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Loss of Stat5a delays mammary cancer progression in a mouse model

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A genetic study was conducted to determine if activated Stat5a contributes to mammary carcinogenesis and to evaluate the mechanism. Similar to human breast cancers, a proportion of mammary adenocarcinomas in the WAP-TAg transgenic mouse model demonstrate constitutive Stat5a/b and Stat3 activation. Stat5a activation is linked to mammary epithelial cell survival and differentiation, and proliferation in hematopoetic cell lineages. Breeding WAP-TAg mice to mice carrying germ-line deletions of the Stat5a gene generated mice with reduced levels of Stat5a. Hemizygous loss of the *Stat*5a allele significantly reduced levels of Stat5a expression without altering mammary gland development or transgene expression levels. In comparison to mice carrying two wild-type *Stat*5a alleles, hemizygous loss of the *Stat*5a allele reduced the number of mice with palpable tumors at 7 months of age (67% from 100%, $P<0.05$), resulted in smaller tumors at 7 months of age (3.8 cm^3 from 7.6 cm^3, $P=0.003$), delayed first tumor appearance (208 days from 188 days, $P=0.01$), and increased the apoptotic index in the adenocarcinomas (4.3 ± 0.3 from 1.2 ± 0.2, $P=0.016$). Neither cell proliferation nor differentiation in the cancers was altered. Decreasing Stat5a activation levels could be a therapeutic approach for reducing survival of breast cancer cells. *Oncogene* (2002) **21,** 4335–4339. doi:10.1038/sj.onc.1205484

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Signal transducer and activator of transcription (Stat) 5a is a tyrosine phosphorylated signaling protein that lies downstream of the prolactin-signaling pathway in normal mammary epithelial cells (Hennighausen *et al*., 1997a). 5b is a closely related protein arising from gene duplication (Liu *et al*., 1995). Both proteins can be tyrosine phosphorylated and activated by prolactin in mammary epithelial cells but Stat5a is the more abundant protein and plays the more prominent role in mammary gland development (Hennighausen *et al*., 1997b; Liu *et al*., 1997). Once activated by tyrosine phosphorylation, Stat proteins are translocated from the cytoplasm to the nucleus where they can act as transcription factors (Bromberg and Darnell, 2000; Turkson and Jove, 2000). In normal mammary epithelial cells Stat5a promotes cell survival and its loss leads to accelerated apoptosis during mammary gland involution (Humphreys and Hennighausen, 1999). Stat5a/b are survival factors for early hematopoietic progenitor cells including lymphoid, myeloid and erythroid lineages (Bunting *et al*., 2002; Snow *et al*., 2002; Socolovsky *et al*., 2001; Kieslinger *et al*., 2000). Activation of Stat5a/b contributes to the development of acute and chronic myeloid leukemias (Birkenkamp *et al*., 2001; Maru, 2001; Sternberg *et al*., 2001; Coffer *et al*., 2000; Gouilleux-Gruart *et al*., 1997).

Stat5 is activated in some human breast cancer cell lines and primary breast cancers (Canbay *et al*., 1997; Richer *et al*., 1998; Watson and Miller, 1995). In some cases this activation is prolactin dependent (Canbay *et al*., 1997). However, activation of Stat5a by members of the epidermal growth factor receptor family also has been reported (Gallego *et al*., 2001; Olayioye *et al*., 1999; Jones *et al*., 1999). Like EGF, transforming growth factor (TGF) α signals through the Epidermal Growth Factor Receptor (EGFR). Activation of Stat5a by TGFα in the WAP-TGFα transgenic mouse model retards mammary gland involution and promotes the development of hyperplasia (Humphreys and Hennighausen, 1999).

The physiological activity of the prolactin-Stat5a/5b signaling pathway is dose responsive. The impairment of mammary gland development and lactogenesis observed in mice carrying two deleted Stat5a alleles can be rescued by up-regulation of Stat5b activity through serial pregnancies (Liu *et al*., 1998). Mice carrying one intact allele for the prolactin receptor and one deleted allele cannot lactate due to a partial loss of prolactin receptor (Ormandy *et al*., 1997).

