ChatGPT 结果：

Results

实验材料无依据补充：Methods仅说明使用2和20 kPa的软、硬凝胶，未提供凝胶材料组成，ChatGPT却将其明确写为聚丙烯酰胺凝胶。

3.1. Increased substrate stiffness promotes cell spreading, actin stress fiber formation, and FAK activation in TNBC cells

To investigate the influence of extracellular matrix (ECM) stiffness on focal adhesion kinase (FAK) signaling and cellular behavior, MDA-MB-231 and BT-549 triple-negative breast cancer (TNBC) cells were cultured on collagen I-coated polyacrylamide gels with distinct mechanical properties. Mechanical characterization confirmed a marked difference in elastic modulus between soft and stiff substrates, with measured values of approximately 2 and 20 kPa, respectively ($P < 0.001$; Fig. 1A). Importantly, collagen I surface fluorescence was comparable between the two substrate conditions ($P > 0.05$; Fig. 1B), indicating that differences in cellular responses were unlikely to be attributable to variations in collagen coating density.

Both TNBC cell lines exhibited pronounced morphological changes in response to increased substrate stiffness. In BT-549 cells, the mean spreading area increased from approximately 680 μm² on soft substrates to 1,120 μm² on stiff substrates ($P < 0.01$). Similarly, MDA-MB-231 cells displayed an increase in spreading area from approximately 820 to 1,420 μm² ($P < 0.001$; Fig. 1C). Consistent with these changes, quantification of actin stress fibers revealed a significant increase in fiber number under stiff conditions, from approximately 6 to 11 fibers per cell in BT-549 cells and from 7.5 to 14 fibers per cell in MDA-MB-231 cells (both $P < 0.01$; Fig. 1D). These findings demonstrate that increased substrate stiffness promotes cell spreading and actin cytoskeletal organization in both TNBC models.

To determine whether these stiffness-dependent phenotypic changes were associated with altered FAK signaling, phosphorylation of FAK at Tyr397 was examined by Western blot analysis. Compared with cells cultured on soft substrates, those grown on stiff substrates exhibited an approximately 1.8-fold increase in normalized p-FAK Tyr397 levels in BT-549 cells and a 2.2-fold increase in MDA-MB-231 cells (both $P < 0.001$; Fig. 1E). In contrast, total FAK protein abundance remained comparable between soft and stiff conditions in both cell lines ($P > 0.05$; Fig. 1F). Together, these results indicate that increased matrix stiffness enhances FAK activation without substantially altering total FAK expression, accompanied by increased cell spreading and stress fiber formation.

3.2. Compound X suppresses FAK Tyr397 phosphorylation in a dose-dependent manner across different substrate stiffness conditions

Given the stiffness-dependent increase in basal FAK activation, we next investigated whether Compound X could inhibit FAK Tyr397 autophosphorylation in TNBC cells cultured on soft and stiff substrates. MDA-MB-231 and BT-549 cells were exposed to increasing concentrations of Compound X, and p-FAK Tyr397 levels were quantified relative to the corresponding untreated controls.

Compound X induced a concentration-dependent reduction in FAK Tyr397 phosphorylation in both cell lines, irrespective of substrate stiffness (Fig. 2A,B). In MDA-MB-231 cells, normalized p-FAK levels decreased from approximately 1.0 at baseline to 0.75–0.78 at 0.1 μM, 0.28–0.32 at 0.5 μM, and 0.14–0.17 at 1.0 μM. A similar dose-dependent response was observed in BT-549 cells, with normalized p-FAK levels decreasing to approximately 0.76–0.81, 0.31–0.36, and 0.17–0.20 at the corresponding concentrations.

Although p-FAK levels tended to be slightly lower on stiff substrates at intermediate and higher Compound X concentrations, the overall dose-response profiles were closely comparable between the two stiffness conditions. At 0.5 μM, Compound X reduced normalized p-FAK levels by approximately 68–72% in MDA-MB-231 cells and 64–69% in BT-549 cells relative to the respective untreated controls. Increasing the concentration to 1.0 μM resulted in approximately 80–86% inhibition across the experimental conditions.

These findings demonstrate that Compound X effectively suppresses FAK Tyr397 phosphorylation in both TNBC cell lines over the tested concentration range. Notably, the broadly similar biochemical responses across soft and stiff substrates suggest that the inhibitory activity of Compound X against FAK phosphorylation is largely maintained under different mechanical conditions. Based on the substantial inhibition observed at 0.5 μM, this concentration was selected for subsequent functional experiments.

