Isolation and characterization of murine mammary cell lines with differentiated aggressive phenotype

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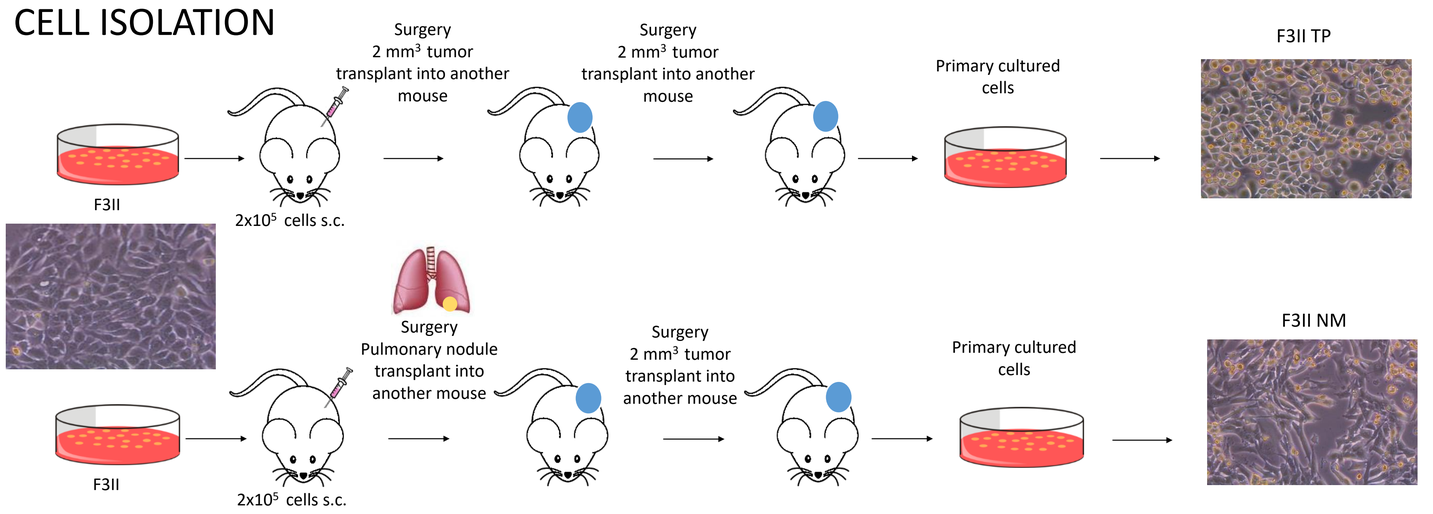
INTRODUCTION

Breast cancer is the first cause of death from female cancer. The recurrence of the disease originated at the level of secondary organs, or metastasis, is responsible for 90% of deaths from cancer. The factors that endow these cells with metastatic functions are largely unknown. One of the limitations in the study of tumor cells with aggressive phenotypes is that cell lines maintained in culture lose this ability to invade and colonize tissues. On the other hand, it has been shown that reinjection of cells in animals can lead to their enrichment with aggressive phenotypes. The aim of this work was the isolation and characterization of different cell populations with differentiated aggressive capacities

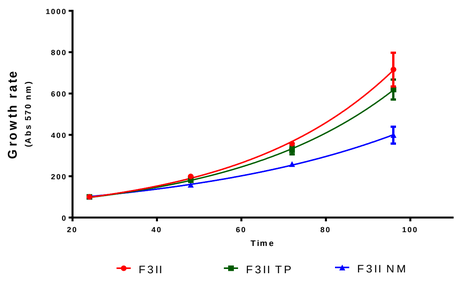
CELL ISOLATION



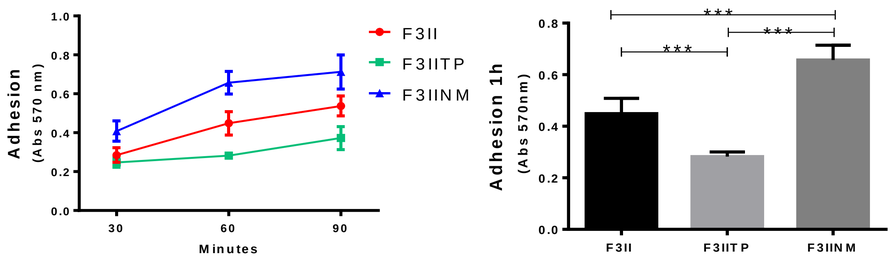
Isolation of F3II TP and F3II NM. 2 x 10^5 F3II cells were inoculated in the subcutis of BALB/c mice. When the tumor reached 2000 mm^3, mice were sacrificed and a fragment of 2 mm^3 of tumor was transplanted by surgery into another mouse. This process was repeated twice. After that, a primary cell culture was done and the F3II TP cell line was established in vitro. Meanwhile, other mouse was sacrificed and a pulmonary nodule of its was taken and transplanted by surgery into another mouse. When the tumor reached 2000 mm^3, mice were sacrificed and a fragment of 2 mm^3 of tumor was transplanted by surgery into another mouse. This process was repeated twice. After that, a primary cell culture was done and the F3II NM cell line was established in vitro.

