Hedgehog and Notch Signaling Regulate Self-Renewal of Undifferentiated Pleomorphic Sarcomas

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Running title: Hedgehog and Notch in Sarcoma

Key Words: Hedgehog, Notch, Undifferentiated Pleomorphic Sarcoma, Tumor Initiating Cell, Xenograft

Grant Support: This work was funded by the Ontario Institute of Cancer Research, the Canadian Cancer Society Research Institute, and the Canadian Institutes of Health Research
Abstract:

Like many solid tumors sarcomas are heterogeneous and include a small fraction of so-called side population (SP) cells with stem-like tumor-initiating potential. Here we report that SP cells from a soft tissue tumor of enigmatic origin termed undifferentiated pleomorphic sarcoma (also known as malignant fibrous histiocytoma or MFH sarcoma) displays activation of both the Hedgehog and Notch pathways. Blockade to these pathways in murine xenograft models this human cancer decreased the proportion of SP cells present and suppressed tumor self-renewal, as illustrated by the striking inability of xenograft tumors subjected to pathway blockade to be serially transplanted to new hosts. In contrast, conventional chemotherapies increased the proportion of SP cells present in tumor xenografts and did not affect their ability to be serially transplanted. SP cells from these tumors displayed an unexpectedly high proliferation rate which was selectively inhibited by Hedgehog and Notch blockade, compared to conventional chemotherapies. Together, our findings deepen the concept that Hedgehog and Notch signaling are fundamental drivers of tumor self-renewal, acting in a small population of tumor-initiating cells present in tumors. Further, our results suggest not only novel treatment strategies for deadly recurrent unresectable forms of this soft tumor subtype, but also potential insights into its etiology which has been historically controversial.

Precis:

Findings suggest not only novel treatment strategies for a soft tumor subtype seen almost exclusively in the elderly, but also possible insights into its enigmatic origins of development which have been historically controversial.
Introduction

Cytological heterogeneity is common in neoplasia (1). The various cellular subpopulations differ in their morphologic, biochemical, genetic, and karyotypic characteristics(2). Subpopulations of cells with an enhanced capacity to initiate tumours when transplanted into immunodeficient mice are termed tumor initiating cells, and are thought to be responsible for self-renewal of the neoplasm. These tumor initiating cells have the ability to recapitulate the original morphology of primary tumors upon serial transplantation in mice and have been identified in a variety of tumor types(3-8). Sarcomas are composed of a heterogeneous population of cells with mesenchymal characteristics. They contain a putative tumor initiating cell subpopulation that is identified by their ability to efflux Hoechst333242 dye. Such cells fall to the side of the main cell population when examined using flow cytometry, and as such are termed side population (SP) cells(9). Only a small minority of cells from sarcomas are SP cells, yet these cells have the capacity to recapitulate the original heterogeneous cellular morphology of the primary tumors upon serial transplantation in NOD/SCID mice(9). Both tumor initiating cells and SP cells are hypothesised to play a role in the resistance to therapies. Neural and breast cancers that are resistant to conventional radiation and chemotherapy often contain a higher proportion of tumor initiating cells(10, 11). In some cancer types, tumor initiating cell subpopulations are found to be resistant to conventional chemotherapies(12). The efflux of Hoechst 333242 dye is in part regulated by ABC transporters, and these transporters also can efflux chemotherapeutic agents such as doxorubicin (13), vinblastine (14), paclitaxel (15), imatinib mesylate (16), topotecan (17) and
methotrexate (18). This property could make them resistant to therapies, and as such, this subpopulation of cells could be responsible for tumor recurrence following initial treatment.

Tumor initiating cells share characteristics with stem cells as well as with the progenitor cell from which the tumor arises. In the case of sarcomas, such a progenitor cell type could be a MSC (mesenchymal stromal or stem cell). Indeed, previous studies show that mesenchymal tumors can arise from targeting expression of an oncogenic mutation in an MSC population (19-21). Developmentally important signalling pathways act to maintain MSCs in a less differentiated state, and thus might also contribute to the maintenance of tumor initiating cells in sarcomas. Such pathways include the hedgehog signalling pathway and the notch signalling pathways, both of which act to inhibit MSC differentiation (22-24).

Undifferentiated pleomorphic sarcoma (previously termed malignant fibrous histiocytoma), is the most common form of soft tissue sarcoma. The survival rates for undifferentiated pleomorphic sarcoma have changed little in the past decades (25-27) and as such, innovative therapeutic approaches are needed. Because of the frequent occurrence of this tumor and the lack of progress in improving survival, we studied the SP cells from these lesions for characteristics which might be therapeutically targeted. Here we compared the gene expression profiles between SP and non-SP cells in undifferentiated pleomorphic sarcomas to identify molecular mechanisms that could be responsible for the differences in behavior between SP and
non-SP cells, and to identify potential pharmacologic candidates for the targeting this subpopulation of cells.

