Multiple Roles of Perforin in Hampering ERBB-2 (Her-2/neu) Carcinogenesis in Transgenic Male Mice

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MULTIPLE ROLES OF PERFORIN IN HAMPERING ERBB-2 (Her-2/neu)

CARCINOGENESIS IN TRANSGENIC MALE MICE

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Running title: Perforin-mediated surveillance in ErbB-2-transgenic males

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Abstract

Perforin (pfp)-mediated cytotoxicity is one of the principal immunosurveillance mechanisms involved in the fight against cancer. However, its importance in spontaneous epithelial cancer is still poorly defined. Here, we use a realistic mouse model which displays many features that are equivalent to human pathology, to evaluated the role of pfp-dependent immunosurveillance by comparing tumor progression in rat ErbB-2 (neu) transgenic, pfp-proficient (neu+/pfp+) or pfp-deficient (neu+/pfp−) BALB/c male mice. Adult neu+/pfp+ males developed poorly differentiated salivary carcinomas, whereas neu+/pfp− males displayed their salivary carcinomas noticeably earlier and showed zones of more highly differentiated tumor, indicating that pfp-mediated immunosurveillance is able not only to delay the growth kinetic of an aggressive epithelial tumor, but also to shape its histology. The role of pfp-mediated immunosurveillance appeared to be of even more dramatic importance against the less aggressive male mammary carcinomas. In neu+/pfp+ males, the incidence of mammary carcinomas was a sporadic and late event. By contrast in neu+/pfp− males their incidence was four-times higher. This higher cancer incidence was associated with a two-fold higher occurrence of persisting mammary remnants, a major risk factor for mammary cancer in male mice, and one which would appear to be due to pfp’s previously unidentified involvement in male mammary gland rejection during embryogenesis.

This work thus provides further proof of the complex role that the immune system plays in the body and gives new insight into the pathogenesis of epithelial tumors, demonstrating that the penetrance and malignancy of a tumor may be dramatically affected by pfp-dependent mechanisms.
Introduction

Observational studies linked to clinical-outcome analysis in cancer patients that have been carried out in recent years have displayed the prognostic and predictive value of the tumor microenvironment inflammatory state. This has generated a new wave of interest in the immunosurveillance phenomenon (1). Natural immune surveillance against the onset of cancer is one of the most important tenets in experimental tumor immunology (2, 3). Extensive evidence have shown that immunodeficient mice develop more carcinogen-induced and spontaneous cancers than wild-type mice, and that tumor cells from immunodeficient mice are more immunogenic than those from immunocompetent mice (4). Numerous studies have elucidated several molecular (5) and cellular (6, 7) immune surveillance mechanisms that hamper tumor onset and shape its growth. However, as their role in spontaneous epithelial cancer is poorly defined, the importance of immunosurveillance in the control of most common human cancers is still difficult to grasp.

Alterations in the ERBB-2 oncogene and its signal transduction pathways are amongst the causes of epithelial cell neoplastic transformation, carcinoma progression, metastatic spreading and resistance to therapy (8). In human pathology, ERBB-2 (Her-2) overexpression and mutations are evident in 30-40% of epithelial tumors and are associated with the early onset of precancerous lesions, increased metastasis and severe prognoses (9). In human breast cancer, gene amplification and protein overexpression of ErbB-2 are associated with poor prognosis (10). Similarly, high-grade human salivary gland carcinomas harbor increased ErbB-2 protein expression and gene copy number (11).

In rats, a single point mutation in the transmembrane domain favors ERBB-2 homo- and hetero-dimerization and transforms the rat proto-oncogene into a dominant oncogene (neu) (12). In a neu transgenic BALB/c (BALB-neuT) mouse strain, females develop fast-growing mammary carcinomas in all their mammary glands (13). However, mammary neu+ carcinomas in BALB-neuT males are late sporadic events and the prominent cause of death is multifocal poorly differentiated acinic adenocarcinoma that initially involves the parotid and then the submandibular glands (14).
4 weeks of age, males display multiple foci of atypical salivary gland hyperplasia, at the duct and acinic level, that progress to become poorly differentiated carcinomas that are clinically evident at the 26th week of age (14).

Their consistent genetic predisposition to developing lethal carcinoma, their progression through well defined stages and the long-lasting interaction with the host microenvironment make BALB-neuT mice an appealing model for the evolution of the role that immunosurveillance plays in inhibiting neu+ epithelial tumors. The consistent genetic predisposition to developing lethal carcinomas, their progression through well-defined stages and the long-lasting interaction with the host microenvironment make BALB-neuT mice an appealing model for the evaluation of the role that immunosurveillance plays in inhibiting neu+ epithelial tumors. Previous studies on female BALB-neuT mice have shown that despite the absolute penetrance and aggressive tumorigenesis caused by neu transgene expression in the mammary glands and the existence of CD8 T cell central immune tolerance to the rat neu protein product (15, 16), pfp- and complement-mediated immunosurveillance mechanisms significantly impair the onset of mammary carcinomas (17, 18).

