Six1 Overexpression in Mammary Cells Induces Genomic Instability and Is Sufficient for Malignant Transformation

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Abstract

Homeoproteins are transcription factors that act as master regulators of development and are frequently dysregulated in cancers. During embryogenesis, the Six1 homeoprotein is essential for the expansion of precursor cell populations that give rise to muscle and kidney, among other organs. Six1 overexpression is observed in numerous cancers, resulting in increased proliferation, survival, and metastasis. Here, we investigate whether Six1 can play a causal role in mammary tumor initiation. We show that Six1 overexpression in MCF12A mammary epithelial cells promotes multiple properties associated with malignant transformation, including increased proliferation, genomic instability, and anchorage-independent growth. We further show that this transformation is dependent on up-regulation of its transcriptional target, cyclin A1, which is normally expressed in the embryonic mammary gland but dramatically reduced in the adult gland. Six1-transformed MCF12A cells are tumorigenic in nude mice, forming aggressive tumors that are locally invasive and exhibit peritumoral lymphovascular invasion. In human breast carcinomas, expression of Six1 and cyclin A1 mRNA correlate strongly with each other \( P < 0.0001 \), and expression of Six1 and cyclin A1 each correlate with Ki67, a marker of proliferation \( P = 0.041, 0.0001 \), respectively. Together, our data indicate that Six1 overexpression is sufficient for malignant transformation of immortalized, nontumorigenic mammary epithelial cells, and suggest that the mechanism of this transformation involves inappropriate reexpression of cyclin A1 in the adult mammary gland.

Introduction

The homeobox gene superfamily encodes for transcription factors that play important roles in development (1). As "master regulators" of development, homeoproteins control diverse cellular processes by regulating the expression of many downstream target genes. Thus, it is typical for an individual homeoprotein to confer pleiotropic effects on cell behavior. These effects include alterations in proliferation, survival, migration, and invasion among others (2). Because these processes are also all important in human cancers, misexpression of homeobox genes is likely to play a causal role in tumor onset and/or progression. However, although aberrant expression of numerous homeobox genes has been observed in various solid tumor and hematologic malignancies, it is still unclear if such misexpression is generally a cause or consequence of tumorigenesis (3, 4). In support of a causal role for homeoproteins in the initiation of cancer, overexpression of several homeoproteins has been shown to result in oncogenic transformation of human cells (5–7). Most recently, Oct-4 was shown to play a critical role in the genesis of germ cell tumors, in a dose-dependent manner (8). This elegant study provided further insight into how homeobox genes can be oncogenic, yet distinct from "classic oncogenes" in several ways that make them attractive therapeutic targets: they are tissue (and often developmentally) restricted, they can be associated with early stages of carcinogenesis, and they are sensitive to dosage (9). In this paper, we provide evidence that the Six1 homeoprotein plays a causal role in mammary tumor initiation, leading to highly aggressive mammary tumors in vivo.

The Six1 homeoprotein is essential for the development of numerous organs through its effects on cell proliferation, survival, and cellular migration (10–13). Six1 plays a role in the expansion of precursor cell populations during organogenesis, after which its expression is lost in most adult differentiated tissues (14). Aberrant overexpression of Six1 is observed in human cancers, where it leads to increased proliferation, survival, and metastasis (15–17). The pro-proliferative role of Six1 has been shown in breast and ovarian carcinoma, as well as in rhabdomyosarcoma (15–17). In breast cancer cells, Six1 promotes entrance into and progression through S phase and attenuates the DNA damage–induced G2 cell cycle checkpoint (14, 15). Together, these properties of Six1 may contribute to its role in tumorigenesis.

Several of the known transcriptional targets of Six1 play a role in cell growth and proliferation, including cyclin A1, cyclin D1, and c-myc (10, 15, 18). We have previously shown that the embryonic and tissue-restricted cyclin A1 mRNA is regulated by Six1 in the normal mammary gland (15). Both genes are highly expressed in the embryonic mammary gland but are dramatically decreased in the adult-differentiated mammary gland, suggesting a role for Six1 and cyclin A1 in early mammary gland development (15). Interestingly, cyclin A1 is up-regulated by Six1 in breast cancer cell lines and is critical for the Six1-mediated increase in proliferation in these cells (15). Aside from controlling proliferation, cyclin A1 influences numerous other cellular processes including cell survival, DNA repair, and angiogenesis (19–21). Its role in these processes suggests that cyclin A1 misexpression could contribute to several tumorigenic phenotypes that are found in a spectrum of different tumors. Indeed, cyclin A1 is highly expressed in testicular, ovarian,
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and endometrial cancers (22). It is also overexpressed in acute lymphoblastic and myeloid leukemias (23, 24), and expression of cyclin A1 in the myeloid lineage of transgenic mice results in increased myelopoiesis that progresses to full-blown leukemia in 15% of the mice (25). However, although cyclin A1 overexpression has been observed in breast cancer cell lines (15), its role in human breast cancers has never been formally tested.