IN VITRO CHARACTERIZATION

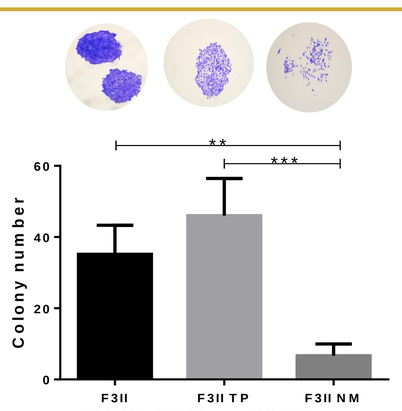
Cell line	Doubling time (h)
F3II	22,80
F3II TP	25,52
F3II NM	31,71



Cell doubling time. 1,5 x10^4 F3II, F3II TP and F3II NM cells were seeded in 12-wells plate. After incubation with DMEM 5% SFB for 24, 48, 72 and 96 h, each plate was stained and fixed with crystal violet - methanol, resuspended in ethanol:acetic solution (3:1) and measured at 570 nm. A growth curve given as 100% measured at 24 h for each treatment was built. Statistical analysis was performed by comparing the non-linear adjustment curves through its parameters.

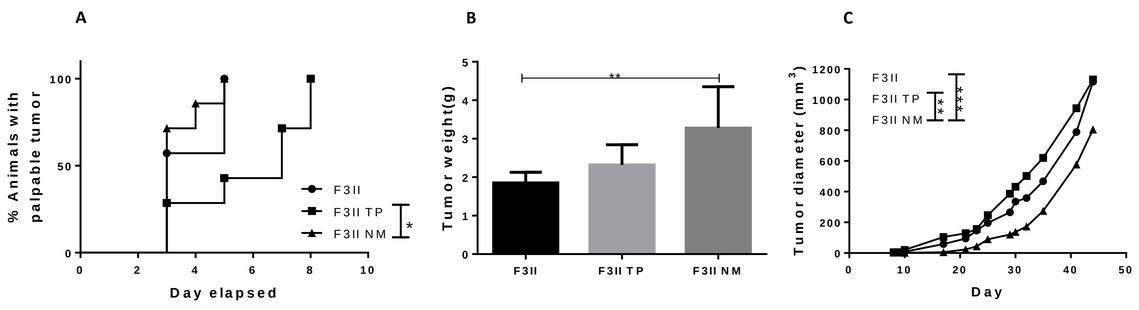


Cell adhesion capacity. 4,5 x10^4 cells F3II, F3II TP and F3II NM cells were seeded in a 96-well plates and incubated for 3 different times (30, 60 and 90 minutes). After washing, adherent cells were fixed and stained with crystal violet – methanol solution, solubilized in ethanol-acetic solution (3:1) and the absorbance was measured at 570 nm. Data represent the means SD± of one representative experiment of at least three independent experiments. **p<0,01. ***p<0,001 ANOVA post test Tukey´s multiple comparison test.



Clonogenic capacity. 250 F3II, F3II TP and F3II NM cells were seeded in 12-wells plate with DMEM 5% SFB. 7 days later, CFU(colony-forming unit) were fixed and stained with methanol – crystal violet solution and counted in triplicates. Representative microphotographs of the different cell lines on the colony formation assay are shown at top of each bar (40X). Results are representative of three independent experiments. **p<0,01. ***p<0,001. Kruskal-Wallis´ test.