We herein compare the ability of pfp-mediated immunosurveillance to inhibit neu+ salivary carcinomas, with high penetrance, and mammary carcinomas with a slower progression and lower penetrance in BALB-neuT male mice.
Materials and Methods

**Mice.** BALB-neuT male mice (13) from Biogem, Ariano Irpino, Italy were crossed with pfp<sup>−/−</sup> BALB/c females (17). Heterozygous pfp<sup>+/−</sup> and neu<sup>+/+</sup> F1 male mice were then backcrossed with pfp<sup>−/−</sup> or pfp<sup>+/+</sup> BALB/c females. The progeny was genotyped to discriminate between normal (neu<sup>−/−</sup>/pfp<sup>+</sup>), pfp<sup>−/−</sup>(neu<sup>−/−</sup>/pfp<sup>−/−</sup>), those heterozygous for the neu transgene (neu<sup>+/−</sup>/pfp<sup>+</sup>), and those heterozygous for the neu transgene and pfp<sup>−/−</sup>(neu<sup>+/−</sup>/pfp<sup>−/−</sup>). A first line was generated from a BALB/c pfp<sup>−/−</sup> female received from the Peter MacCallum Cancer Centre, East Melbourne, Australia that was crossed with a BALB-neuT male re-derived in SPF conditions by Charles River, Calco, Italy, and maintained at the Dept. Clin. Biol. Sci., Orbassano, Italy (DCBS Selection). A second line was generated later at the Mol. Biotechnol. Ctr. (MBC Selection) from a BALB/c pfp<sup>−/−</sup> female originally from the Peter MacCallum Cancer Centre but received from NIAID/NIH, Bethesda, MD where a colony of these mice is maintained, by courtesy of Dr. Robert Wiltrout, that was crossed with a BALB-neuT male re-derived by Biogem, Ariano Irpino, Italy. Tumor onset was evaluated as previously described (13). BALB/c mice knockout for the interleukin (IL)-15 gene (neu<sup>−/−</sup>/IL15<sup>−/−</sup>) were a gift from Dr. Silvia Bulfone-Paus (Research Center Borstel, Germany) to Dr. Pier-Luigi Lollini. All mice were maintained in specific pathogen free conditions (Allentown Caging Equipment, Allentown Inc., Allentown, NJ) and treated in conformity with current European guidelines and policies. The Bioethical Committee of the University of Torino approved the experimental plan.

**Morphologic analysis.** Salivary glands were frozen in a cryo-embedding medium or fixed in formalin and embedded in paraffin for histological and immunohistochemical analyses. Sections were incubated with the following primary antibodies: rabbit polyclonal anti-human Her-2 (A0485; Dako Corporation, Carpentaria, CA), mouse mAb to Proliferating Cell Nuclear Antigen (PCNA) (M0879; Dako Corporation), anti-CD3 (Ab828, Abcam, Cambridge, UK); anti TCR γδ (553175, BD Pharmingen, San Diego, CA). Immunocomplexes were detected using Streptavidin Peroxidase
(Thermo Scientific-Lab Vision Corporation, Freemont, CA) and either the DAB Chromogen System (Dako Corporation) or the Bajoran Purple Chromogen System (Biocare Medical, Concord, CA). The whole mount and immunohistochemical preparations of mammary glands were carried out as previously described (17). For mammary gland reabsorption studies, embryos were removed from the uterus at E14.5 and E16.5, fixed for 1 h in PFA1% and embedded in OCT and cut in serial sections. Male and female embryos were distinguished by PCR as described (19).

Cytometry of tumor infiltrating leukocytes. Freshly isolated tumor specimens of 7-8 mm mean diameter were dissociated as previously described (18) to obtain cellular suspensions that were treated with anti CD16/CD32 (01245B, BD Biosciences, San José), and stained with: anti-CD49b PE (130-091-816), anti-CD45 VioGreen (130-097-294), anti-CD8a VioBlue (130-094-360), anti-CD3e FITC (130-092-962), all from Miltenyi Biotec (Calderara di Reno, Italy), and anti-γδ TCR PE/Cy7 (118124, Biolegend, San Diego, CA). Samples were acquired and analyzed on the CyAn ADP using Summit 4.3 software (DakoCytomation, Heverlee, Belgium).

