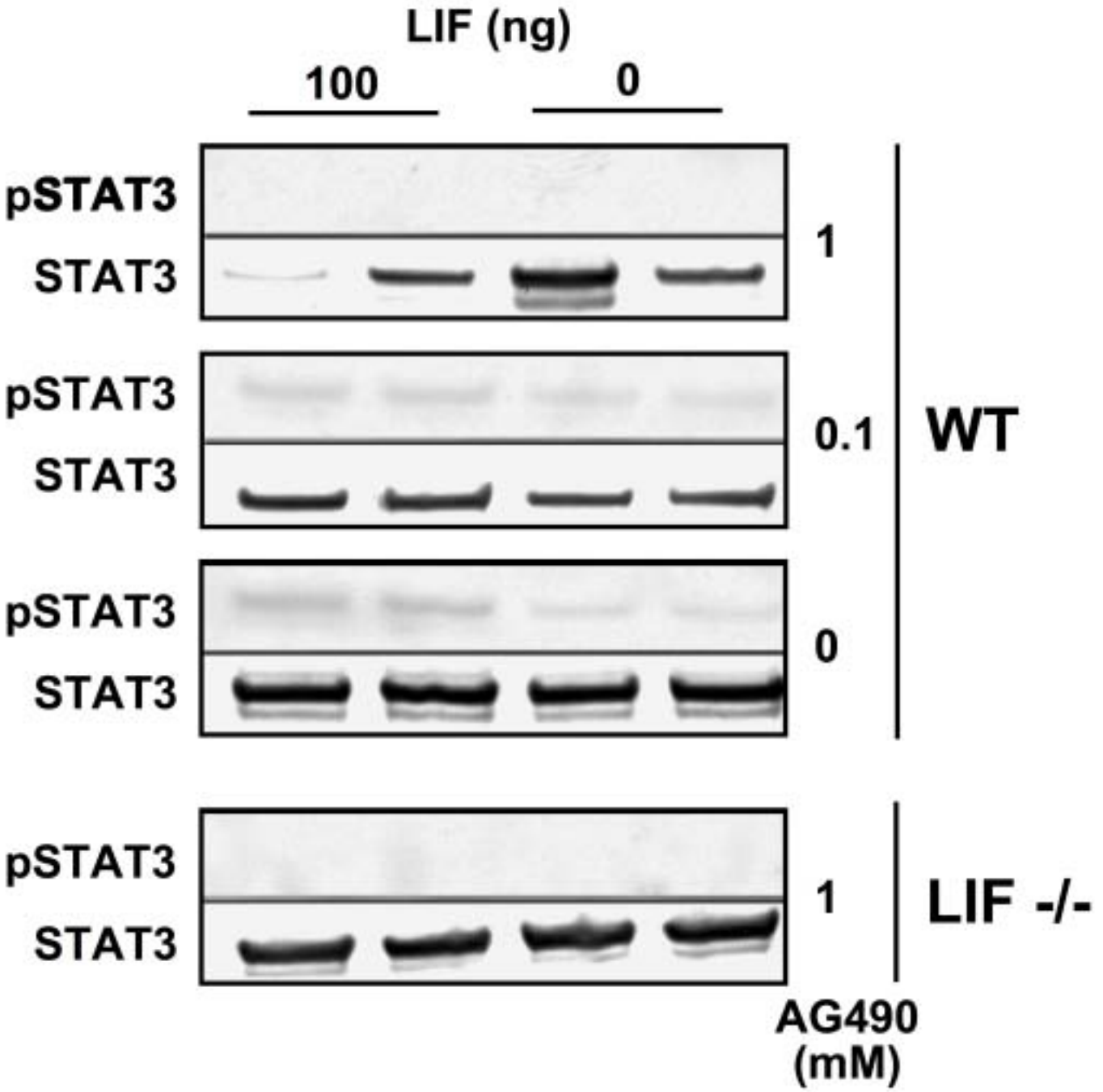
Figure 3: Figure 1 :

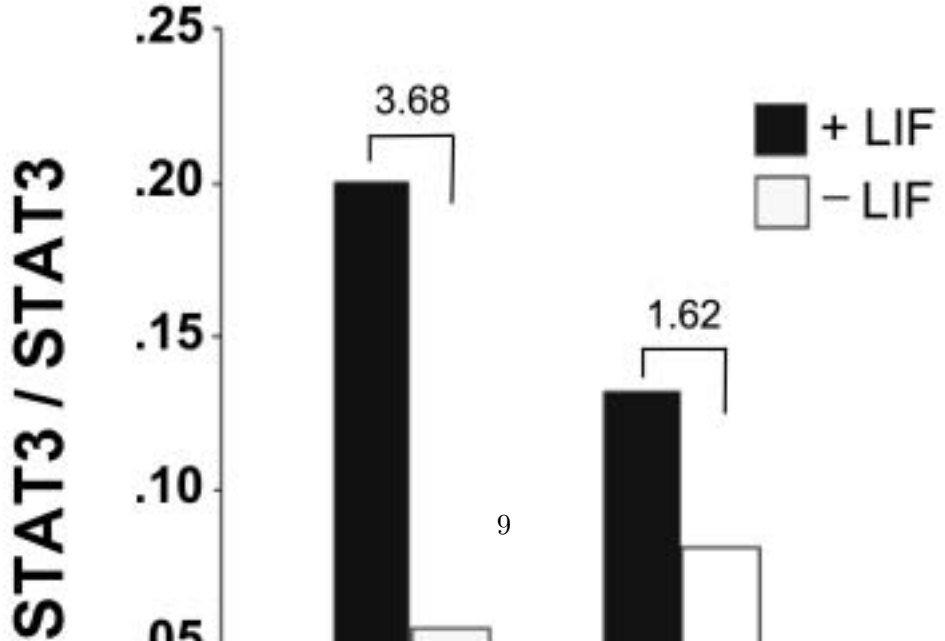
C


Effective Distance / Horn Length
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
DMSO
AG43
AG490
AG1478
U0126
U0124
PB98059
SB233580
SB202474
Jak2
EGFR
Mek1/2
MAPKp38

