The Src family tyrosine kinases are key modulators of cancer cell invasion and metastasis and a number of Src kinase inhibitors are currently in clinical development for the treatment of solid tumours. However, there is growing evidence that Src is also upregulated at very early stages of epithelial cancer development. We have investigated the role of Src in mouse skin, which is one of the most tractable models of epithelial homeostasis and tumorigenesis. We found that Src protein expression and activity was regulated during the normal hair cycle and was increased specifically during the proliferative anagen phase and also in response to the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). AZD0530, a selective Src inhibitor, prevented the TPA-induced proliferation of basal keratinocytes both in vivo and in vitro. Moreover, treatment with AZD0530 reduced papilloma formation following the well-established 7,12-dimethylbenz(a)anthracene (DMBA) skin carcinogenesis protocol but did not inhibit the subsequent proliferation of the papillomas. Furthermore, AZD0530 did not alter the malignant conversion of papillomas to squamous cell carcinoma suggesting a role for Src in early tumour development in the skin carcinogenesis model, rather than at later stages of tumour progression. Src expression and activity were also seen in human actinic keratoses that are hyperproliferative pre-malignant skin lesions, indicating that Src may also play a role in the early stages of human skin tumour development. Thus, Src inhibitors such as AZD0530 may therefore have chemopreventative properties in patients with hyperproliferative epidermal disorders.

Introduction
Src family kinases (SFKs) are a family of nine non-receptor tyrosine kinases of which Src is the prototype. Src tyrosine kinase expression is frequently elevated in a number of epithelial tumours including colon, breast and pancreas compared with the adjacent normal tissues (1–3). In colon cancer, there is increased Src expression in pre-neoplastic lesions and this has been linked to malignant potential (4–7). Further increases in Src expression are also seen in metastatic tissues, and activating mutations have been found in a small subset of metastatic colon tumours suggesting an additional role for Src in tumour metastasis (8–10). Much interest has therefore evolved around the development of Src kinase inhibitors for the treatment of cancer, although the exact role of Src in tumour progression actually remains unclear (11,12). Src is involved in many aspects of tumour cell behaviour such as proliferation, survival, angiogenesis, migration, invasion and metastasis and a number of small molecule inhibitors are showing potential as both anti-proliferative and anti-invasive agents in preclinical studies in different solid tumour types (13–14). However, nothing is known about the effects of Src kinase inhibitors at the very early stages of disease and also in normal tissue homeostasis.

The skin provides a tractable system in which to study epidermal homeostasis and early events associated with the development of tumours. As increased Src expression and activity has been reported in hyperproliferative epidermal disorders and pre-malignant lesions (15,16), we have used the well-established two-stage mouse skin carcinogenesis model to study the effects of AZD0530 (20), a potent Src kinase inhibitor that is currently in clinical development for the treatment of solid malignancies, on tumour development in the skin. In this model, treatment of dorsal skin with the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) results in H-Ras mutations within the bulge stem cells of the hair follicle. Subsequent treatment with multiple applications of the tumour promoter phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) induces clonal expansion of these initiated cells giving rise to a number of benign, hyperplastic skin lesions or papillomas some of which go on to form carcinomas (21).

Increased Src expression and activity was associated with areas of increased proliferation in the skin and AZD0530 treatment resulted in an inhibition of keratinocyte proliferation and a reduction in the number of papillomas that formed. There was no effect on the malignant conversion of the papillomas suggesting that the predominant role of Src in the skin carcinogenesis model is at the early stages of tumour development.

Materials and methods

Animals
FVB mice were obtained from Harlan, UK. All experiments were carried out in accordance with the United Kingdom Animal Scientific Procedures Act (1986). To induce anagen, dorsal skin was treated topically twice weekly with 6.25 µg of TPA (Sigma, Poole, UK) in 150 µl of acetone for 2 weeks. AZD0530 (provided by AstraZeneca, Alderley Park, Macclesfield, UK) was given by daily gavage as indicated at a concentration of 10 mg/kg using 0.5% hydroxypropylmethylcelulose/0.1% polysorbate as a vehicle.