Bone marrow transplantation and NK cell depletion. Eight-week-old neu+/pfp⁺ male mice were exposed to a total dose of 7.8 Gy whole-body irradiation using a Gilardoni RADGIL X-ray generator (Gilardoni S.p.A, Lecco, Italy). Twenty-four hours after irradiation, mice intravenously received 15 x 10⁶ bone marrow cells (BMC) isolated from the femur and tibia of neu⁻/pfp⁻ or neu⁻/pfp⁺ males and were then monitored for the appearance of salivary carcinoma. Non transplanted, irradiated control mice died within 2 weeks. To verify bone marrow engraftment, mice were bled and peripheral blood cells were stained with Ab anti-CD45 (130-091-811; MiltenyiBiotec), anti-B220/CD45R (558108), anti-CD90.1/Thy1.2 (553003), anti CD8a (553033), anti-CD49b (553858) (all from BD Pharmingen), anti-CD11b (101206), anti-CD4 (100528) (all from Biolegend) and analyzed by flow cytometry one month after the injection of BMC. Only transplanted mice displaying lymphoid and myeloid cell levels comparable to neu⁻/pfp⁺ mice were evaluated. NK cell
depletion was performed on neu+/pfp+ males irradiated and reconstituted with BMC from neu+/pfp+ mice, starting from 30 days after BMC transplantation, and on untreated 6-week old neu+/pfp+ males. Mice received intraperitoneal injections of 0.2 ml of phosphate-buffered saline containing a 1:20 dilution of anti-asialo GM1 rabbit anti-serum (Wako Pure Chemicals Industries, Osaka, Japan) on days 0, 1 and 2 and then once a week until the salivary carcinoma became palpable. Controls were injected with normal rabbit serum (NRbS) (GIBCO, Grand Island, NY).

Statistical analysis. Statistical differences were evaluated using GraphPad software 5.0 (GraphPad Inc. San Diego, CA). The Mantel-Cox log-rank test was used to assess the differences in tumor incidence and overall survival. Differences in the frequency of tumors and mammary gland remnants were evaluated using the chi-square test with Yates’ correction. All the other statistical differences were assessed using the two-tailed unpaired Student’s t test.
Results

Pfp hampers the onset and shape the histology of neu⁺ salivary carcinomas in male mice.

Salivary carcinomas are the prominent cause of death in neu⁺ males. The faster progression of these tumors in neu⁺/pfp⁻ as compared to neu⁺/pfp⁺ males that was initially observed in a small number of males from the DCBS Selection (SupplementalFig. 1) spurred us to study the kinetics of these carcinomas in a larger number of males from the MBC Selection. Salivary carcinomas became palpable significantly earlier in neu⁺/pfp⁻ as compared to neu⁺/pfp⁺ mice (Fig. 1A; p < 0.0001). While salivary carcinomas appeared from the 26th week of age in neu⁺/pfp⁺ males, neu⁺/pfp⁻ males had already started to develop salivary tumors at the 18th week. Similarly, overall survival was significantly shorter in neu⁺/pfp⁻ than in neu⁺/pfp⁺ male mice (Fig. 1B; p < 0.0001).

At 8 weeks of age, both neu⁺/pfp⁺ and neu⁺/pfp⁻ males displayed small foci of atypical hyperplasia surrounded by numerous reactive cells in the salivary intercalated ducts and the serous acini (Fig. 2A and Fig. 2B, respectively). In neu⁺/pfp⁺ males the carcinomas that became palpable from the 26th week of age appeared to have been uniformly generated by the confluence and evolution of multiple foci of ductal hyperplasia, and were finally composed of solid lobules of uniform cells with no glandular differentiation (Fig. 2C), high mitotic activity (Fig. 2D), and high neu expression (Fig. 2E). By contrast, the quick outgrowth of poorly differentiated (Fig. 2F), highly proliferative (Fig. 2G), and highly neu⁺ (Fig. 2H) carcinomas in neu⁺/pfp⁻ males was accompanied by the expansion of several areas of variously differentiated acinic carcinomas characterized by zones with clear cells (Fig. 2I) and others with large cells, resembling normal serous cells, organized in small nodules (Fig. 2L). Both areas were characterized by low proliferative activity (Fig. 2J and Fig. 2M) and high neu expression (Figure 2K and Fig. 2N).