**Materials and Methods**

**Primary Tumors**

Undifferentiated pleomorphic sarcoma tumor tissue was obtained at the time of the initial biopsy. Pathology was verified by an independent experienced musculoskeletal pathologist, and for the absence of staining for routine markers use to identify sarcoma subtypes. The samples were mechanically dissociated and then digested using 10mg/ml of collagenase IV (Worthington), 2.4 U/ml of Dispase (Becton Dickinson), and 0.05% trypsin (Wisent), with constant rotation 37°C for 60 minutes. Cells were then centrifuged at 1400 rpm for 10 minutes and washed three times in PBS. After washing, cells were strained through 70μm filters, and collected cells were counted and either directly used for SP analysis or for xenograft injections into mice.

**Xenografts in immunodeficient mice**

After dissociation of primary tumors, the harvested cells were collected and re-suspended at the concentrations of 1 million cells per 50μl of DMEM and chilled on ice. This cell suspension was mixed with an equal volume of ice-cold Matrigel (Becton Dickinson) and 10,000 cells were
subcutaneously injected into eight to ten week old NOD/SCID mice. Each mouse was engrafted at a single injection site. After injection, the mice were observed for up to 15 weeks.

**Flow Cytometry**

For SP assays, cells were treated either alone with 2.5 \( \mu \text{g/ml} \) of Hoechst 33342 dye (Sigma), or in combination with 50 \( \mu \text{M} \) of verapamil (Sigma), for 90 minutes at 37\(^\circ\)C. The cells were then counterstained with 1\( \mu \text{g/mL} \) of propidium iodide (PI, Molecular Probes) and PI positive (non-viable) cells were excluded from analysis. To detect for SP, cells were analyzed by using a dual wavelength analysis (blue, 424-444nm; red, 675nM) after excitation with 350nm UV light (MoFlow, Cytomation or LSRII, Becton Dickinson). In all cells isolated from xenografts, murine cells were first excluded using staining with a biotin conjugated anti-mouse H-2k antibody (BD pharmaningen) at a concentration of 1:2000 for 15 minutes at 4\(^\circ\)C, and streptavidin PE-Cy7 conjugate (Invitrogen) at the concentration of 1:2000 for 15 minutes at 4\(^\circ\)C (28). The proportion of annexin V stained cells was analyzed in the various cell populations as previously reported (29) using an annexin V-FITC antibody (Invitrogen). To measure cell proliferation, BrdU at concentration of 100\( \mu \text{g/kg} \) was injected intraperitoneally into mice bearing human tumor xenografts. Sorted cells were prepared as cytospins and fixed with FixDenat (Roche Applied Sciences) for 40 mins at RT. The cells were then incubated in a mixture of mouse anti-BrdU antibody (1:100 dilution, DAKO cytomation) and DNAase-1 (1u/ul, invitrogen) at 4C in a humidified chamber. A biotinylated secondary antibody (Vector laboratories) and avidin-linked peroxidase (Vectastain Universal Elite ABC kit, Vectorlabs) were used to detect binding of the antibody.
**Primary cell cultures from xenografted tumors**

Individual cells were isolated from the tumor mass from cells two undifferentiated pleomorphic sarcomas that were established as xenografts in mice using the identical procedure as for isolation of the primary cells. The anti-mouse H-2k antibody (BD pharmingen) was used to exclude cells from the mouse as described above. The cells were plated into individual culture dishes, and initially maintained in DMEM plus 5% fetal calf serum. To verify that the cell cultures were representative of undifferentiated pleomorphic sarcoma, 10,000 cells were transplanted into nod-scid mice which were observed for ten weeks, then sacrificed. Histology showed that the tumors that developed from the cell cultures were representative of the primary tumor (Supplemental Fig. 1A and B). GLI2 is a potent activator of hedgehog mediated transcription, mice lacking Gli2 show a similar phenotype to that seen in mice lacking hedgehog ligands, and its overexpression causes neoplasia in transgenic mice(30). As such we used overexpression of GLI2 to activate hedgehog mediated transcriptional activity in the cell cultures. Lipofectamine™ (Invitrogen) was used to transfect cells with either pCMV/SV-FLAG-GLI2, or an empty vector as previously reported(31). Western analysis using an antibody to FLAG was used to confirm successful transfection and expression. The culture media was changed to media lacking serum for 24 hours, after which they were then treated with cycloamine, DAPT (both at 10^{-4} mol/L), or carrier for 3 days in the serum free media. At the end of the treatment, the cells were analyzed for the level of expression of Hh and Notch target genes, and the proportion of cells sorting to the SP. Cell culture experiments were performed in triplicate for each tumor cell culture.
Expression Analysis