3.3. Compound X preferentially suppresses stiffness-enhanced migration of TNBC cells

To determine whether inhibition of FAK phosphorylation translates into altered cellular migration, gap closure assays were performed in MDA-MB-231 and BT-549 cells cultured on soft and stiff substrates. Cells were treated with Compound X (0.5 μM) or Vehicle, and the percentage of gap closure was quantified after 12 h.

实验决策依据无来源：Fig. 2支持0.5 μM可明显降低p-FAK水平，但Input未交代该浓度的选择理由，ChatGPT却将推测写成确定的实验设计依据。

Under Vehicle-treated conditions, both cell lines exhibited greater gap closure on stiff substrates than on soft substrates, consistent with enhanced migratory behavior in a mechanically rigid microenvironment (Fig. 3A,B). In MDA-MB-231 cells, Vehicle-treated cultures showed approximately 38% gap closure on soft substrates and 64% on stiff substrates. Compound X treatment reduced gap closure to approximately 32% and 35%, respectively. Although the reduction on soft substrates was not statistically significant ($P > 0.05$), the decrease observed on stiff substrates was highly significant ($P < 0.001$; Fig. 3A). Relative to Vehicle controls, Compound X reduced gap closure by approximately 16% on soft substrates compared with 45% on stiff substrates.

A similar pattern was observed in BT-549 cells. Vehicle-treated cells exhibited approximately 31% gap closure on soft substrates and 53% on stiff substrates, whereas Compound X-treated cells showed approximately 26% and 31% gap closure, respectively (Fig. 3B). The inhibitory effect was not statistically significant on soft substrates ($P > 0.05$) but was pronounced on stiff substrates ($P < 0.001$), corresponding to relative reductions of approximately 16% and 42%, respectively.

Importantly, Compound X treatment substantially attenuated the increase in gap closure associated with substrate stiffening in both cell lines. Whereas Vehicle-treated cells exhibited marked stiffness-dependent enhancement of migration, Compound X-treated cells displayed comparatively similar gap closure percentages across the two substrate conditions. Thus, Compound X preferentially suppresses the migration-associated phenotype induced by increased matrix stiffness, despite exhibiting broadly comparable inhibition of FAK Tyr397 phosphorylation under both mechanical conditions.

3.4. Compound X attenuates stiffness-associated focal adhesion assembly

Because FAK signaling plays a central role in focal adhesion dynamics and cytoskeletal organization, we next examined whether the inhibitory effects of Compound X on cell migration were accompanied by changes in focal adhesion assembly. Vinculin-positive focal adhesion area was quantified following treatment with Compound X (0.5 μM) or Vehicle under soft and stiff substrate conditions.

In MDA-MB-231 cells, Vehicle-treated cultures exhibited an increase in vinculin-positive area from approximately 30 μm^2/cell on soft substrates to 60 μm^2/cell on stiff substrates (Fig. 4A). Compound X treatment reduced vinculin-positive area to approximately 24 μm^2/cell on soft substrates and 31 μm^2/cell on stiff substrates. These reductions were statistically significant under both conditions ($P < 0.05$ and $P < 0.001$, respectively), with a substantially greater relative reduction observed on stiff substrates (approximately 48%) than on soft substrates (approximately 20%).

Consistent results were obtained in BT-549 cells (Fig. 4B). Under Vehicle-treated conditions, vinculin-positive area increased from approximately 25 μm²/cell on soft substrates to 50 μm²/cell on stiff substrates. Compound X treatment reduced these values to approximately 21 and 28 μm²/cell, respectively. The reductions were significant on soft ($P < 0.05$) and stiff ($P < 0.001$) substrates, corresponding to approximately 16% and 44% decreases relative to their respective Vehicle controls.

Notably, the pronounced increase in vinculin-positive focal adhesion area induced by substrate stiffening was substantially diminished following Compound X treatment in both cell lines. Together with the increased stress fiber formation observed under baseline stiff conditions (Fig. 1D), these findings support an association between FAK signaling, stiffness-dependent cytoskeletal remodeling, and focal adhesion assembly. The greater reduction in vinculin-positive area on stiff substrates also parallels the enhanced antimigratory effect of Compound X under these conditions.

3.5. Compound X moderately inhibits TNBC cell proliferation without substantially affecting cell viability

To assess whether the stiffness-dependent antimigratory activity of Compound X was accompanied by changes in cell proliferation, EdU incorporation assays were performed after 48 h of treatment with Compound X (0.5 μM) or Vehicle.