Because Six1 is overexpressed in a number of cancers where it regulates proliferation, survival, and metastasis, and because Six1 transcriptionally activates cyclin A1, which can lead to leukemias in mice, we asked whether it could play a causative role in breast tumor development. Here, we show that Six1 expression in MCF12A-immortalized mammary epithelial cells confers multiple properties associated with malignant transformation including increased cell proliferation, genomic instability, anchorage-independent growth, and in vivo tumor formation with invasive properties. We further show that Six1-induced transformation of these cells is dependent on up-regulation of its transcriptional target cyclin A1. Finally, we show that both Six1 and cyclin A1 are coordinately expressed in human breast cancer, and that their overexpression significantly correlates with increased tumor cell proliferation. Together, these data show that inappropriate expression of Six1 in mammary epithelium is sufficient for malignant transformation, and that this effect is mediated at least in part through up-regulation of the transcriptional target cyclin A1.

Materials and Methods

Cell culture, generation of stable cell lines. MCF12A human mammary epithelial cells were cultured, as previously described (26), in either 5% horse serum or 5% fetal bovine serum (FBS). NIH3T3 mouse fibroblasts were cultured as described (27). Stable clones in both of these cells lines were generated by transfection with a Six expression plasmid SIXFL (14) or pcDNA3.1(CAT) (Invitrogen), followed by selection in 400 μg/ml G418. Individual clones were selected and screened for Six1 expression by Northern blot analysis.

Western blotting, kinase assays, and real-time PCR. Western blotting was performed on nuclear extracts as previously described (28). Immunocomplex kinase assays, RNA isolation, and quantitative reverse transcription-PCR were performed as previously described (15).

Proliferation assays. For cell growth assays, MCF12A cells were incubated for 24 h in serum-free medium and then released into the cell cycle by adding back either normal growth medium with 5% horse serum or growth medium containing 0.5% horse serum. At time points after release, triplicate wells for each cell line were stained with 1% toluidine blue in 0.5% borax for 5 min and then lysed with 1% SDS. Absorbance was read at 630 nm to determine relative cell number. Bromodeoxyuridine (BrdUrd) incorporation was performed, as described (15), 24 h after release from a serum-starved synchrony.

Comet assays. To assess relative levels of DNA breaks in MCF12A stable clones, comet assays were performed as previously described (29). Electrophoresis was performed on the agarose-embedded cells in the alkaline rinse solution at a constant 20 V (~40 milliamps) for ~25 min. Images of the embedded cells and comet tails were acquired using a spot camera attached to a fluorescent microscope. Comet tails were analyzed using CometScore software. At least 25 randomly selected comet tails were analyzed for each cell line, and the olive and tail moment was calculated.

Spectral karyotype analysis. Cells were allowed to grow until ~60% confluent and were harvested after mitotic arrest with colcemid (0.05 μg/ml) for 2 h. Hypotonization was performed with 0.075 mol/L KCl, and a 3:1 mixture of methanol and glacial acetic acid was used for fixation. Cell suspensions were dropped and 3-d-old slides digested for 3 min at 37°C in 0.01% of pepsin in 01N HCl and sequentially washed in PBS/MgCl₂, 1% formaldehyde/PBS/MgCl₂, and PBS (pH 7.2). After dehydration, the slides were denatured in 70% Formamide/2× SSC (pH 7.0) at 70°C for 1.5 min and the denatured spectral karyotype (SKY) probe (Applied Spectral Imaging-ASI) was applied. Hybridization in a dry chamber at 37°C was allowed to occur for 48 h. The reporter tags avidin and digoxigenin were detected by Cy5 and Cy5.5-conjugated antibodies according to the manufacturer’s recommendations. Image acquisition was performed using a SD300 Spectracube system (ASI) mounted in an Olympus BX60 microscope with a custom-designed optical filter (SKY-1; Chroma Technology). Cytogenetic abnormalities were designated according to the ISCN 2005 (30).