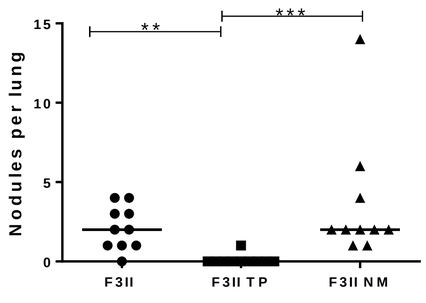
IN VIVO CHARACTERIZATION



D

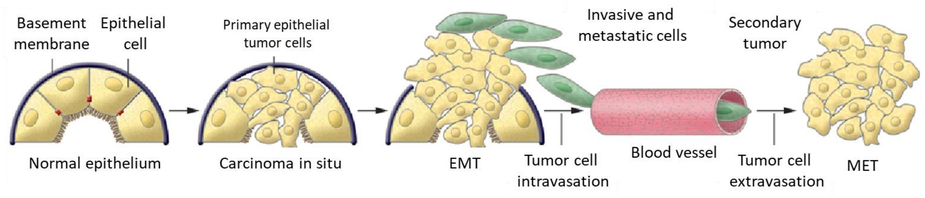
Cell line	Mice with spontaneous metastasis
F3II	4
F3II TP	0
F3II NM	0

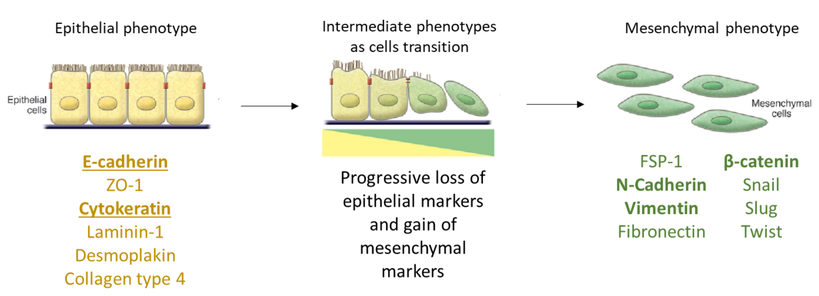
Tumor progression. 2x10^5 F3II, F3II TP and F3II NM were inoculated in the subcutis of BALB/c mice. **(A)** The presence of palpable tumor was registered.*p<0,05 Logrank test. **(B)** y **(C)** Tumors were measured thrice a week with caliper and tumor volume was calculated. Mice were sacrificed when the tumor volume was 2000 mm^3. Statistical analysis were conducted on tumor growth rates calculated from day 10 onwards. **p<0,01. ***p<0,001 ANOVA post test Tukey´s multiple comparison test. Tumor weight **p<0,01 Kruskal-Wallis test. **(D)** The table shows the number of mice with spontaneous metastasis. Experimental group n=7



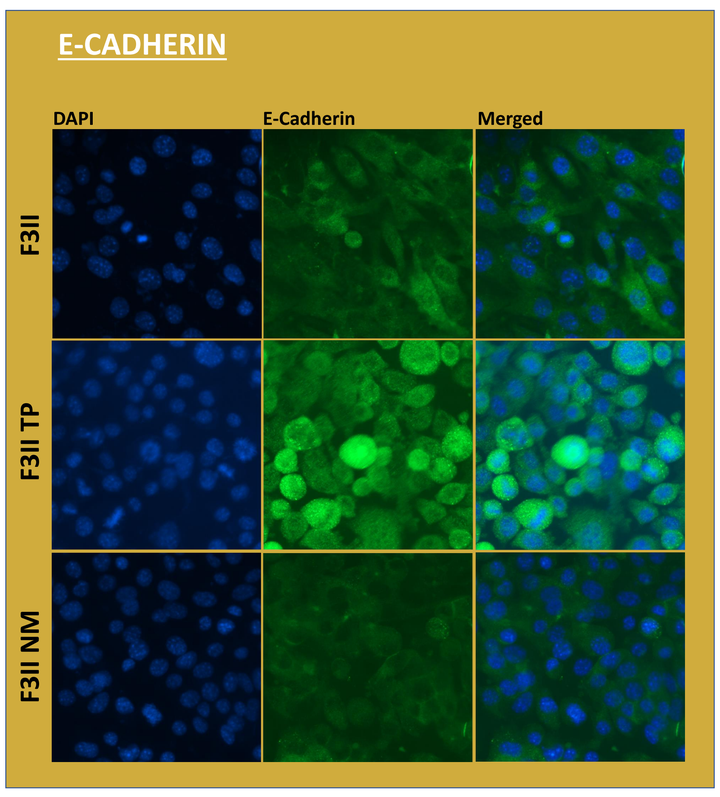
Experimental lung colonization assay. 2 x 10^5 F3II, F3II TP and F3II NM cells were injected in the lateral tail vein of mice. 21 days later, mice were sacrifice. The number of lung nodules per animal is represented on the graph. Data are expressed as scatter dot plot and median (experimental groups n=10). **p<0,01. ***p<0,001. Kruskal-Wallis test