Chemical carcinogenesis
Chemical carcinogenesis using DMBA and TPA was performed on individual study groups of 20 female 8-week-old animals as described previously (22). The number of benign and malignant tumours was recorded weekly for 45 weeks after DMBA treatment (single dose of DMBA at 25 µg in 150 µl of acetone). No increase in the number of benign tumours was seen after week 20. Groups receiving AZD0530 (10 mg/kg) or the corresponding vehicle were treated by daily gavage from the day of DMBA treatment. To determine the effect of AZD0530 on the carcinoma conversion frequency, papilloma bearing mice were treated with AZD0530 (25 mg/kg) from week 13 and the conversion frequency calculated as the number of carcinomas formed as a percentage of the number of papillomas per mouse in each group.

Immunohistochemistry and quantification of mitosis
Dorsal skin samples were harvested from mice and fixed in neutral-buffered formaldehyde 10% vol/vol (Surgipath Europe Ltd, Peterborough, UK) and then embedded in paraffin. Five micron sections were used for hematoxylin–eosin staining, quantification of mitotic figures and immunohistochemistry. The slides were de-paraffinized, hydrated through increasing alcohol gradients and immersed in 10 mM sodium citrate, pH 6, for epitope retrieval using a 2 min microwave pulse under pressure. Primary antibodies were diluted in Tris-buffered saline/0.01% Tween: Src [1:150, Cell Signalling Technology (Danvers, MA) or 1:100 (clone 2-17), Cancer Research UK (London, UK)], Src pY419 [1:100, Cell Signalling Technology (Danvers, MA) and Kit67 [1:100, Vector Labs (Peterborough, UK) for mouse samples or Dako Cytomation (Glastrup, Denmark) for human samples]. Slides were washed twice in Tris-buffered saline/0.01% Tween followed by visualization using a Cytomation Envision+ kit (Dako Cytomation, Glastrup, Denmark). Histological sections of nine papillomas (four vehicle and five AZD0530 treated) were

Abbreviations: DMBA, 7,12-dimethylbenz(a)anthracene; PBS, phosphate-buffered saline; SFK, Src family kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate.
examined and the number of mitotic figures counted in each papilloma and expressed as the average number of mitoses per high-power field ($\times100$ magnification). Up to 25 high-power fields were examined in each case.

Isolation of primary keratinocytes

Tails were taken from adult 6- to 8-week-old mice, cut lengthwise and the skin was removed. Skins were washed in sterile phosphate-buffered saline (PBS) prior to overnight incubation in dispase (Roche, Basel, Switzerland) (8 mg/ml in PBS). The epidermis was removed, cut into several pieces and trypsinized (0.025% in phycoerythrin buffer) for 10 min at 37°C followed by gentle vortex to remove the cells. Twenty percent of fetal bovine serum in Dulbecco’s modified Eagle’s medium was then added to neutralize the trypsin and the sample put through a cell strainer (70 µm, Becton Dickinson, Oxford, UK). The sample was spun at 1000 r.p.m. for 5 min, the pellet washed with PBS, spun again at 1000 r.p.m and the pellet resuspended in keratinocyte basal medium with the addition of KGM singlequestes (Clonetics, Slough, UK). Cells were plated on collagen I-coated cellware (Becton Dickinson, Oxford, UK).

Proliferation assay

Keratinocytes were grown in 96-well plates and treated with drug vehicle (dimethyl sulfoxide), TPA (20 ng/ml) or a combination of TPA and AZD0530 (1 µM) or TPA and PP2 (10 µM, Calbiochem, San Diego, CA). A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay was carried out as described previously (17) every 24 h after treatment. Results are mean ± SD of four replicate wells taken from a represent experiment in a series of three.