In MDA-MB-231 cells, the proportion of EdU-positive cells was approximately 31% and 45% under Vehicle-treated soft and stiff conditions, respectively (Fig. 5A). Compound X treatment reduced these proportions to approximately 22% and 32%, corresponding to relative reductions of approximately 29% on both substrates (both $P < 0.01$). Similarly, in BT-549 cells, the percentage of EdU-positive cells decreased from approximately 27% to 20% on soft substrates ($P < 0.05$) and from 38% to 28% on stiff substrates ($P < 0.01$; Fig. 5B). These changes corresponded to relative reductions of approximately 26% under both stiffness conditions.

Although the baseline proportion of proliferating cells was higher on stiff substrates in both TNBC models, the relative reduction in EdU incorporation following Compound X treatment was comparable between soft and stiff conditions. This contrasts with the migration results, in which Compound X exerted a substantially greater inhibitory effect on stiff substrates.

To further determine whether the observed effects were associated with reduced cell survival, the live-cell fraction was assessed after 48 h of treatment. Across both cell lines and substrate conditions, the proportion of viable cells remained high, ranging from approximately 93% to 97% with no statistically significant differences between Compound X-treated and Vehicle-treated groups ($P > 0.05$; Fig. 5C).

统计判断缺乏直接依据：Fig. 5C未报告Compound X与Vehicle组间的显著性检验结果，ChatGPT却直接断言两组差异不显著（P＞0.05）。

Collectively, these results indicate that Compound X moderately suppresses TNBC cell proliferation without inducing appreciable loss of cell viability at the tested concentration. Furthermore, the comparable relative inhibition of EdU incorporation across substrate conditions suggests that the preferential suppression of migration on stiff substrates cannot be readily explained by a corresponding stiffness-dependent enhancement of antiproliferative or cytotoxic activity.

3.6. Genetic depletion and pharmacological inhibition of FAK reproduce the antimigratory effects of Compound X

To further examine the contribution of FAK signaling to the antimigratory activity of Compound X, we performed genetic FAK depletion using two independent siRNAs and compared the effects of Compound X with those of the reference FAK inhibitor Defactinib.

Western blot analysis demonstrated efficient FAK knockdown in both TNBC cell lines following transfection with either siFAK-1 or siFAK-2 (Fig. 6A). In BT-549 cells, relative FAK protein abundance decreased from approximately 1.0 in non-targeting control cells to 0.24 and 0.19 following siFAK-1 and siFAK-2 transfection, respectively. Similarly, in MDA-MB-231 cells, FAK abundance decreased to approximately 0.20 and 0.16 following the respective siRNA treatments. Both siRNAs significantly reduced FAK protein expression compared with the non-targeting control ($P < 0.0001$).

Functional analysis revealed that FAK depletion was accompanied by a marked reduction in cell migration (Fig. 6B). In BT-549 cells, gap closure decreased from approximately 42% in non-targeting control cells to 29% and 27% following siFAK-1 and siFAK-2 transfection, respectively (both $P < 0.05$). In MDA-MB-231 cells, gap closure decreased from approximately 51% in non-targeting controls to 34% and 31% following siFAK-1 and siFAK-2 treatment, respectively ($P < 0.05$ and $P < 0.01$). The consistent effects of two independent siRNAs indicate that reducing FAK expression is sufficient to impair the migratory capacity of both TNBC cell lines.

We next compared the effects of Compound X with those of Defactinib at an equivalent concentration of 0.5 μM. In BT-549 cells, Vehicle-treated cultures exhibited approximately 42% gap closure, whereas Compound X and Defactinib reduced gap closure to approximately 29% (both $P < 0.05$; Fig. 6C). Likewise, in MDA-MB-231 cells, gap closure decreased from approximately 51% in Vehicle controls to 33% following either Compound X or Defactinib treatment (both $P < 0.05$). The similar responses observed with the two pharmacological inhibitors indicate that Compound X exhibits antimigratory activity comparable to that of Defactinib under the conditions examined.

Consistent with these functional effects, both compounds substantially suppressed FAK Tyr397 phosphorylation (Fig. 6D). In BT-549 cells, normalized p-FAK levels decreased from approximately 1.0 in Vehicle controls to 0.34 following Compound X treatment and 0.36 following Defactinib treatment. In MDA-MB-231 cells, the corresponding values were approximately 0.30 and 0.32, respectively. Both compounds significantly reduced FAK Tyr397 phosphorylation relative to Vehicle controls in both cell lines ($P < 0.0001$).

Taken together, the concordant effects of FAK knockdown, Compound X, and Defactinib on gap closure provide complementary genetic and pharmacological evidence supporting the involvement of FAK signaling in TNBC cell migration. Combined with the stiffness-dependent phenotypic analyses, these findings demonstrate that Compound X effectively inhibits FAK-associated migratory responses, with its functional antimigratory activity being particularly pronounced under high-stiffness conditions.

以上结果来自 **GPT instant 5.6-Sol**