Results

Six1 expression promotes proliferation in mammary epithelial cells. Six1 is expressed in ~50% of primary breast cancers and is known to have a pro-proliferative and prosurvival role in cancer (15–17). Therefore, we sought to examine whether Six1 plays a causal role in promoting mammary tumorigenesis. To this end, we expressed Six1, or chloramphenicol transferase (CAT) as a
negative control, in MCF12A human mammary epithelial cells. These are immortalized but nontumorigenic mammary epithelial cells capable of forming organized epithelial structures when grown in three-dimensional culture (26). Western blot analysis shows that the Six1-transfected clones (12A-Six1) overexpress Six1 protein compared with the CAT-transfected controls (12A-Ctrl; Fig. 1A, inset).

Because the pro-proliferative role of Six1 has been well-documented in both development and cancer, we first confirmed that Six1 also promotes proliferation of MCF12A cells. As expected, the 12A-Six1 clones grew more rapidly in culture (Fig. 1A), progressed more quickly into S phase and through the cell cycle (Fig. 1B), and showed increased proliferation as measured by BrdUrd incorporation after release from serum starvation (Fig. 1C).

We have previously shown that cyclin A1 is transcriptionally up-regulated by Six1, and that it mediates the proliferative effect of Six1 in breast cancer cells (15). In agreement with these results, cyclin A1 mRNA levels were increased when Six1 was overexpressed in MCF12A cells, and this increase resulted in an increase in cyclin A1–associated, but not cyclin A2–associated, kinase activity (Fig. 1D). Together, these experiments show that Six1 overexpression increases the proliferative rate of nontransformed mammary epithelial cells as well as cancerous mammary epithelial cells.

**Six1 expression promotes genomic instability in mammary epithelial cells.** Genomic instability is a feature of virtually all cancer cells and is proposed to be a basic mechanism underlying tumorigenesis as well as tumor progression (35). Multiple cell cycle checkpoints are critical to maintaining genomic stability by controlling cell cycle progression in response to cellular signals including those induced by DNA damage (36). Six1 expression in breast cancer cells attenuates the G2-M DNA damage checkpoint (14), suggesting that it may increase susceptibility to the accumulation of unrepaired DNA lesions. Additionally, high expression of a transcriptional target of Six1, cyclin A1, can decrease efficiency of DNA repair (19). Therefore, we sought to determine whether Six1 expression in mammary epithelial cells increases genomic instability, providing an additional mechanism by which Six1 could promote tumorigenesis.

Because we predicted that increased expression of cyclin A1 and attenuation of the G2-M checkpoint could cause an increase in unrepaired DNA lesions, we first assayed Six1-overexpressing cells for an increase in DNA breaks using the comet assay (29). We found that 12A-Six1 clones exhibit a statistically significant increase in tail moment compared with the control cells (Fig. 2A), indicative of a higher number of DNA breaks. Because an increase in DNA double-strand breaks (DSB) can promote genomic instability, leading to increased chromosomal breaks and translocations, we next examined these cells for such gross chromosomal abnormalities by spectral karyotype (SKY) analysis (Fig. 2B).

SKY analyses showed that MCF12A cells are hypotriplid with clonal rearrangements involving chromosomes 1, 2, 3, and 8, and numerous numerical imbalances including extra copies of chromosomes 7 and 13. The three 12A-Ctrl lines tested were similar to the untransfected MCF12A cells (Fig. 2Ba and b). In addition to the described anomalies, all three 12A-Six1 clones examined showed two to four new clonal structural abnormalities and two to five distinct single-cell abnormalities (Fig. 2Bc-d and C). In particular, the 12A-Six1-2 clone displayed numerous structures resembling microchromosomes with no visible arms (Fig. 2Bc-d and D). These findings support the hypothesis that Six1 overexpression leads to genomic instability.