Salivary carcinomas are infiltrated by pfp-dependent effector immune cells.
Various effectors immune cells are pfp-dependent (CD8⁺αβ T, NK, NKT, and γδ T cells), most of which are CD3⁺. In fact, infiltrating cells in salivary carcinomas from both neu⁺/pfp⁺ and neu⁺/pfp⁻ mice were prominently CD3⁺, as shown by immunohistochemistry (Fig. 3A and Fig. 3B). A cytofluorimetric analysis was also performed to evaluate the relative amount of pfp-dependent effector immune cells in salivary tumors. As shown in Figure 3C, CD8⁺αβ T, γδ T, and NK cells (identified as CD3⁺ CD49b⁺) were detected in the salivary tumors of both neu⁺/pfp⁻ and neu⁺/pfp⁺ mice, whereas NKT cells (identified as CD3⁺ CD49b⁺) were present at very low percentages. The negligible presence of NKT cells suggests that these cells cannot be the major effectors that hamper neu⁺ salivary carcinogenesis, while CD8⁺αβ T, γδ T, and NK cells might all contribute to this immunosurveillance phenomenon.

T cells are not the principal players in immunosurveillance fight against neu⁺ salivary carcinomas.

To assess which pfp-dependent effector cell population was principally involved in hampering neu⁺ salivary carcinogenesis, we decided to perform bone marrow chimeras by reconstituting lethally irradiated 8-week old neu⁺/pfp⁺ male mice with BMC from either neu⁻/pfp⁻ or neu⁻/pfp⁺ male donors; some of the neu⁻/pfp⁺ reconstituted mice were also depleted of NK cells. Since the generation of γδ T cells mainly occurs during the fetal life, BMC reconstituted mice have CD8⁺αβ T and NK cells, but are virtually devoid of γδ T cells (Supplemental Fig. 2). Salivary carcinoma onset occurred significantly earlier (p < 0.0001) in mice reconstituted with neu⁻/pfp⁺ BMC (Fig. 4A) as compared to untreated neu⁺/pfp⁺ mice (Supplemental Fig. 3). These accelerated kinetic is due to the lethal irradiation and the time required by transplanted BMC to reconstitute the mouse, but a possible contribution from the lack of γδ T cells cannot be ruled out. Nevertheless, salivary carcinomas arising in males reconstituted with neu⁻/pfp⁺ BMC were uniformly poorly differentiated carcinomas with severe atypical features and a high proliferative rate (Fig. 4B and Fig. 4C), thus very similar to those that arise in untreated neu⁺/pfp⁺ males (Fig. 2C-E). Moreover,
when mice reconstituted with neu+/pfp+ BMC where depleted of NK cells, they displayed significantly anticipated salivary carcinoma onset \( (p = 0.02) \) as compared to not-depleted ones. Here, the kinetics was very similar to what found in mice reconstituted with neu+/pfp- BMC (Fig. 4A). The histological features of the salivary carcinomas that arose in mice reconstituted with neu+/pfp+ BMC and depleted of NK cells (Fig. 4D-G) and those that arose in mice reconstituted with neu+/pfp- BMC (not shown) were also very similar. Besides poorly differentiated (Fig. 4D) and highly proliferative areas (Fig. 4E), they also displayed several areas of differentiated acinic carcinomas with clear cell features, pronounced glandular differentiation (Fig. 4F), and lower proliferative rate (Fig. 4G), as found in untreated neu+/pfp- mice (Fig. 2F-N). Taken together, these data suggest that the main players in the immunosurveillance against neu+ salivary carcinomas may be NK cells, but certainly not CD8αβ T cells, and probably not γδ T cells either.

**Salivary carcinogenesis is delayed by NK cells.**

To better evaluate the role of NK cells, neu+/pfp+ males were depleted of NK cells via the chronic administration of anti-asialo GM1 antibodies. Salivary carcinoma onset was significantly faster in these mice than in neu+/pfp+ males treated with the control isotype and similar to that of neu+/pfp- mice (Fig. 5A, \( p = 0.003 \) and \( p = 0.4 \), respectively). Moreover, salivary carcinomas that arose in NK cell-depleted neu+/pfp+ males display areas with poor differentiation, high proliferation and neu expression (Fig. 5B-D) together with others with various degree of differentiation, lower proliferative rate and high neu expression (Fig. 5E-J). These results confirm the key role of NK cells not only in making the kinetic of salivary carcinogenesis slower but also in doing the immune selection of premalignant cells that will become true cancer in salivary glands.