D


LIF -/-
AG490


LIF (ng)
100
0
pSTAT3
STAT3
1
pSTAT3
STAT3
0.1
WT
pSTAT3
STAT3
0
pSTAT3
STAT3
1
LIF -/-
AG490
(mM)


.25
.20
.15
.10
.05
STAT3 / STAT3
3.68
1.62
+ LIF
− LIF

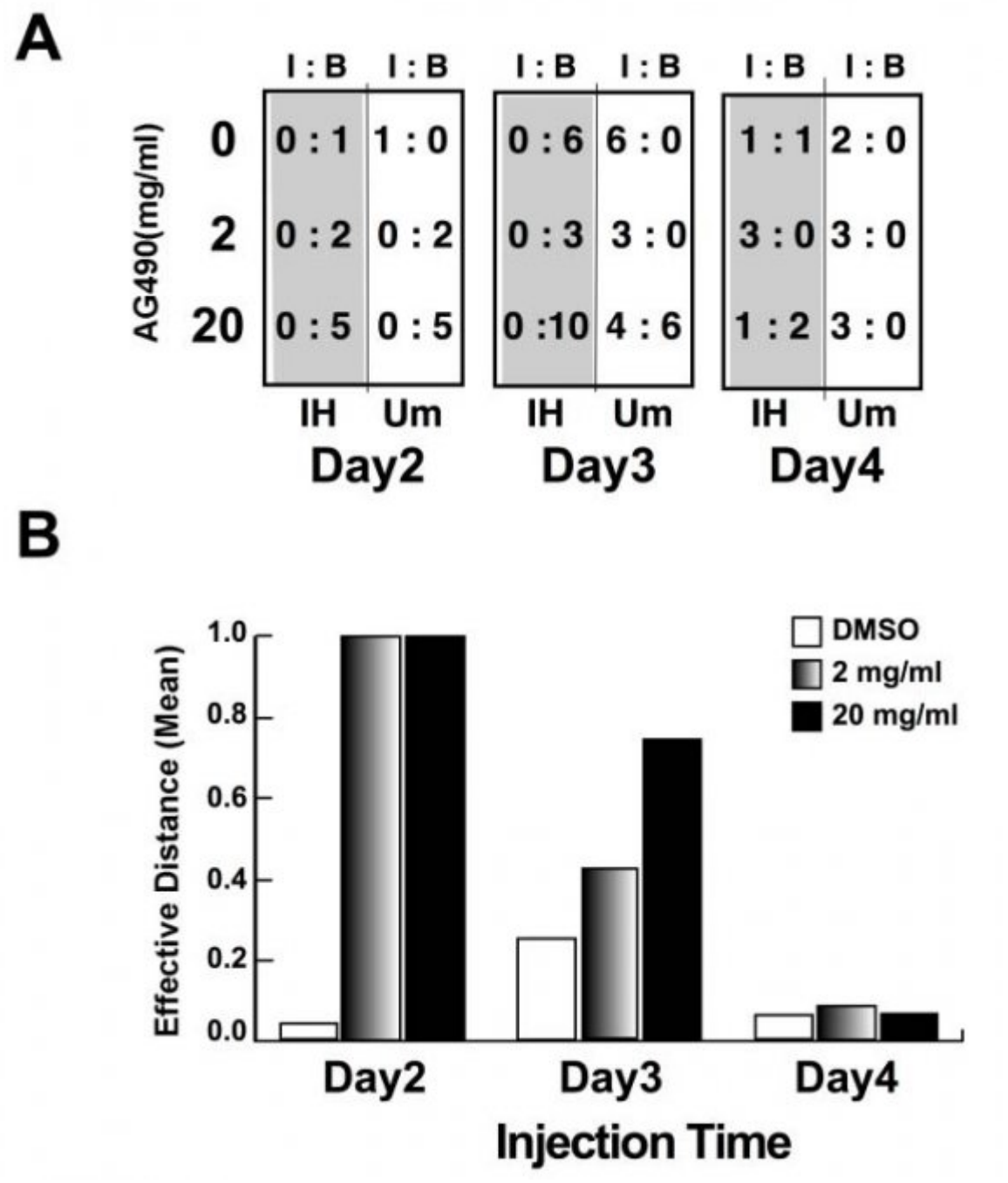

A
I : B
I : B
I : B
I : B
I : B
I : B
AG490(mg/ml)
0
2
20
0 : 1
1 : 0
0 : 2
0 : 2
0 : 5
0 : 5
0 : 6
6 : 0
0 : 3
3 : 0
0 :10
4 : 6
1 : 1
2 : 0
3 : 0
3 : 0
1 : 2
3 : 0
IH
Um
IH
Um
IH
Um
Day2
Day3
Day4
B
Effective Distance (Mean)
1.0
0.8
0.6
0.4
0.2
0.0
DMSO
2 mg/ml
20 mg/ml
Day2
Day3
Day4
Injection Time

Figure 6:

	transcription degenerative ulceration, and failure of uterine implantation. J Exp Med 194: 189-203. PMCID: PMC2193459	(STAT) signaling joint disease,	results in gastrointestinal
Year 2016 (D D D D) K	16. Proc Natl Acad Sci U S A 104: 19357-19362. PMCID: PMC2148294		

Figure 7:

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