**Six1 induces transformation of mouse fibroblasts.** Our results indicate that Six1 promotes multiple properties in...
mammary epithelial cells that are associated with cancer onset and/or progression, including enhancement of proliferation and increased genomic instability. We thus asked whether Six1 could in fact play a causative role in tumor initiation. To this end, we assessed Six1-expressing cell lines for the transformation-associated phenotype of colony growth in soft agar. Because murine cells are more easily transformed than their human counterparts (37), we first tested whether Six1 is capable of transforming mouse fibroblasts. Six1, or CAT as a negative control, was stably expressed in NIH3T3 mouse fibroblasts (Fig. 3A). Similar to what occurs in

Figure 2. Six1 expression increases DNA breaks and genomic instability in MCF12A mammary epithelial cells. A, Six1 increases the incidence of DNA breaks. Comet assays were performed with 12A-Six1 and 12A-Ctrl cells. Twenty-five randomly selected comet tails were analyzed for both tail moment (shown) and olive moment (data not shown). Similar increases were observed for both measurements in the 12A-Six1 cells, compared with the 12A-Ctrl cells, indicating an increased number of DNA breaks. Error bars, 95% confidence interval of the mean. Statistical significance was determined using an unpaired t test to compare all the 12A-Ctrl values to all the 12A-Six values. B to D, SKY analysis shows increased genomic rearrangements in 12A-Six1 cells compared with 12A-Ctrl cells. Ba and b, untransfected MCF12A and 12A-Ctrl clones were found to be hypotriploid as illustrated by a 12A-Ctrl clone in inverted 4,6-diamidino-2-phenylindole (DAPI; Ba) and SKY (Bb) images, with clonal rearrangements involving chromosomes 1, 2, 3, and 8, and extra copies of chromosomes 7 and 13. Bc and d, the three 12A-Six1 clones examined showed two to four new clonal structural abnormalities and two to five distinct single-cell abnormalities (Bc and d). The 12A-Six1-2 clone displayed numerous structures resembling microchromosomes with no visible arms, shown by inverted DAPI (Bc) and SKY (Bd) images. C and D, the karyotype of the same 12A-Six-2 cell is shown in (C) with the numerous minichromosomes shown separately in D.
mammary epithelial cells, Six1 expression in NIH3T3 fibroblast cells induces expression of cyclin A1 (data not shown). This indicates that Six1 is transcriptionally active in fibroblasts and that it can activate this same target gene in both cell types. When 3T3-Six1 cells were plated in soft agar, they grew robustly, indicative of malignant cellular transformation. In contrast, 3T3-Ctrl cells were unable to grow under the soft agar conditions tested (Fig. 3B).

Six1 induces transformation of mammary epithelial cells dependent on cyclin A1. Although primary rodent fibroblasts are easily transformed with as few as two genetic alterations, human cells are more resistant to transformation (37, 38). Therefore, we next determined whether Six1 overexpression is also sufficient to induce the transformation of immortalized mammary epithelial cells. The ability to transform mammary epithelial cells has only been described for one other homeobox gene, HoxA1 (7). Strikingly, we found that Six1, like HoxA1, is able to transform mammary epithelial cells, leading to marked colony growth in soft agar (Fig. 3C and D).

As outlined above, cyclin A1 has been implicated in the regulation of numerous processes associated with tumorigenesis (19–21, 39), and its overexpression in the myeloid lineage of transgenic mice leads to myeloid leukemia (25). We therefore hypothesized that cyclin A1 might, in part, mediate the ability of Six1 to transform mammary epithelial cells. To test this hypothesis, we used RNA interference to stably knock down cyclin A1 expression in 12A-Six1 cells. Each 12A-Six1 clone was transfected with retrovirus-expressing shRNA-targeting cyclin A1 mRNA or a nontargeting control shRNA. The cyclin A1 shRNA reduced cyclin A1 mRNA expression in each of the three 12A-Six1 clones compared with the control shRNA (data not shown). Importantly, this reduced expression of cyclin A1 resulted in a significant decrease in cyclin A1–dependent kinase activity, with no effect on cyclin A2–dependent kinase activity (Fig. 4A). As expected, knockdown of cyclin A1 also decreased proliferation of the 12A-Six1 clones (Fig. 4B). When the 12A-Six1/cyclin A1 knockdown cells were plated in soft agar, colony growth of all three clones was almost completely inhibited (Fig. 4C). These results indicate that up-regulation of cyclin A1 is necessary for Six1-induced transformation of MCF12A cells.