Some human breast cancers and breast cancer cell lines also demonstrate constitutive activation of another Stat family member, Stat3 (Berclaz *et al*., 2001; Bowman *et al*., 2000). In normal mammary epithelial cells Stat3 activation induces apoptosis (Chapman *et al*., 1999). But in the breast cancer cell

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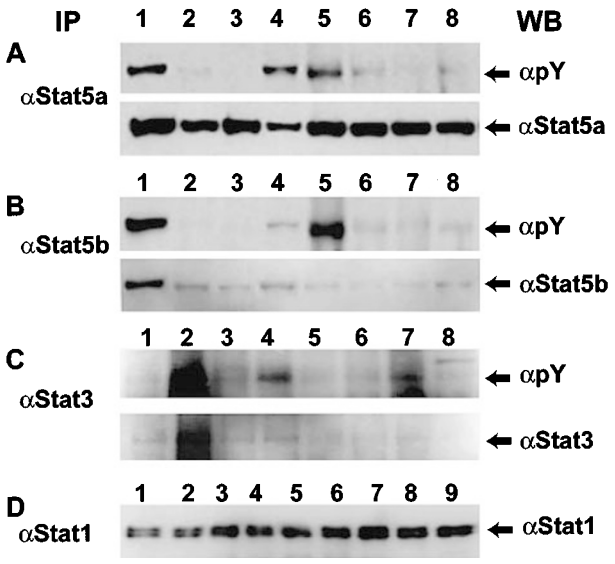

Figure 1 A high percentage of adenocarcinomas from WAP-TAg mice carrying two wild-type *Stat5*a alleles demonstrate tyrosine phosphorylation and activation of Stat5a/5b and Stat3. Stat1 is present but is not activated. (**A**) Analysis of Stat5a protein levels and phosphorylation by immunoprecipitation and Western blot. Lane 1: Positive control, lactating mammary tissue. Lanes 2–8: Seven representative adenocarcinomas from seven individual mice. Varying levels of Stat5a phosphorylation were found in six of the adenocarcinomas shown (lanes: 2, 4–8). A total of 51 individual adenocarcinomas were analyzed for the presence and tyrosine phosphorylation of Stat5a. All adenocarcinomas demonstrated the presence of Stat5a. Tyrosine phosphorylated Stat5a was detected in 44 (86%) of the adenocarcinomas examined. (**B**) Analysis of Stat5b protein levels and phosphorylation by immunoprecipitation and Western blot. Lane 1: Positive control, lactating mammary tissue. Lanes 2–8: Seven representative adenocarcinomas from seven individual mice, the same samples analysed in (**A**). In comparison to Stat5a expression levels, Stat5b expression levels appeared lower but a high percentage of adenocarcinomas demonstrating Stat5a tyrosine phosphorylation also exhibited Stat5b tyrosine phosphorylation (lanes 4–8). A total of 31 individual adenocarcinomas were examined for the presence and tyrosine phosphorylation of Stat5b. All adenocarcinomas demonstrated the presence of Stat5b. Stat5b was tyrosine phosphorylated in 19 (61%) of the adenocarcinomas examined. No adenocarcinomas demonstrated tyrosine phosphorylation of Stat5b in the absence of Stat5a tyrosine phosphorylation. (**C**) Analysis of Stat3 protein levels and tyrosine phosphorylation by immunoprecipitation and Western blot. Lanes 1–8: Eight representative adenocarcinomas from eight individual mice. Varying levels of Stat3 phosphorylation and activation were found in seven of the eight adenocarcinomas shown (lanes 1–7). A total of 16 individual adenocarcinomas were examined for the presence and tyrosine phosphorylation of Stat3. Fourteen (88%) demonstrated the presence of Stat3. Stat3 was tyrosine phosphorylated in all of the adenocarcinomas that expressed Stat3. Twelve adenocarcinomas were examined for both Stat5a and Stat3. Nine (75%) demonstrated tyrosine phosphorylation of both Stat5a and Stat3. Two adenocarcinomas demonstrated Stat5a tyrosine phosphorylation in the absence of Stat3 and one adenocarcinoma demonstrated Stat3 tyrosine phosphorylation in the absence of Stat5a/5b tyrosine phosphorylation. (**D**) Analysis of Stat1 protein levels by immunoprecipitation and Western blot. Lane 1: Positive control, lactating mammary tissue. Lanes 2–9: Eight representative adenocarcinomas from eight individual mice. Stat1 is present (lanes 1–9) but not tyrosine phosphorylated (data not shown). A total of 15 individual adenocarcinomas were examined for the presence and tyrosine phosphorylation of Stat1. Stat1 expression was detected in all 15 adenocarcinomas but there was no tyrosine phosphorylation of Stat1 detected (data not shown). Adenocarcinomas were dissected out at the time of necropsy and isolated from surrounding non-cancerous mammary tissue. Proteins were extracted from frozen mammary tissue and adenocarcinomas and processed for immunoprecipitation and Western blotting as previously published (Li *et al*., 1997) αpY: antibody specific for tyrosine phosphorylation (05321, Upstate Biotechnology, Lake Placid, NY, USA). αStat5a, αStat5b, αStat3, and αStat1: antibodies specific for Stat5a (Liu *et al*., 1996) (sc-1656, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Stat5b (Liu *et al*., 1996), Stat3 (sc-7179, Santa Cruz Biotechnology, Inc.) and Stat1 (sc-346, Santa Cruz Biotechnology, Inc.), respectively