**Pfp blocks neu+ mammary carcinomas in male mice.**

The onset of neu+ mammary carcinomas is a late event displayed by less than 20% of one-year-old neu+/pfp+ males that survive salivary carcinomas. As surprisingly higher incidence of
mammary cancer was first seen in a small number of neu+/pfp− males from the DCBS selection (data not shown). When this observation was reexamined on a large number of males from the MBC selection it was evident that more than 70% of neu+/pfp− males display a mammary carcinoma within one year of age (Fig.6A). Whole mount analysis of the most technically accessible 2nd, 3rd and 4th mammary glands showed that microscopic mammary carcinomas were evident in 8% of 25-30 week-old neu+/pfp− males, but in over 36% of age-matched neu+/pfp+ males. This reveals that, even at this earlier time, a fourfold higher carcinoma incidence is microscopically evident in neu+/pfp− than in neu+/pfp+ males (Fig.6B, p = 0.003). These carcinomas homogeneously consist in solid nodular aggregates that arise from ducts of persisting mammary remnants (Fig.6C and Fig.6D) composed of moderately differentiated atypical ductal neu+ cells surrounded by delicate vascularized stromal bundles and a moderate inflammatory infiltrate (Fig.6E-H).

**Impaired mammary gland reabsorption in pfp deficient male embryos.**

The triggering of androgen receptors causes the regression of mammary sprouts that disappear during late embryogenesis (E15.5) in most male mouse glands (20). An evaluation of the frequency of the persistence of glandular sprouts in the mammary areas from 20-30-week-old males showed that these were evident in only 6.5% of mammary areas in neu+/pfp+ mice, but in about 14.3% neu+/pfp− mice (Fig.7A). Persistent mammary sprouts were also evident in 3.3% of the mammary areas of neu−/pfp+ males and in 9% of neu−/pfp− mice. A similar increase in the persistence of mammary remnants was also found in neu−/IL15− males (Fig.7A) that are typically devoid of skin intraepithelial γδ T cells (21, 22). These data show that an impaired regression of rudimentary mammary tissue is connected with both the deprivation of pfp and intraepithelial γδ T cells. Immunohistochemical analysis of mammary buds in male and female embryos of 14.5 and 16.5 days, showed the presence of γδ T cells only around male mammary buds, confirming the possible role of these cells in male mammary gland reabsorption (Fig. 7B and Fig. 7C). The
impaired reabsorption of mammary remnants observed in pfp⁻ males fits in well with the reduced involution of mammary glands after lactation in pfp⁻ females (Supplemental Fig. 4).

The defective regression of mammary sprouts appears to be a major risk factor for mammary cancer in adult males. However in neu⁺/pfp⁺ males, only 30% of mammary remnants were associated with microscopic carcinomas, which instead were present in 75% of mammary sprouts persisting in neu⁺/pfp⁻ males (Fig. 7D). These data show that the protection associated with the expression of the pfp gene derives from two distinct effects; a reduction in the number of males at risk due to the persistence of mammary remnants, and an efficient block of carcinogenesis in those adult neu⁺ males that have mammary remnants.
Discussion

We have recently exploited the genetic predestination of female BALB-neuT mice to mammary carcinogenesis (23) to assess the weight of pfp-mediated (17) and complement-mediated (18) natural immunosurveillance towards neu+ mammary carcinomas. We have now extended our observation to the role of pfp-mediated mechanism in the surveillance of salivary and mammary carcinomas that arise in BALB-neuT males (14). The data obtained show that the pfp deficiency: i) reduces the tumor-free survival of these mice; ii) allows for the growth of more differentiated salivary tumor histotypes and iii) impairs male mammary sprout reabsorption during embryogenesis.

The slow growing salivary carcinomas are a common cause of death in adult neu+/pfp+ males. About 70% of them die before reaching one year of age because of salivary tumor outgrowth. Salivary carcinogenesis is more aggressive in neu+/pfp males. These pfp-deficient mice display shorter disease free times and overall survival, with about 95% of them dying by one year of age. BMC reconstitution of lethally irradiated neu+/pfp+ males excluded T cells performing a prominent role in this pfp-mediated phenomenon, while NK depletion experiments pointed to the central role of NK cell-expressed pfp in the inhibition of salivary carcinomas. These findings fit in well with our previous observation on the similar key role NK cells play in the control of mammary carcinogenesis in neu+/pfp+ females (17) and with emerging data from epithelial human tumors that demonstrate a significant correlation between the reduced expression of pfp by NK cells and cancer progression in patients with pancreatic, gastric and colorectal cancers (24).