Western blotting

Keratinocytes were washed twice with PBS and lysed in RIPA buffer [50 mM Tris–HCl, pH 7.4, 150 mM sodium chloride, 5 mM ethylene glycol-bis(aminoethyltheretracetate acid, 0.1% sodium dodecyl sulphate, 1% Nonidet P-40 and 1% deoxycholate] with inhibitors [10 mM pyrophosphate, 100 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 100 µM sodium orthovanadate, 10 µg/ml leupeptin and 10 µg/ml benzamidine (all Sigma, Poole, UK)]. Clarification was by high-speed centrifugation (13 000 r.p.m. at 4°C for 15 min). Twenty to forty micrograms of cell lysates were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, transferred to nitrocellulose and immunoblotted as described previously (17).

Human actinic keratoses and squamous cell carcinomas

Eleven archival, formalin fixed, paraffin-embedded actinic keratoses were examined. Lesions were from photo-exposed skin sites of 10 immunosuppressed

Fig. 1. Src expression in mouse skin. (A) Dorsal skin sections from P21 (telogen) and P28 (anagen) mice stained with haematoxylin and eosin (left panels) or Src antibody (right panels). Scale bar 250 µm. (B) Dorsal skin sections from untreated (top panels) and TPA-treated (bottom panels) mice stained with antibodies for Src, activated Src (pY419) or Ki67. Scale bars 100 µm. Higher magnification images (lower panel) show Src localized to the matrix (broken arrows) and the outer root sheath (solid arrows).
renal transplant recipients; four female, six male with a mean age of 56.2 years (range 37–69). Skin squamous cell carcinoma samples (30) of which six had adjacent normal skin samples were taken from a tissue array (Super Bio Chips, Caltag-MedSystems Ltd, Botolph Claydon, UK).

Results

Src expression and activity is increased during anagen
Initially, we examined the expression of Src in dorsal skin during different stages of the first post-natal hair cycle as described in (23). In the resting or telogen follicles (P21), Src expression was low. However, upon onset of anagen (P28), when cells are proliferating, there was a large increase in Src expression (Figure 1A). These levels of Src expression were maintained throughout anagen and were lost as the follicles receded during catagen (data not shown). Anagen can also be induced following treatment with the tumour promoter TPA. This was seen as increased Ki67 positivity in the hair follicles and basal keratinocytes, together with a thickening of the epidermal layer (Figure 1B). TPA treatment resulted in a substantial increase in Src expression within the hair follicles predominantly in the rapidly dividing matrix region (Figure 1B, broken arrows) and also the outer root sheath or bulge region of the follicle (Figure 1B, solid arrows). The antibody used predominantly recognizes Src; however, we cannot rule out the possibility that it reacts with other Src family members expressed in the skin such as Fyn and Yes. We therefore used a Src-specific antibody and showed the same localization of Src in the matrix and outer root sheath of the anagen follicles (supplementary Figure 1 is available at Carcinogenesis Online). The increased expression of Src was accompanied by an increase in Src activity as measured by phosphorylation of Src on tyrosine 419, the autophosphorylation site (Figure 1B). The sequence surrounding the autophosphorylation site in Src is conserved between family members and as such the antibody used in these studies will react with other activated Src family members. So, Src levels and activity are increased as the follicles enter anagen that correlates with a rapid initiation of proliferation within the skin suggesting a link between increased Src expression and activity and the proliferative capacity of keratinocytes in vivo.

AZD0530 inhibits keratinocyte proliferation
As Src was increased in the skin following TPA-induced anagen, we addressed whether the Src kinase inhibitor AZD0530 could inhibit the