lines studied, constitutive activation of Stat3 is associated with cell growth and survival (Garcia *et al*., 2001). Stat1 has not been identified as having an oncogenic role in breast cancer (Levy and Gilliland, 2000; Schaber *et al*., 1998; Shang *et al*., 1999).

To test the hypothesis that activated Stat5a increases survival of mammary cancer cells a study was performed using genetically manipulated mouse models. The WAP-TAg mouse model of mammary cancer progression was selected because 86% of the adenocarcinomas that develop in this transgenic model demonstrated activation of Stat5a. Coincident activation of Stat5b was found in 61% of these adenocarcinomas and activation of Stat3 was found in 75% of these adenocarcinomas. In contrast, Stat1 was expressed but not activated in the adenocarcinomas (Figure 1).

A selective reduction in Stat5a expression levels was introduced into the WAP-TAg transgenic mouse model by breeding WAP-TAg transgenic mice with mice carrying a germ-line deletion of the *Stat*5a gene. Female mice with two different genotypes were generated: Stat5a+/+/WAP-TAg with wild-type levels of Stat5a expression and Stat5a+/−/WAP-TAg with reduced levels of Stat5a expression (Figure 2A). Each mouse underwent one pregnancy at the age of two months. During initiation of cancer progression by expression of TAg, these two genotypes exhibited equivalent levels of mammary gland development, equal numbers of precancerous cells, and WAP-TAg transgene expression (Figure 2A). Stat5a−/−/WAP-TAg mice were not included in the study because complete loss of Stat5 led to impaired mammary gland development (Liu *et al*., 1997), reduced numbers of precancerous cells, and reduced levels of transgene expression and could not be considered developmentally equivalent to the Stat5a+/+/WAP-TAg mice (data not shown).

Cancer progression was delayed significantly in mice that demonstrated reduced levels of Stat5a expression (Stat5a+/−WAP-TAg). Consistent with earlier reports, one hundred percent of WAP-TAg mice carrying two wild-type *Stat*5a alleles developed palpable mammary tumors by the age of 7 months (Li *et al*., 2000). In contrast, only 67% of the Stat5a+/−/WAP-TAg mice developed palpable tumors by 7 months of age (Figure 3A) (Chi square, $P<0.05$). Moreover, in