Six1-transformed mammary epithelial cells form highly aggressive, invasive tumors. To show that Six1 can transform MCF12A cells in vivo, we injected 2 × 10⁶ 12A-Six1 or 12A-Ctrl cells into the mammary fat pad of nude mice. Six1 expression induced tumor formation in vivo in all mammary glands injected with 12A-Six1 cells (15 of 15 mice, using 5 mice per each of the 3 clonal isolates), whereas no tumors were observed in the control glands injected with 12A-Ctrl cells (0 of 15 mice, using 5 mice per each of the 3 clonal isolates; Fig. 5A; Supplementary Table S1). On pathologic examination, these tumors resembled high-grade infiltrating ductal carcinoma (Bloom-Richardson grade III of III), characterized by poor differentiation, marked nuclear pleomorphism, and high mitotic activity (Fig. 5Ca and b). In addition, the tumors exhibited local invasion, both of normal mammary epithelial structures and surrounding fibroadipose tissue (Fig. 5Cc). Importantly, invasion of peritumoral lymphatic vessels by tumor cells was observed (Fig. 5Cd), indicating that the Six1-transformed cells are capable of carrying out early steps in the metastatic process (vascular invasion). Thus, strikingly, we find that Six1 not only induces transformation of MCF12A cells in vitro but also leads to the formation of biologically aggressive tumors in a mouse xenograft model. The vasoinvasion that we observed in these tumors is notable, as other transformation-inducing homeobox genes can give rise to locally invasive, but not vasoinvasive, tumors (7, 40).

Oncogenes that induce proliferation typically also induce apoptosis (41) but in ovarian cancer and during development Six1, promotes both proliferation and survival (11, 12, 16). To examine proliferative and apoptotic rates in these Six1-induced

Figure 3. Six1 confers the ability to form colonies in soft agar in fibroblasts and mammary epithelial cells. A, Western blot analysis shows Six1 protein expression in NIH3T3 cells stably transfected with a plasmid expressing Six1 (3T3-Six1) or CAT as a negative control (3T3-Ctrl). Six1 expression in NIH3T3 fibroblasts (B) and in MCF12A mammary epithelial cells (C and D) confers the ability to grow colonies in soft agar. Each cell line was plated in triplicate wells in 0.4% agar and reproduced in multiple experiments. D, representative regions for each clone are shown at higher magnification (~25–30% of each well), highlighting the absence of colonies for 12A-Ctrl clones compared with the robust colony growth in all 12A-Six1 wells.
tumors, we immunostained the tumors with antibodies against Ki67, as a marker of proliferating cells, and for activated caspase-3, as a marker of apoptotic cells. In addition, we immunostained the tumors for Six1 as a marker of proliferating cells and for activated caspase-3.

As previously reported (32), Six1 expression was examined by in situ hybridization and scored as undetectable (0), weak staining (1), and strong staining (2). These staining intensities were considered no expression (0), low expression (1), and overexpression (2). Serial sections from these same tumors were examined for proliferative potential by immunostaining for Ki67 expression. We found that tumors that overexpressed Six1 showed statistically significant increases in percent Ki67–positive cells compared with both the nonexpressing and low-expressing tumors (Fig. 6A and B). Furthermore, there is a significant correlation between Six1 expression level and percent Ki67 staining (P < 0.0001; Supplementary Table S2). These data are consistent with the notion that Six1 overexpression results in highly proliferative breast tumors. Because we have shown that cyclin A1 is a critical mediator of the Six1 effect on both proliferation (15) and transformation (Fig. 4), we further examined these tumor samples for cyclin A1 expression by in situ hybridization. Cyclin A1 mRNA, which is normally not expressed post puberty in the mammary gland (15), was scored as undetectable (0) or expressed (1). As expected, we observed that only a small percentage of the benign breast tissue samples expressed cyclin A1 (3 of 15 or 20%). In contrast, the percentage of breast tumors expressing cyclin A1 (62 of 106 or 58%) is very similar to the percentage that express high levels of Six1 (55 of 106 or 52%; Supplementary Table S2). Importantly, the same tumors that overexpress Six1 generally also show aberrant cyclin A1 expression (Fig. 6A). Of the 76 tumor samples that overexpressed Six1, 66 of them (87%) expressed cyclin A1. When we compared cyclin A1 expression across Six1 nonexpressors, low expressors, and high expressors, we found that the percentage of tumors expressing cyclin A1 increased significantly with increasing Six1 expression (Fig. 6C; P < 0.0001), and that Six1 overexpression strongly correlated with cyclin A1 expression in a statistically significant manner (P < 0.0001; Supplementary Table S2). In addition, those tumors that expressed cyclin A1 had significantly more proliferating cells, as measured by Ki67 staining (Fig. 6A and D), and cyclin A1 expression and Ki67 expression showed a