The role of NK cells was not limited to affecting the pace of cancer progression, but also sculpted the histotype of salivary carcinomas that become clinically evident. The transforming neu transgene is expressed not only by several ductal cells but also by some cells of serous acini in the salivary glands of male mice during puberty. Areas of atypical hyperplasia of ductal and acinar structures are microscopically evident in the earlier stages of carcinogenesis. However, only poorly differentiated monomorphic neu+ carcinomas of ductal origin sneak through the pfp-dependent
mechanisms. By contrast, salivary carcinomas that become clinically evident in neu\(^+\)/pfp\(^-\) males display multiple minor areas of acinic neoplasia with a higher degree of differentiation and lower proliferative rate among the large areas of poorly differentiated carcinomas. Similarly, when lethally irradiated neu\(^+\)/pfp\(^+\) males were reconstituted with neu\(^-\)/pfp\(^+\) BMC, only poorly differentiated carcinomas were able to expand. By contrast, when lethally irradiated neu\(^+\)/pfp\(^+\) males were reconstituted with neu\(^-\)/pfp\(^-\) BMC, or with neu\(^-\)/pfp\(^+\) BMC but were depleted of NK cells, areas of neoplasia with a higher degree of differentiation and lower proliferative rate were again found to be components of the outgrowing tumors. Finally, areas of more highly differentiated neoplasia were also able to expand when otherwise untreated neu\(^+\)/pfp\(^+\) males were depleted of NK cells. These data further support the importance of NK cells not only in immunosurveillance, but also in the immunoediting of epithelial cancer (25).

This pfp-dependent selection of tumor histotype also corroborates data on neoplastic stem cells’ increased ability to give rise to tumors when injected into immunocompromised, as compared to normal, mice (26, 27). While a few neoplastic stem cells are able to give rise to a tumor in mice with an efficient immune system, many others are able to outgrow in those that are immunocompromised. This implies that malignant cells with stem cell properties are endowed with a different degree of penetrance, suggesting that the cell differentiation expressed by tumors may not only be the result of the transformation of cells with distinctive stem cell features, but also of the action of transforming stimuli on differentiated cells that are able to regain stem cell functions. Those with a higher degree of differentiation, which are therefore less aggressive, such as transformed salivary acinar cells, may be blocked by immune surveillance mechanisms, while those that are less differentiated, such as mammary and salivary transformed ductal cells, are only delayed in their progression.

Lastly, pfp-deficient BALB-neuT males also display a four times higher incidence of mammary carcinomas, showing the importance of pfp-dependent immunosurveillance against aggressive tumors with low penetrance. This surprising finding may result from both immune
mechanisms and altered pfp-dependent mammary morphogenesis. In both neu\(^+/\)pfp\(^+\) and neu\(^-/\)pfp\(^-\) males, moderately differentiated neu\(^+\) cells that originate from the ducts of the rudimental mammary gland sprouts make mammary carcinomas. Consequently, the persistence of these rudiments in a few areas in male mammary pads is a major risk factor. Microscopic examination of a large number of mammary areas showed that the frequency of persisting mammary remnants was almost twice as high in neu\(^+/\)pfp\(^-\) than in neu\(^+/\)pfp\(^+\) males. A higher frequency of persisting mammary remnants was also evident in neu\(^-/\)pfp\(^-\) males as compared to neu\(^-/\)pfp\(^+\) males. The physiological regression of rudimental male mammary gland sprouts takes place during late embryogenesis (E15.5) (20). Present data indicate that this regression is impaired in the absence of pfp, independently of the expression of the neu transgene.

\(\gamma\delta\)T cells appear to be the main pfp\(^+\) cell population responsible for the reabsorption of mammary gland rudiments in male embryos. These cells appear from E14 (28), being the predominating pfp\(^+\) cell population in these stages of fetal development. Functionally competent pfp\(^+\) NK cells only appear from E16.5 (29), when the reabsorption of male mammary gland rudiments is complete. The observation of similar persisting mammary gland remnant frequency in neu\(^-/\)IL15\(^-\) and neu\(^-/\)pfp\(^-\) male mice corroborates the central role \(\gamma\delta\) T cells play in the reabsorption of the male mammary gland. In effect, IL-15 KO mice are devoid of skin intraepithelial \(\gamma\delta\) T cells since IL15-mediated signals are required for their development (21, 22).

While the fact that pfp may play a role in placental morphogenesis and in the maintenance of a microenvironment that is biased towards Th2 cytokine production (30, 31) has previously been recognized, its role in mammary regression during male embryogenesis has not yet been established. Moreover, the impairment in the regression of mammary rudiments in pfp\(^-\) males and the defective involution of mammary glands after milking observed in pfp\(^-\) females, suggest that pfp mediated mechanisms play a more diffuse role in mammary gland remodeling. The presence of numerous \(\gamma\delta\) T cells in lactating mammary glands (32), once again points to the possible involvement of these lymphocytes in mammary tissue remodeling.
Since the frequency of male mice with at least one mammary area containing mammary remnants is almost twice as high among neu+/pfp than in neu+/pfp+ males, the lack of pfp appears to play a marked role in increasing the probability of developing mammary cancer. The fact that the incidence of mammary cancer is four times higher in neu+/pfp− males than in neu+/pfp+ males suggests that the higher risk of mammary cancer, caused by defective mammary rudiment regression, is further enhanced by the lack of pfp-dependent immunosurveillance. In effect, the number of mammary remnants associated with a microscopic tumor is about 2.5 times higher at weeks 20-30 of age in neu+/pfp− as compared to neu+/pfp+ males.