Tumor tissues were cryopreserved in liquid nitrogen and RNA was extracted using Trizol (Invitrogen) and quality assessed with a Bioanalyzer (Agilent Technologies). In the case of sorted cells, they were centrifuged at 1200 rpm for 10 minutes, and RNA was extracted from cell pellets using RNeasy Mirco Kit (Qiagen). cDNA was generated using Superscript II (Invitrogen). For real-time RT-PCR, gene human specific Taq-Man fluorogenic probes for GLI-1, PTCH1, HES-1, HEY-1, and HEY-2 were used. We performed standard quantitative RT-PCR reactions for all probes on the ABI 7900HT (Applied Biosystems) system. Asparagine synthetase (AS) and glyceraldehyde-3-phosphate (GAPDH) were used as internal control genes. For gene profiling, cDNA was generated and hybridized onto Affymetrix Human Genome U133 Plus 2.0 gene chips. The expression of SP cells from each tumor was then normalized to the expression of the corresponding non-SP cells. A gene list was constructed by selecting genes that were regulated in the same direction (SP vs. non-SP) in at least 8 out of the 10 sample pairs with a fold change greater than 1.25. This list was examined using Genespring® GX software (32), to identify differentially regulated pathways. Expressions of individual differentially regulated genes were verified using quantitative RT-PCR.

Pharmacologic treatment of mice

Triparanol was delivered via oral gavaging at a dose of 400mg/kg,. Cyclopamine or DAPT were delivered via intraperitoneal injection at 10mg/kg each day. Doxorubicin or cysplatinum were delivered via intraperitoneal injection at a dose of 5mg/kg or 8mg/Kg respectively each day. The drugs were administered for a total of three weeks. 24 hours after the final treatment the mice
were euthanized and xenografts were harvested and weighed. Tumors were dissected free from surrounding tissues and weighed. Sections of the tumors were then fixed in formalin for histology or cryopreserved in liquid nitrogen for RNA extraction, while the rest of the tumors were prepared as individual cells using the same method described for primary tumors. In some of the mice BrdU was injected 6 hours before sacrifice. Some of the harvested cells were used for molecular analysis, as well as for xenograft injections into additional immunodeficient mice.

Secondary xenografts from tumors from the mice were established from suspensions of 10,000 cells in a similar manner as for the primary tumors. After injection, the mice were observed for up to 20 weeks to assess for tumor formation. At the end of the 20 weeks, the mice were euthanized and any tumors that developed were counted.

**Immunohistochemistry**

Tumor samples were formalin fixed and paraffin embedded. Serial sections of 5 μM were deparaffinized, rehydrated through an alcohol gradient to water and subject to heat induced antigen retrieval in 10 mM citrate for 20 min. Endogenous peroxide activity was blocked with 3%(v/v) peroxide in methanol for 15 min at room temperature and nonspecific binding was blocked with 2% (v/v) normal horse serum (Vectorlabs) and 2% (w/v) BSA (Sigma Aldrich) in PBS for 30 min. Primary antibody to human HES1 (Novus Biologicals Cat#NBP1-47791, Littleton, CO) or human GLI1 (R&D systems Cat# AF3324, Minneapoolis, MN) was incubated overnight @ 4°C. Biotinylated secondary antibody and avidin-linked peroxidase (Vectastain Universal Elite ABC kit, Vectorlabs) were used to detect binding of the antibody. Normal rabbit
and goat serum were used on control sections. Antibody binding was detected with DAB (Vectorlabs). TUNEL staining was used to detect apoptotic cells in tissue sections, using the Apoptag-Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon International). Proliferation was evaluated by immunostaining using antibodies against Ki-67 (DakoCytochemical).

Results

The Hh and notch signalling pathways are activated in SP cells from undifferentiated pleomorphic sarcomas.

To examine if there are genes that are differentially regulated in SP cells in undifferentiated pleomorphic sarcomas, we compared gene expression profiles between SP and non-SP cell populations from ten primary tumors. The tumor cells were mechanically and enzymatically dissociated into individual cells, hematopoietic cells depleted, and tumor cells sorted after Hoechst dye staining into SP and non-SP fractions as previously reported (9). cDNA was generated from total RNA extracted from an equal number of cells from each population, which was hybridized onto Affymetrix Human Genome U133 Plus 2 gene chips. Genes differentially regulated between the SP and non-SP in the array were verified using real time RT-PCR for twenty genes in each sample. The data from the arrays is deposited in the GEO database.
(accession numbers GSE24199 and GSE32375). A gene list comparing expression levels between the SP and non-SP population was constructed by selecting genes that were differentially regulated in the same direction in at least eight of the ten pairs with a change of greater than 1.25 fold (Supplemental data). Analysis of the gene list using Genespring® GX software (32), identified that the Hedgehog, Notch, Wnt, TGF-beta, Interleukin, Kit, EGF, and Alpha6 Beta4 Integrin pathways were significantly differentially regulated between the SP and non-SP population (p values 0.01, 0.02, 0.03, 0.03, 0.04, 0.05, 0.05, and 0.05 respectively).