![Fig. 2. AZD0530 inhibits keratinocyte proliferation. (A) Five-week-old mice were treated with vehicle or AZD0530 by daily gavage for 2 weeks or treated with TPA to induce anagen in combination with vehicle or AZD0530. Dorsal skin sections were taken and stained with Ki67 antibody. Representative images are shown for each treatment group. Scale bars 50 μm. The number of Ki67-positive basal keratinocytes were counted and expressed as a percentage of the total number of basal keratinocytes in each section (at least 200). Results are expressed as the mean ± SD from three mice. (B) Keratinocytes in culture were treated with vehicle, TPA or TPA in combination with AZD0530 or PP2 and proliferation measured over 3 days. Values are mean ± SD from quadruplicate wells taken from a representative experiment in a series of three. (C) Keratinocytes were treated with AZD0530 for 24 h before preparation of cell lysates and western blot analysis with the indicated antibodies.](image-url)
increased proliferation associated with the onset of anagen. Mice were treated with AZD0530 prior to TPA treatment. There was a significant reduction in the number of Ki67-positive basal keratinocytes in AZD0530-treated animals although this was only evident after TPA treatment (Figure 2A). There was also a reduction in the TPA-induced thickening of the epidermal layer in the AZD0530-treated animals (Figure 2A). We also looked at the effects of AZD0530 on the proliferation of primary basal keratinocytes in culture. As is seen in vivo, TPA treatment resulted in enhanced proliferation of keratinocytes in culture and treatment with AZD0530 could block this by ~40% (Figure 2B), demonstrating that the drug had an inhibitory effect on epidermally derived keratinocytes when placed in culture. No
AZD0530 does not prevent malignant conversion. (A) The carcinoma conversion frequency was calculated as the number of carcinomas formed as a percentage of the number of papillomas in vehicle- and AZD0530-treated animals. (B) The number of papillomas was counted in each treatment group over 20 weeks and the results presented as the average number of papillomas per mouse. Daily AZD0530 treatment was started at week 13 (shown by arrow).

To distinguish between the effect of AZD0530 on early events and those directly linked to malignant conversion, AZD0530 treatment was started in another cohort of mice when papillomas had already formed (week 13) and the number of carcinomas counted. In the control group, 13 animals developed carcinomas compared with 11 in the AZD0530-treated animals. The carcinoma conversion frequency, which is a measure of the number of carcinomas as a function of the total number of papillomas for each mouse, was unaltered between the vehicle (median = 3.571) and AZD0530 (median = 3.448)-treated animals groups ($P = 0.7820$) (Figure 4A). Furthermore, fewer new papillomas formed after AZD0530 treatment was started at week 13 (Figure 4B) resulting in a significant difference in the number of papillomas in the AZD0530-treated animals compared with control animals at the end of the experiment ($P = 0.0054$). Thus, new papilloma formation was blocked in animals treated with AZD0530 and taken together, this supports a role for Src activity in the early stages of tumour development.

AZD0530 reduces chemically induced papilloma formation in mice

Given the above results, we hypothesized that AZD0530 treatment may reduce the number of papillomas that develop in the DMBA/TPA skin carcinogenesis model due to an inhibition of the TPA-induced clonal expansion of the DMBA-initiated progenitor cells. We therefore treated animals with AZD0530 and the number of papillomas was monitored over a 20 week period. There was a reduction that was statistically significant, in the number of papillomas that developed per mouse in the AZD0530-treated animals (Figure 3A) but there was no difference in the number of animals that developed papillomas or the time of onset of papilloma formation (results not shown). Using Ki67 as a marker of proliferation, we found no visible difference in proliferation of the papillomas in the AZD0530-treated animals compared with controls (Figure 3B). This was confirmed by counting mitotic figures in the papillomas (Figure 3C). A loss of Src autophosphorylation on tyrosine 419 confirmed inhibition of Src activity in the papillomas of mice treated with AZD0530 (Figure 3D). Thus, AZD0530 does not inhibit proliferation of established papillomas and the data suggest a role for AZD0350 in suppressing expansion of initiated cells in response to TPA treatment resulting in a reduction in the development of new papillomas.