**Six1 expression correlates with markers of proliferation in human breast cancers.** To investigate whether Six1 expression is associated with highly proliferative breast cancer clinically, we used tissue microarrays of human breast tumor samples to examine Six1 expression concurrent with proliferative markers. Parallel slides were analyzed for Six1, cyclin A1, and Ki67 expression. Data for all three variables were obtained from 130 samples (either primary tumors or metastatic lesions) on the arrays, and these samples were used for all subsequent analyses. Primary tumors from 106 patients were classified based on previous pathologic diagnosis as infiltrating ductal adenocarcinoma (88), adenocarcinoma (4), carcinoma (4), lobular carcinoma (4), scirrhous carcinoma (1), medullary carcinoma (2), squamous cell carcinoma (1), and papillary carcinoma (2). Metastatic lesions were analyzed from lymph nodes (14), liver (4), bone marrow (1), stomach (1), axillary fat (1), lung (1), chest wall (1), and ovary (1).

As previously reported (32), Six1 expression was examined by in situ hybridization and scored as undetectable (0), weak staining (1), and strong staining (2). These staining intensities were considered no expression (0), low expression (1), and overexpression (2). Serial sections from these same tumors were examined for proliferative potential by immunostaining for Ki67 expression. We found that tumors that overexpressed Six1 showed statistically significant increases in percent Ki67–positive cells compared with both the nonexpressing and low-expressing tumors (Fig. 6A and B). Furthermore, there is a significant correlation between Six1 expression level and percent Ki67 staining (P < 0.0001; Supplementary Table S2). These data are consistent with the notion that Six1 overexpression results in highly proliferative breast tumors. Because we have shown that cyclin A1 is a critical mediator of the Six1 effect on both proliferation (15) and transformation (Fig. 4), we further examined these tumor samples for cyclin A1 expression by in situ hybridization. Cyclin A1 mRNA, which is normally not expressed post puberty in the mammary gland (15), was scored as undetectable (0) or expressed (1). As expected, we observed that only a small percentage of the benign breast tissue samples expressed cyclin A1 (3 of 15 or 20%). In contrast, the percentage of breast tumors expressing cyclin A1 (62 of 106 or 58%) is very similar to the percentage that express high levels of Six1 (55 of 106 or 52%; Supplementary Table S2). Importantly, the same tumors that overexpress Six1 generally also show aberrant cyclin A1 expression (Fig. 6A). Of the 76 tumor samples that overexpressed Six1, 66 of them (87%) expressed cyclin A1. When we compared cyclin A1 expression across Six1 nonexpressors, low expressors, and high expressors, we found that the percentage of tumors expressing cyclin A1 increased significantly with increasing Six1 expression (Fig. 6C; P < 0.0001), and that Six1 overexpression strongly correlated with cyclin A1 expression in a statistically significant manner (P < 0.0001; Supplementary Table S2). In addition, those tumors that expressed cyclin A1 had significantly more proliferating cells, as measured by Ki67 staining (Fig. 6A and D), and cyclin A1 expression and Ki67 expression showed a

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**Figure 4.** Six1-induced transformation of MCF12A cells is dependent on cyclin A1. **A,** stable knockdown of cyclin A1 expression via shRNA decreases cyclin A1–dependent, but not cyclin A2–dependent, kinase activity. Immunoprecipitations (IP) were performed with antibodies to cyclin A1 or cyclin A2 and then used for in vitro kinase assays with histone H1 as a substrate. Autoradiographs are shown of phosphorylated histone H1. **B,** cyclin A1 knockdown decreases proliferation of 12A-Six1 clones. BrdUrd incorporation assays were performed 24 h after release from serum starvation. **C,** knockdown of cyclin A1 reverses Six1-induced transformation of MCF12A cells. Colony assays were performed 24 h after release from serum starvation.
statistically significant correlation \((P = 0.014; \text{Supplementary Table S2})\). Together, these clinical data suggest that Six1 induces proliferation via cyclin A1 in human breast tumors, as we have observed in breast cancer cells \textit{in vitro} (15). Combined with our findings that Six1 expression can transform mammary epithelial cells in a cyclin A1–dependent manner (Figs. 3 and 4), our data suggest that Six1-induced expression of cyclin A1 may have a role both in tumor cell initiation and in continued tumor cell proliferation.