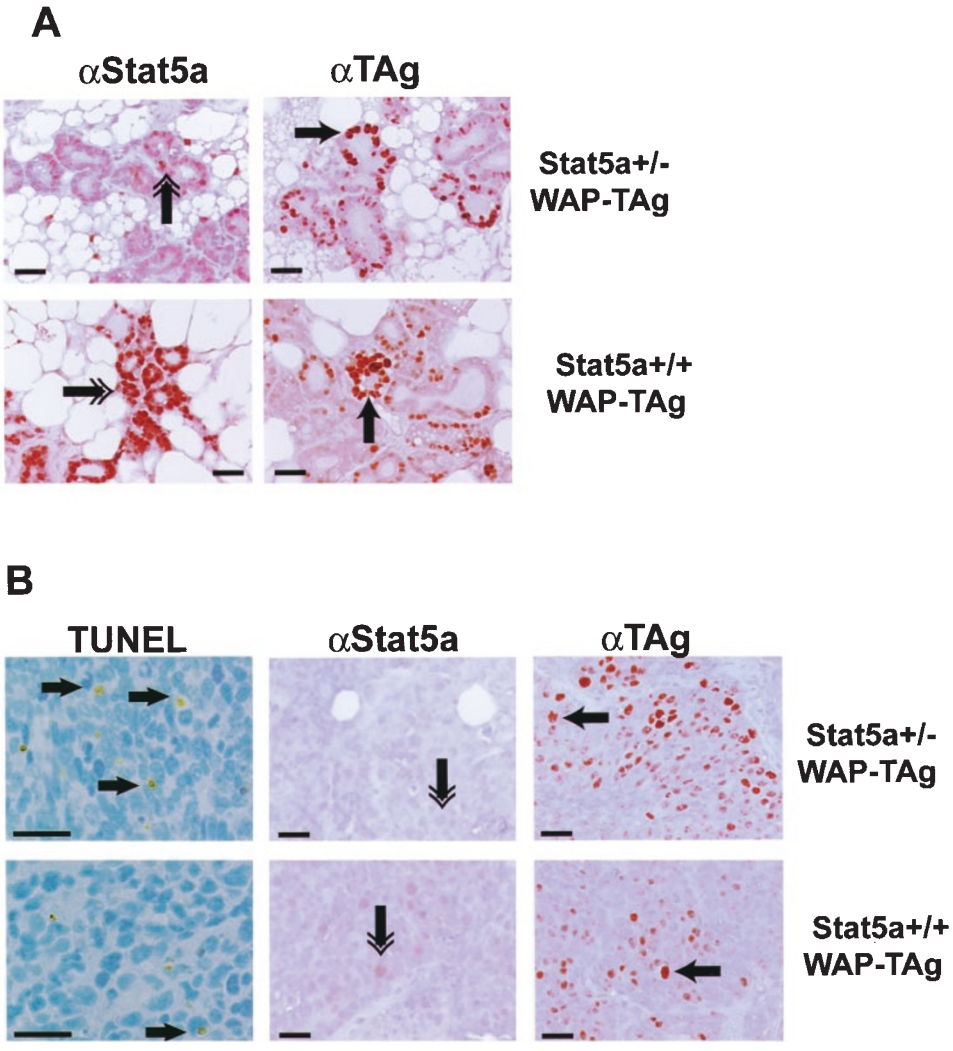

Figure 2 (**A**) Expression of Stat5a and TAg in pre-cancerous mammary tissue from Stat5a+/−/WAP-TAg and Stat5a+/+/WAP-TAg mice. Activated and phosphorylated Stat5a is nuclear localized and indicated by the vertical arrows. Stat5a expression levels but not TAg expression levels are reduced in precancerous mammary tissue from Stat5a+/−/WAP-TAg mice. TAg is nuclear localized and is indicated by rightward horizontal arrows. Mammary gland development is equivalent in the presence of one as compared to two functional *Stat*5a alleles consistent with previous reports (Liu *et al*., 1997). Mammary tissue was taken at day 18 pregnancy. All mice underwent only one pregnancy. (**B**) *In situ* assay for apoptosis and expression of Stat5a and TAg in mammary adenocarcinomas from Stat5a+/−/WAP-TAg and Stat5a+/+/WAP-TAg mice. The percentage of cells exhibiting apoptosis are increased, Stat5a expression levels are reduced, TAg expression levels are not reduced in adenocarcinomas from Stat5a+/−/WAP-TAg mice. Activated and phosphorylated Stat5a is nuclear localized as indicated by the vertical arrows. Leftward horizontal arrows indicate mammary epithelial cells undergoing apoptosis. Rightward horizontal arrows indicate mammary epithelial cells exhibiting nuclear staining for TAg. Tissues collected in 10% neutral buffered formalin, fixed at 4°C overnight, washed in 70% ethanol, encased in a polypropylene-embedding cassette, embedded in paraffin, cut in 5 μm sections, deparaffinized with xylene and rehydrated in a graded series of ethanol washes. Immunohistochemistry for Stat5a and TAg: Endogenous peroxidase activity was quenched by 3% H_2O_2 for 10 min and antigens were unmasked by boiling the sections for 1 min in 10 mM citric acid. Immunohistochemical staining was performed with a 1 : 250 dilution of anti-Stat5a (sc-1656, Santa Cruz Biotechnology, Inc.) and with a 1 : 25 dilution of anti-SV40TAg antibody (sc-147, Santa Cruz Biotechnology, Inc.) using the HistoMouse-SP Kit according to the manufacturer's protocol (Zymed Laboratories, Inc., South San Francisco, CA, USA) and counterstained with hematoxylin. *In situ* assay to identify apoptotic cells: Sections treated with Autozyme (Biomeda, Foster City, CA, USA) for 20 min at 37°C and quenched with 0.15% H_2O_2 for 5 min. Apoptotic cells were identified using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Intergen Company, Purchase, NY, USA). Sections were equilibrated with buffer, incubated with TdT for 40 min at room temperature, washed with wash stop buffer for 30 min, and incubated with anti-digoxigenin for 30 min at room temperature. Color was developed using 0.05% 3,3′-dimethylaminoazobenzene 0.01% H_2O_2 diluted in 0.1 M Tris-HCl (pH 7.5) and sections were counterstained with methyl green. Bars, 20 μm