A number of papillomas that form in the chemical carcinogenesis model convert to squamous cell carcinomas, and we saw an overall reduction in the number of mice that developed carcinomas in the AZD0530 treatment group (Figure 3E). Analysis of the data using the Pearson’s chi-square test showed a significant difference in the number of carcinomas that developed between the control and AZD0530-treated groups ($P = 0.001$) and between the vehicle- and AZD0530-treated groups ($P = 0.003$) but not between the no treatment and vehicle-treated groups ($P = 0.504$). Although there was a delay in the onset of carcinoma conversion in the AZD0530-treated animals, this did not reach statistical significance (Figure 3E).

Src expression in human actinic keratoses and squamous cell carcinomas

To assess whether Src was associated with the early stages of human skin carcinogenesis, we looked at Src expression in a number of human actinic keratoses that are pre-malignant skin lesions with potential to progress to squamous cell carcinoma. Immunohistochemical analysis of normal human skin showed Src staining predominantly in the basal layer of the epidermis (Figure 5 C and D). As the cells migrate upwards and become more differentiated, the staining intensity was reduced. The zone of Src expression (or the number of cells expressing Src) was increased in the actinic keratosis with more extensive staining seen throughout the lesions, which was predominantly membranous (Figure 5G and I). Src activity was also found throughout the lesions and was both membranous and cytoplasmic (Figure 5H and J). Using Ki67 as a marker of proliferation, the actinic keratoses showed increased and disordered proliferation compared with normal skin. Ki67-positive cells were present in both basal and suprabasal layers (compare Figure 5B and F). Thus, although the intensity of Src staining in individual cells was not increased in the actinic keratoses, expression was seen throughout the lesion unlike the restricted pattern of staining seen in normal skin. A similar pattern of expression was found in all the samples that we looked at and the staining pattern in additional lesions can be found in supplementary Figure 2 (available at Carcinogenesis Online).

Src expression was also examined in a panel of 30 human skin-derived squamous cell carcinomas (Figure 6). Src was expressed uniformly throughout the carcinomas: in well-differentiated squamous cell carcinomas, Src staining was membranous with little or no cytoplasmic staining (Figure 6A), moderately differentiated tumours showed both membranous and cytoplasmic staining (Figure 6B),
whereas in poorly differentiated tumours the staining was predominantly cytoplasmic (Figure 6C). In six cases, normal adjacent skin sections were also available and although the level of expression (staining intensity) in individual cells was not increased in the corresponding squamous cell carcinomas as compared with the normal tissue, Src expression was seen throughout the tumours rather than the restricted expression of Src seen in normal skin (Figure 7). The 30 tumours were scored for Src expression using a three-point system with expression being distributed between low, medium and high intensities (supplementary Figure 3 is available at Carcinogenesis Online). High levels of Src expression in the tumours did not always correlate with increased activity as measured by phosphorylation of Src on tyrosine 419 (results not shown). There was also no correlation between tumour grade and levels of Src expression.

**Discussion**

Although there are reports of elevated Src expression in pre-malignant lesions in the epidermis and colon and also hyperproliferative disorders such as psoriasis (4,18,19), the biological importance of these observations and whether it represents a potential therapeutic target has never been addressed. We have used mouse skin as a model that allowed us to examine the effect of a clinically relevant Src-selective kinase inhibitor, AZD0530, not only in tumour development but also normal epithelial homoeostasis. We show that Src expression and activity correlates with the proliferative capacity of skin keratinocytes and that treatment with AZD0530 inhibits keratinocyte proliferation both *in vivo* and *in vitro*. Furthermore, we show that treatment with AZD0530 can inhibit the formation of papillomas in the mouse skin carcinogenesis model but not proliferation of established papillomas,
suggesting that AZD0530 can target the very early transition to hyperplasia. As there was a reduction in the number of papillomas that form rather than a complete block, AZD0530 may suppress expansion of a subset of target cells to form papillomas. It is well established that different subsets of papillomas exist in this model and that target cells for initiation can reside in both the bulge region within the hair follicle and also the interfollicular region (25,26). As Src is predominantly expressed within the bulge region, it is possible that the effects, if AZD0530, are restricted to these cells. Further studies are required to establish this.