**Discussion**

Dysregulation of homeobox genes is commonly observed in human cancers. Although these powerful developmental regulators...
are known to affect numerous processes important in both embryogenesis and tumorigenesis (4), a causal role for homeo-
proteins in tumor initiation and/or progression remains contro-
roversial. The evidence presented in this article that Six1 induces
proliferative activity, genomic instability, and anchorage-indepen-
dent growth in mammary epithelial cells in culture; that it induces
aggressive mammary tumors in vivo; and that its expression
correlates with indices of proliferative activity in human breast
tumors, strongly support an oncogenic role for this gene.
Furthermore, our current data, coupled with previously published
work (15), show that Six1 promotes proliferative activity by up-
regulating Cyclin A1, an embryonic and tissue-restricted cyclin
implicated in cell cycle progression and DNA repair (15, 19, 39, 42).
In addition, both Six1 and cyclin A1 expression strongly correlate
with high levels of proliferation in human breast tumors. Together,
these data support a causal role for Six1 in human breast tumor
initiation and progression, at least in part through increasing
cellular proliferation via up-regulation of cyclin A1.

Six1 is known to induce proliferation and inhibit apoptosis in
a number of contexts both in normal development (10, 12)
and tumorigenesis (16). Although important for the expansion of
progenitor cell populations during embryonic development, an
increase in proliferation coupled with a decrease in apoptosis
would be expected to have dire consequences in an adult
epithelium. Indeed, Six1 expression not only transforms mammary
epithelial cells but also results in tumors that resemble high-grade
human mammary carcinomas, displaying poor differentiation, high
proliferative and low apoptotic rates, invasion into normal
mammary architecture, and vaso-invasion into adjacent lymphatic
vessels. Thus, Six1 not only promotes mammary tumor initiation

Figure 6. Six1 expression correlates with cyclin A1 and Ki67 expression in human breast tumors. Breast tumor tissue arrays were examined for expression of
Six1, Ki67, and cyclin A1 expression. A, representative examples are shown of tumors with no Six1 expression (0; Pt. Ah2), low Six1 expression (1; Pt. Ah3), and
Six1 overexpression (2; Pt. Be6) with their corresponding cyclin A1 and Ki67 levels. Also shown is an example of normal mammary epithelial tissue (normal).
Within each image, the inset number indicates the assigned score for Six1 (0, 1, or 2) or cyclin A1 (0 or 1) staining, or the percent Ki67 staining for that tissue section.
Both Six1 and cyclin A1 levels were examined by in situ hybridization; the negative controls performed with sense probes were completely blank. Ki67 levels were
examined by immunohistochemistry. B, increasing expression of Six1 (from no expression (0), to low expression (1), to overexpression (2)) is associated with an
increasing proliferative rate, as measured by percent Ki67 staining. Points, mean; bars, SE. Error bars, 95% confidence interval of the mean. Statistical significance
between groups was determined by unpaired t test; two-tailed P values are indicated in the figure where significant. C, the percentage of tumors expressing
cyclin A1 increases significantly with increasing Six1 expression. There was a 36.1% increase from nonexpressors (0) to low expressors (1) and a 45.1% increase
from low (1) to overexpressors (2; 0, n = 18; 1, n = 36; 2, n = 76). The statistical significance of the differences between these proportions was calculated by the
2 test based on a contingency table. D, tumors that express cyclin A1 have significantly higher proliferative rates, as measure by Ki67 staining. The data are
represented as described for A.
but also is likely to contribute to subsequent stages of tumor progression. Such a role for Six1 in tumor progression is supported by observations that it is critical for invasion and metastasis in rhabdomyosarcoma (17), and is associated with increased stage in this tumor as well as poor prognosis in both ovarian and hepatocellular carcinoma (16, 43). In breast cancer, Six1 is overexpressed in 50% of primary tumors and in a much higher 90% of metastatic lesions (14, 32), supporting a role for this gene in metastasis. Indeed, our Six-induced MCF12A tumors invade into the lymphovascularature, an early step in the metastatic cascade. Although we did not observe organ metastases in this model, the study was not designed to examine metastasis, and thus, it remains a possibility that Six1 not only initiates breast cancer but transforms them into aggressive, metastatic tumors.