the Stat5a+/−/WAP-TAg mice that developed a palpable tumor by 7 months of age, the mean age at first palpable tumor was older than that of mice carrying two wild-type *Stat*5a alleles (Figure 3B) (ANOVA, $P=0.01$), and the mean tumor burden volume was reduced (Figure 3C) (ANOVA, $P=0.003$). Adenocarcinomas that develop in the WAP-TAg mouse model can be categorized into five

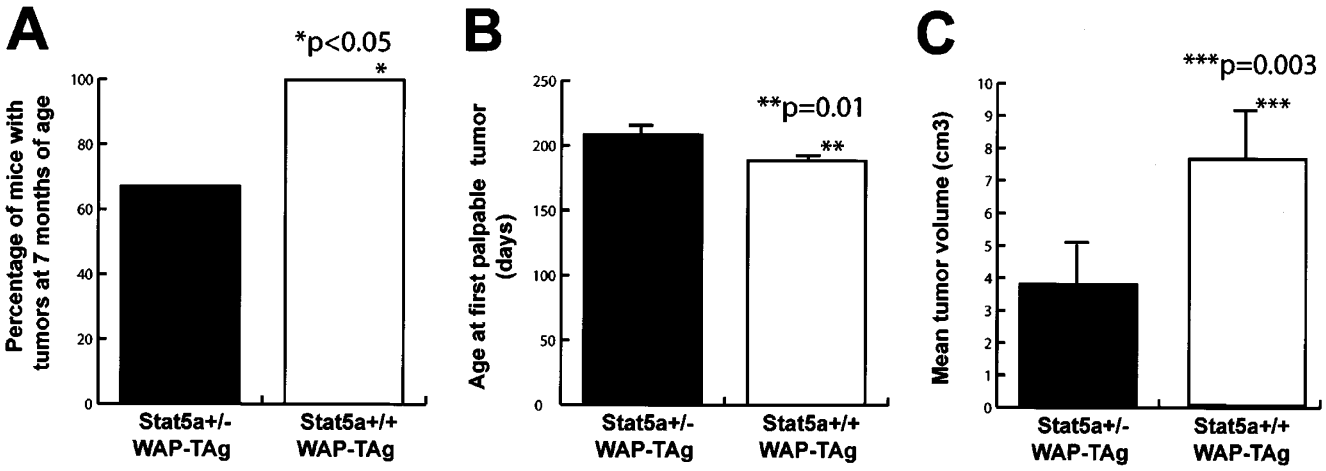

Figure 3 (**A**) The percentage of mice demonstrating palpable adenocarcinomas by seven months of age was reduced in Stat5a+/−/WAP-TAg mice (67%) as compared to Stat5a+/+/WAP-TAg mice (100%), Chi square $P<0.05$. Stat5a+/−/WAP-TAg, $n=29$. Stat5a+/+/WAP-TAg mice, $n=11$. (**B**) In those mice that developed palpable tumors by seven months of age, the number of days to first palpable tumor was increased in Stat5a+/−/WAP-TAg mice (208 days) as compared to Stat5a+/+/WAP-TAg mice (188 days), ANOVA $P=0.01$. Stat5a+/−/WAP-TAg, $n=18$. Stat5a+/+/WAP-TAg mice, $n=11$. (**C**) In those mice that developed tumors by 7 months of age, total tumor volume was reduced in Stat5a+/−/WAP-TAg mice (3.8 cm^3) as compared to Stat5a+/+/WAP-TAg mice (7.6 cm^3), ANOVA $P=0.003$. Stat5a+/−/WAP-TAg; $n=24$. Stat5a+/+/WAP-TAg mice, $n=18$. Mice were generated by breeding WAP-TAg transgenic mice to mice carrying a germ-line deletion of the *Stat*5a gene to generate female mice carrying two wild-type *Stat*5a alleles (Stat5a+/+/WAP-TAg mice) or one wild-type *Stat*5a allele and one inactivated *Stat*5a allele (Stat5a+/−/WAP-TAg mice). Each mouse underwent a single pregnancy. Mice were then followed for the development of palpable tumor, euthanized and necropsied when either the largest palpable tumor was greater than 1 cm^3 in size or the mouse reached seven months of age, whichever came sooner. At necropsy all ten mammary glands were examined and the number and size of each visible tumor was measured and recorded. Some mice developed more than one tumor. Histological analysis of the tumors demonstrated that 100% were adenocarcinomas