It has been known for many years that Src is required for the cell cycle progression of non-transformed cells in vitro. Early studies showed that Src is required for growth factor-induced mitogenesis of quiescent fibroblasts in a pathway that involves transcriptional activation of Myc (27–29). Furthermore, activated Src abrogates the requirement of Myc in the G<sub>0</sub>-G<sub>1</sub> transition in fibroblasts (30). Activation of Myc in keratinocytes also mobilizes stem cells (31,32) and Myc-deficient mice are resistant to DMBA/TPA-induced skin carcinogenesis (33) but it is not known whether activation of Src is linked to Myc during the normal mobilization of stem cells from a quiescent state in the skin.

As well as assessing the effects of a clinically relevant drug in these studies, the use of the Src kinase inhibitor AZD0530 has allowed us to overcome the problems of redundancy and embryonic lethality when the ubiquitous SFKs, Src, Fyn and Yes are simultaneously deleted in mice (34). However, as AZD0530 inhibits the activity of all three kinases (IC<sub>50</sub> Src = 2.2 nM; Yes = 4 nM and Fyn = 10 nM against isolated enzyme preparations), it does not allow us to determine which individual kinases are responsible for the effects we have observed. Furthermore, the sequence surrounding the autophosphorylation site in Src is conserved between family members and as such the antibody used in these studies will react with other activated Src family mem-

Fig. 6. Src expression in human skin squamous cell carcinomas. (A–C) Human skin squamous cell carcinomas stained with haematoxylin and eosin (left hand panels) or an antibody to Src (right hand panels). (A) Well-differentiated, (B) moderately differentiated and (C) poorly differentiated squamous cell carcinoma. Scale bars 100 μm.

Fig. 7. Src expression in human skin squamous cell carcinomas and normal adjacent skin. (A–D) Human skin stained with an antibody to Src and the corresponding squamous cell carcinomas from the same patients are shown (A’–D’). Scale bars 200 μm.
TPA-treated skin (results not shown). Reports of Fyn-specific effects in keratinocytes in vitro and in vivo indicate that there may be functions attributable to individual family members (35,36) and experiments are currently underway to address this.

We saw no role for Src in the later stages of tumour progression which is in line with other studies in the skin where activation of endogenous SFKs, either through conditional inactivation of Csk, the endogenous negative regulator of SFKs, or overexpression of individual Src family members, gave rise to epidermal hyperplasia but not to spontaneous carcinomas (37–40). K5-Csk mice did form spontaneous papillomas; however, these did not convert to carcinomas (37). Over expression of Src can be sufficient in some circumstances to induce the formation of spontaneous squamous cell carcinomas (39), but a significant number of these were at sites of wounding suggesting that Src co-operates with other pathways such as epidermal growth factor receptor signalling or inflammatory signals to induce malignant conversion (39–41).

Numerous reports have demonstrated that the proliferation of the majority of epithelial tumours is not dependant on Src kinase activity (17,42,43). Furthermore, the ability of Src kinase inhibitors like AZD0530 to inhibit the migration and invasion of tumour cell lines (44) (and our unpublished data) suggests that they may be effective anti-invasive agents. However, we have identified a role for Src in the proliferation of epidermal keratinocytes and the development of pre-cancerous lesions. Taken together with the observation that Src is expressed throughout very early pre-malignant lesions in human skin, it is therefore intriguing to speculate that as well as the predicted anti-invasive effects of AZD0530, it may also have effects at very early stages of disease initiation and perhaps be a useful chemopreventative agent in skin cancer in high risk groups. The actinic keratosis samples that were used in this study were taken from immune-compromised patients following renal transplantation. This cohort of patients is more susceptible to developing such lesions that can convert to squamous cell carcinomas and may therefore benefit from such chemopreventative strategies. A greater understanding of the pathways that Src regulates in vivo will undoubtedly aid the clinical development of this exciting class of new therapeutics that targets SFKs.

Supplementary material
Supplementary Figures 1, 2 and 3 can be found at http://carcin.oxfordjournals.org/

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