In conclusion, our data support the existence of a critical role played by pfp-expressing NK cells in both immunosurveillance and immunoediting of neu+ epithelial cancers. They markedly delay the onset of mammary and salivary carcinomas and their growth rate, but also sculpt the differentiation stage of salivary carcinomas. In addition, our findings have unveiled the previously unsuspected involvement of pfp in male mammary gland reabsorption during embryogenesis, suggesting the existence of a possible role for γδ T cells in this phenomenon. All together these findings provide further proof of the multiple roles that pfp-mediated mechanisms fulfill in cancer control and may be important for the development of NK cell-based therapeutic strategies.
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Footnotes

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2Abbreviations used in this paper: neu, rat ERBB-2 oncogene; pfp, perforin; BALB-neuT mice: neu transgenic BALB/c mice; neu+/pfp+ mice, wild type BALB/c mice; neu-/pfp- mice; BALB/c mice pfp knockout; neu+/pfp+ mice, BALB-neuT mice; neu+/pfp- mice, BALB-neuT mice pfp knockout; neu-/IL15- mice, BALB/c mice IL-15 knockout; PCNA, Proliferating Cell Nuclear Antigen; BMC, bone marrow cells.

3The authors have no financial conflict of interest.
References


Captions to figures

Figure 1. Salivary carcinogenesis in neu⁻/pfp⁻ males mice is more aggressive as compared to that of neu⁺/pfp⁺ male mice. (A) Early onset and higher incidence of salivary tumors in neu⁺/pfp⁻ males (n = 38; black solid line) as compared to neu⁺/pfp⁺ males (n = 68; black dotted line). (B) Survival time of neu⁺/pfp⁻ (n = 27; black solid line) and neu⁺/pfp⁺(n = 50; black dotted line) males. ***, p< 0.0001, Mantel-Cox log-rank test.

Figure 2. Pfp deficiency permits the growth of less aggressive, more differentiated tumor histotypes. (A and B) Hematoxylin and eosin staining of salivary glands from 8 weeks old neu⁺/pfp⁺ (A) and neu⁺/pfp⁻ (B) male mice. Very similar areas of tubular (ductal) (green contour) and acinar (white contour) atypical hyperplasia are evident in both neu⁺/pfp⁺ and neu⁺/pfp⁻ male
mice. Nodular aggregates of cells resembling cells of intercalated ducts and pseudo-acinar structures composed of enlarged and atypical cells with acinar features are evident. Numerous infiltrating inflammatory cells are in close contact with the hyperplastic lesions (black arrows). (C-N) Distinctive features of salivary carcinomas in 26 to 34-week-old neu+/pfp+ (C-E) and neu+/pfp− (F-N) male mice. In neu+/pfp+ male mice sheets of poorly differentiated duct cells with severe atypical features homogeneously form clinically evident salivary carcinomas (C) with high proliferative activity (D). Clinically evident carcinomas in neu+/pfp− mice display a similarly, predominant poorly differentiated histotype (F) associated with large areas of clear cell (I) and well-differentiated (L) acinic carcinomas whose proliferative activity (J, M) is lower of that of poorly differentiated tumors (G). All the carcinoma histotypes are formed of highly neu+ cells (E, H, K, N). Results are representative of 9 neu+/pfp+ and 3 neu+/pfp− salivary glands analyzed. Magnification x400; insets x1000.

Figure 3. Pfp-dependent effector immune cells equally infiltrate salivary tumors of both neu+/pfp− and neu+/pfp+ male mice. (A and B) Immunohistochemical staining of 16 week old neu+/pfp+ (A) and neu+/pfp− (B) salivary tumors for CD3 showing a significant amount of infiltrating CD3
reactive cells surrounding the hyperplastic lesions. Results are representative of 9 neu+/pfp+ and 3 neu+/pfp− salivary glands analyzed. Magnification x400. (C) Cytofluorimetric analysis of T (CD3+ CD8+), NK (CD3− CD49b+), NKT (CD3+ CD49b+) and γδ T (CD3+γδ+) cells in the salivary tumors of neu+/pfp− or neu+/pfp+ demonstrated that these cells infiltrate both tumors. Graph shows the percentage ± SEM of CD45+ cells expressing the different markers in neu+/pfp− (n = 3; black bars) or neu+/pfp+ (n = 3; white bars) salivary tumors. Three independent experiments were performed and a representative one is shown.