Six1 expression in breast cancer cells is known to attenuate the G2-M DNA damage checkpoint (14), potentially leading to genomic instability (44). Indeed, our data show that inappropriate expression of Six1 results in an increase in DNA breaks and genomic instability, prevalent features of human breast cancers (45). The mechanism by which Six1 induces genomic instability is likely to be complex. Various functions of Six1, in addition to its regulation of the G2 checkpoint (14), can impinge on the integrity of the genome. For example, Six1 induces the expression of several known cell cycle regulators, including cyclin A1 (15), c-myc (10), and cyclin D1 (18). Thus, it is plausibly that unscheduled expression of these cell cycle regulators (as a consequence of Six1 overexpression) may drive the cell cycle inappropriately, bypassing checkpoints, and resulting in genomic instability. In addition, cyclin A1, c-myc, and cyclin D1 may all play roles in genomic instability that extend beyond their ability to control the cell cycle (19, 46, 47). Although we have not observed significant changes in either c-myc or cyclin D1 in MCF12A mammary epithelial cells (these targets of Six1 were identified in other cell types, suggesting cell type–specific regulation of these genes), we have observed significant changes in the levels of cyclin A1 in mammary epithelial cells. The Six1-mediated increase in cyclin A1 expression is of particular interest as in addition to regulating the cell cycle, cyclin A1 has been implicated in DSB repair. Whereas low levels of cyclin A1 are known to mediate DSB repair, high levels of cyclin A1 inhibit it (19). Thus, inappropriate induction of cyclin A1 in response to Six1 overexpression may result in decreased DSB repair, in addition to accelerated cell cycle progression, further contributing to genomic instability.

Importantly, our data show that the tissue and developmentally restricted cyclin A1 is necessary for Six1-induced transformation, and that Six1 and cyclin A1 are coordinately expressed in human breast tumors where they correlate with an increased proliferative index. The role of cyclin A1 in transformation is likely due to its ability to increase cell proliferation (15), in a manner similar to the somatic cyclin A2, as both human A-type cyclins can activate cyclin–dependent kinase (cdk)2 (48). Although not yet shown for cyclin A1, cyclin A2 is a known target of adhesion-dependent signals that control cell proliferation, contributing to its ability to transform cells (49). In support of a critical role for cyclin A/cdk2 interactions in transformation, treatment of cells with Cdk2 inhibitors leads to the loss of anchorage-independent growth mediated by Ros protein tyrosine kinase (50). Therefore, it is possible that misexpression of cyclin A1 also leads to proliferation in the absence of adhesion, a hallmark of malignant transformation.

Of interest, cyclin A1 has properties distinct from cyclin A2 that arguably make it a more interesting oncogene than cyclin A2 (24). For example, cyclin A1 has a unique role in DSB repair (19) that may further contribute to its protumorigenic functions. Most importantly, unlike cyclin A2, cyclin A1 is not expressed in the majority of adult somatic cells (24). Instead, its expression is restricted to early embryogenesis (42) and to hematopoietic progenitors and germ line cells (24). In the mammary gland, cyclin A1 levels are high in the embryonic gland but are dramatically reduced and almost completely absent in the adult virgin and pregnant mammary gland, mirroring the expression pattern of Six1 (15).

In summary, homeobox genes are powerful developmental regulators affecting many cellular processes including proliferation, survival, migration, invasion, and neovascularization (2). It has been postulated that deregulation of homeobox genes leads to an out of context activation of their developmental functions, contributing to tumor initiation and progression (3). Here, we present strong evidence that Six1 overexpression is oncogenic in mammary epithelial cells, promoting proliferation, as it does in development, and promoting genetic instability. Strikingly, Six1-transformed cells form highly aggressive and invasive tumors when injected into the mouse mammary gland, suggesting that Six1 promotes not only tumor initiation but also tumor progression. Overall, our data suggest that aberrant expression of Six1 in breast cells actively promotes tumorigenesis by reactivation of a developmental program out of context. Because this pathway is silent in most normal adult cells, Six1, cyclin A1, or other developmentally and tissue-restricted molecules within this pathway may serve as excellent therapeutic targets to inhibit breast cancer progression.

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