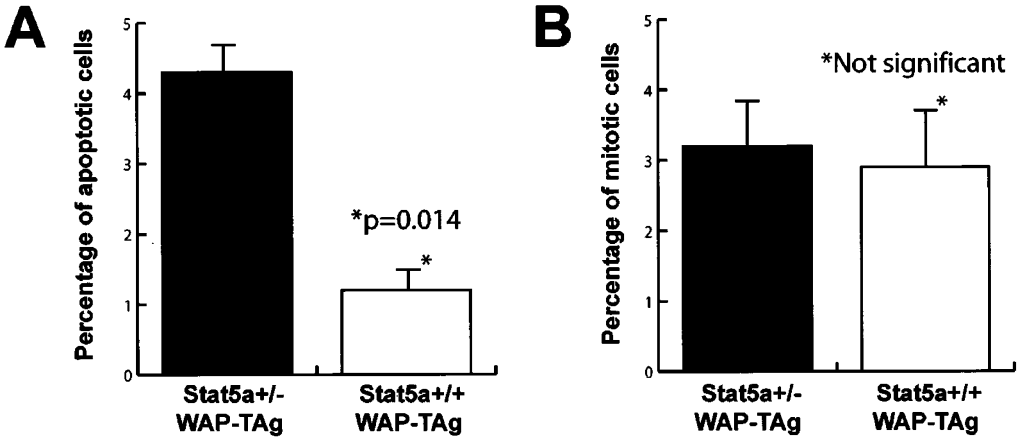

Figure 4 (**A**) The percentage of apoptotic cells was increased in mammary adenocarcinomas from Stat5a+/−/WAP-TAg (4.3%±0.3) as compared to Stat5a+/+/WAP-TAg mice (1.2%±0.2), Mann–Whitney, $P=0.014$. (**B**) There was no significant difference in the percentage of mitotic cells in mammary adenocarcinomas from Stat5a+/−/WAP-TAg (3.2%±0.7) as compared to Stat5a+/+/WAP-TAg mice (2.9%±0.9), Mann–Whitney, $P=0.647$. The percentages of apoptotic and mitotic cells were determined and calculated as previously published (Furth *et al*., 1999). A minimum of 1000 cells was counted for each sample. Percentage of apoptotic cells: Stat5a+/−/WAP-TAg; $n=5$. Stat5a+/+/WAP-TAg mice, $n=4$. Percentage of mitotic cells: Stat5a+/−/WAP-TAg; $n=5$. Stat5a+/+/WAP-TAg mice, $n=6$

different grades ranging from relatively well (Grade 1) to poorly (Grade 5) differentiated. In previous studies 69% of the adenocarcinomas were relatively poorly differentiated Grade 4–5 tumors (Furth *et al*., 1999). Loss of Stat5a did not alter this distribution. Grade 4–5 adenocarcinomas represented 67% of the adenocarcinomas categorized in the Stat5a+/−/WAP-TAg mice and 75% of the adenocarcinomas categorized in the Stat5a+/+/WAP-TAg mice.

To test the hypothesis that a reduction in Stat5a led to increased levels of apoptosis and decreased mammary cancer cell survival, an *in situ* assay for the identification of apoptotic cells was performed and the apoptotic indices were compared in Grade 4–5

adenocarcinomas from Stat5a+/−WAP-TAg and Stat5a+/+/WAP-TAg mice (Figure 2B). The mean apoptotic index was higher in Stat5a+/−/WAP-TAg as compared to Stat5a+/+/WAP-TAg mice (4.3 ± 0.3 compared to 1.2 ± 0.2, Mann–Whitney $P = 0.014$) (Figure 4A). In contrast there was no significant difference in either the mitotic indices (Figure 4B), levels of TAg expression, or state of differentiation (Figure 2B). Levels of Stat5a expression were lower in the Stat5a+/−/WAP-TAg as compared to Stat5a+/+/WAP-TAg mice (Figure 2B).

In summary, loss of Stat5a impaired cancer cell survival and delayed cancer progression in a mouse model of mammary cancer progression. Decreasing Stat5a activation levels could represent a therapeutic approach for reducing survival of breast cancer cells.

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