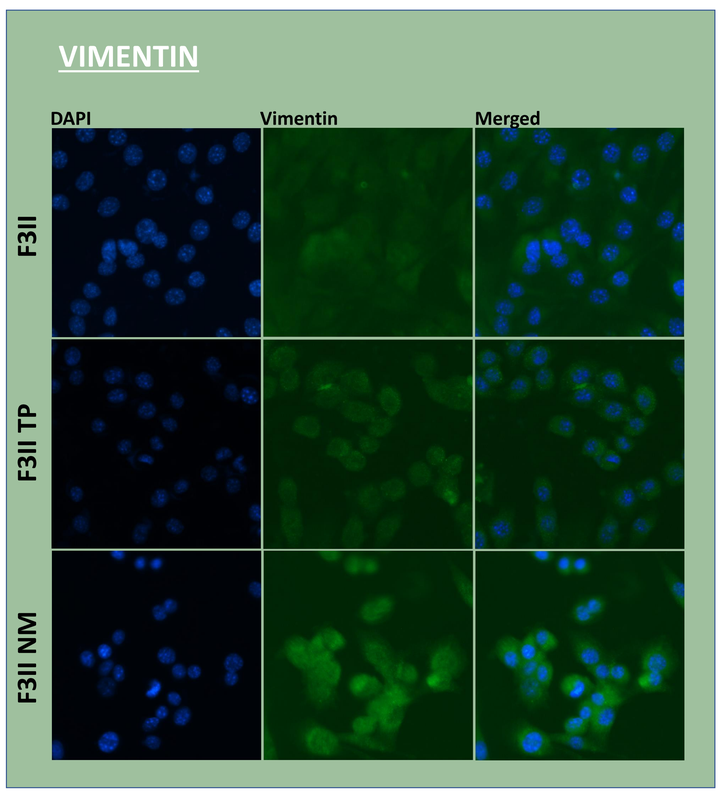
MOLECULAR CHARACTERIZATION

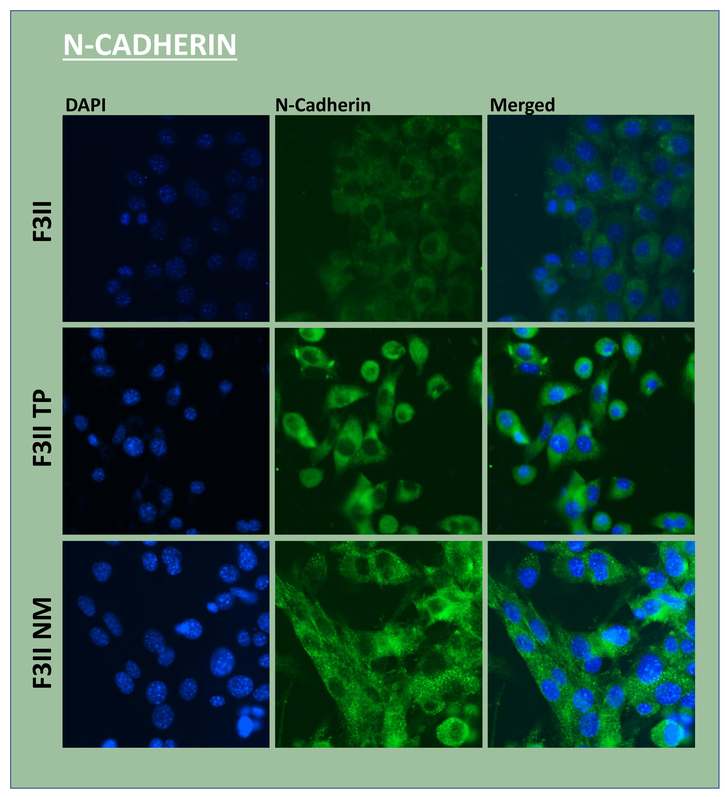


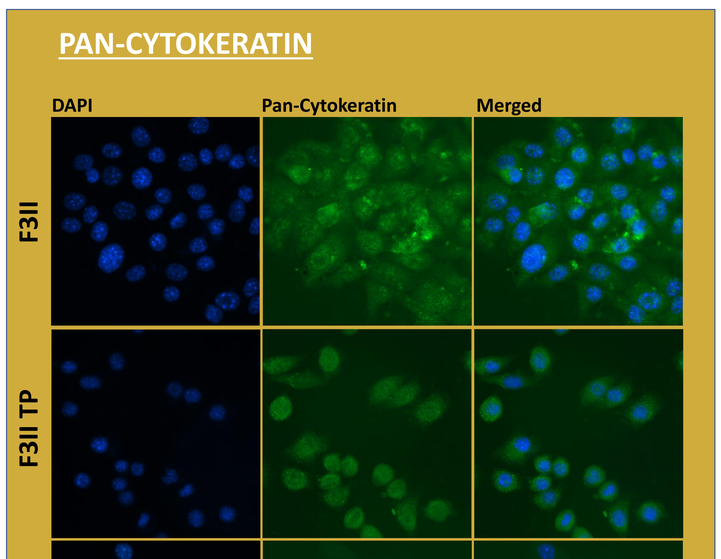


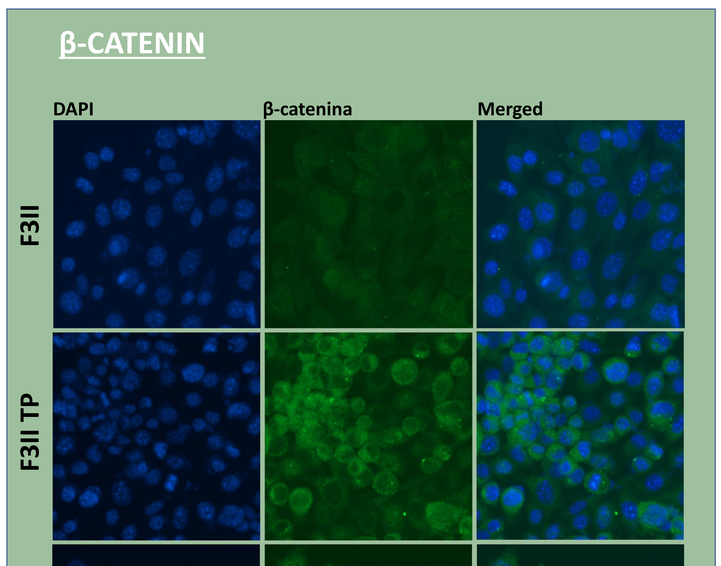
Epithelial mesenchymal transition. The first stage of metastatic process involves epithelial cells losing their polarity and detaching from the basement membrane. The next step involves epithelial mesenchymal transition (EMT) and an angiogenic switch, faciliting the malignant phase of tumor growth. This EMT enable cancer cells to enter the circulation and exit the blood stream at a remote site, where they may grow and form micro and macro metastases, which may involve METs (Mesenchymal epithelial transition) and thus a reversion to an epithelial phenotype. Some of the epithelial and mesenchymal cell markers used by EMT researchers are listed. The ones that are highlighted were used to identify this markers in F3II, F3II TP and F3II NM cell lines. (Modified of Kalluri, et al. 2009)











Epithelial and mesenchymal markers expression in F3II, F3II TP and F3II NM cell lines. The expression of the epithelial markers, E- cadherin and Pan-cytokeratin, and the mesenchymal markers, vimentin, β-catenin and N-cadherin was determinated by indirect immunofluorescence with specific antibodies. Left panels: DAPI; middle panels: marker expression; right panels: merged. (200X)

CONCLUSION

- We isolated two different cell line, F3II TP and F3II NM that not only differ between themselves, they are different from the parental line F3II.
- The in vitro characterization demonstrate that the cell lines have different doubling time, adhesion and clonogenic capacity. F3II NM presented major adhesion capacity and lower clonogenic potential
- The three lines showed different tumoral progression in vivo. They differed in tumor latency, tumor weight, progression and number of pulmonary nodules.
- F3II, F3II TP and F3II NM presented different epithelial-mesenchymal phenotype. This could explain they behaviors in vivo and in vitro

	E-Cadherina	Pan-Cytokeratin	Vimentin	β-catenin	N-cadherin
F3II	↑	↑	↓	↓	↓
F3II TP	↑↑	↑	↑	↑	↑
F3II NM	↓	↓	↑↑	↑	↑