**Figure 4. Salivary carcinogenesis onset is affected by NK cells.** (A) A Kaplan-Meier curve showing the incidence of salivary tumors in neu+/pfp+ male mice reconstituted with neu+/pfp+ BMC (n = 26; black dotted line) or with neu−/pfp− BMC (n = 29; black solid line) or with neu+/pfp+ BMC and treated with anti-asialo GM1 antibodies (n = 7; grey solid line). *, p = 0.02; Mantel-Cox log-rank test. (B-G) Histology and immunohistochemistry showed that salivary carcinomas in neu+/pfp+ males reconstituted with neu+/pfp+ BMC are poorly differentiated with severe atypical features (B) and a high proliferative rate (C) whereas those in males reconstituted with neu+/pfp+ BMC and treated with anti-asialo GM1 antibodies display a predominant poorly differentiated histotype (D) and a high proliferative rate (E) associated with areas of more differentiated acinic carcinoma with clear cell features (F) and lower proliferative activity (G). Results are representative of 4 salivary tumors analyzed for each experimental group. Magnification x400.
Figure 5. Pfp-mediated NK cell activity delays the onset and changes the differentiation of neu+ salivary carcinomas. (A) Kaplan-Meier curve comparing the incidence of salivary carcinomas in neu+/pfp+ male mice treated with anti-asialo GM1 (n = 6; grey solid line) and untreated neu+/pfp+ male mice (n = 5; black solid line) with that of neu+/pfp+ males treated with the control isotype (n = 9; black dotted line). **, p = 0.003, *, p = 0.01, Mantel-Cox log-rank test. (B-J) Histological and immunohistochemical analysis shows that salivary carcinomas arising in neu+/pfp+ males treated with anti-asialo GM1 display poorly differentiated areas (B) with a high proliferative activity (C) and high neu expression (D) coexisting with moderately (E) and well differentiated areas with tubular aspects (H) with lower proliferative activity (F, I) and high neu expression (G, J). Magnification x400.
Figure 6. Pfp deficiency enhances the frequency of mammary tumors in neu+ males. (A) A Kaplan-Meier curve showing male mammary tumor incidence in neu+/pfp+ (n = 68; black dotted line) and neu+/pfp− (n = 37; black solid line) mice. ***, p<0.0001, Mantel-Cox log-rank test. (B) Tumor frequency in neu+/pfp+ (n = 50; white bar) and neu+/pfp− (n = 36; black bar) males measured by whole mount analysis of the 2nd, 3rd and 4th mammary glands. **, p = 0.003, two-tailed unpaired Student’s t test. (C-H) Morphological features of representative mammary tumors in neu+/pfp+ and neu+/pfp− male mice. (C and D) Whole mount images of tumor masses (white asterisks) developed in mammary gland remnants of neu+/pfp+ (C) and neu+/pfp− (D) males consisting of a few, poorly arborized, ducts (arrows). In both groups of mice, neoplastic epithelial cells give rise to aggregates of monomorphic moderately differentiated duct atypical cells surrounded by delicate vascularized stromal bundles (E and F). Immunohistochemistry reveals homogeneous neu positivity (G and H). Whole mount magnification x40; histology and immunohistochemistry magnification x400, insets x1000.
Figure 7. Defective regression of mammary sprouts and potential role of γδ T cells in the involution of mammary glands in male mice. (A) Frequency of mammary remnants found in the areas of 2nd, 3rd, and 4th mammary glands in 20-30 week-old male mice, as assessed by whole mount (neu+/pfp+ n = 292; neu+/pfp− n = 196; neu−/pfp+ n = 150; neu−/pfp− n = 168; neu−/IL15− n = 126). *, p < 0.05, **, p < 0.01, chi-square test with Yates’ correction. (B and C) Immunohistochemistry for γδ T cells in female (B) and male (C) mammary bud at E14.5. The bud is fully formed. The epithelial cells are arrayed in an inverted bulb shape. The mesenchymal cells are arranged in four to five layers in a radial fashion around the epithelial cells. A couple of γδ T cells (arrows) are visible only close the male bud. Magnification x400. (D) Frequency of mammary remnants displaying microscopic tumors in whole mount examined from 20-30 week-old neu+/pfp+ (n = 50) and neu−/pfp− (n = 36) male mice. **, p< 0.01, chi-square test with Yates’ correction.