Because the Notch and Hedgehog (Hh) pathways are implicated in tumorigenesis, will maintain MSCs in a less differentiated state, and can be targeted using a pharmacologic strategy, we selected them for further analysis. The expression of transcriptional targets of the Hh pathway (GLI1, PTCH1, and HHIP) and notch signalling (HES1, HEY1, and HEY2) were compared between the SP and non-SP populations using real time RT-PCR from five additional tumors. There was an up-regulation in the expression of GLI1, PTCH1, and HHIP (Figure 1A), and an fold up-regulation in the expression of HES1, HEY1, and HEY2 (Figure 1B) in the SP fraction compared to the non-SP fraction, verifying that Hh and Notch pathways are activated in the SP cells. Since Hoechst dye treatment alone can regulate gene expression, we tested additional tumor cultures and found no change in expression of these genes tested with Hoechst dye itself.
Since we found that the Hh and notch pathways were activated in SP cells compared to non-SP cells, we then examined tissue sections from undifferentiated pleomorphic sarcomas to determine if we could detect a subpopulation of cells expressing Hh and notch target genes in-situ. Tissues sections from tumors were examined using immunohistochemistry for GLI1 or HES1. For both proteins, we identified a subset of cells, representing roughly 3% of the total number of tumor cells stained using these antibodies (Fig. 1C and 1D). Because the expression of these genes is upregulated in SP cells, this subpopulation of positively stained cells likely represents cells that sort to the SP from the undifferentiated pleomorphic sarcomas.

*Inhibition of Hh or notch signalling reduces the proportion of SP cells in undifferentiated pleomorphic sarcomas.*

The SP is enriched for tumor initiating potential in sarcomas, and the proportion of cells in this subpopulation does not substantially vary between the primary tumor and tumors that develop from serial transplantations of isolated cells into immunodeficient mice (9). To determine if the modulation of Hh or Notch signalling might alter the proportion of cells that sort to the SP, we established undifferentiated pleomorphic sarcoma xenografts in NOD/SCID mice and treated the mice with agents which inhibit activation of these pathways. Serial dilutions of the undifferentiated pleomorphic sarcoma cells were performed and implanted into the NOD/SCID mice, and it was found that 10,000 undifferentiated pleomorphic sarcomas cells were the minimum number that would reproducibly engraft and form a tumor in the immunodeficient
mice within four weeks. As such, 10,000 tumor cells from five separate tumors were injected into a subcutaneous location along with matrigel into at least twenty NOD-SCID mice for each tumor, to establish a single xenograft tumor in each mouse. Four weeks following transplantation a tumor could be identified under the skin by palpation in the mice. Some of the mice were used for treatment with conventional chemotherapeutic agents. The remainder were then treated with one of two agents that block hedgehog signalling (Triparanol (33, 34) or cyclopamine (35)); an agent that blocks Notch signaling (DAPT (36, 37)); or a vehicle as a control. Four mice were used for each treatment group, and in tumors in which there were not enough cells to establish grafts in more than twenty mice, we did not include treatment with cyclopamine. Thus, we only have data from two of the primary tumors treated using cyclopamine. After three weeks of treatment, the mice were sacrificed and the tumors harvested. Total RNA was extracted from the tumor tissue, and real-time RT-PCR analysis using human specific primers was used to verify that triparanol or cyclopamine inhibited the expression of downstream transcription targets in the Hedgehog signalling pathway (Fig. 2A), and that DAPT successfully inhibited the expression of downstream transcription targets in the Notch pathway (Fig. 2B). To examine the effect of blockade of the Hh and notch pathways on the proportion of cells that are maintained in the SP, we performed SP analysis on cells obtained from the harvested xenograft tumours post-treatment. For each tumor, the proportion of SP cells was significantly reduced compared to tumors treated with carrier only (Fig. 2 C and D). Data from the use of cyclopamine was similar to that obtained using triparanol, resulting in a 62% reduction in the proportion of cells which sorted to the SP.
Hh or notch signalling can have paracrine effects on tumor growth, by regulating non-cancerous stromal cell activity and secondarily altering tumor cell behavior, and stromal cells could express these ligands influencing tumor cell growth (38,39). To determine if Hh or notch signalling modulation has autocrine effects on undifferentiated pleomorphic sarcoma cells, we examined sarcoma cells from two tumors established as xenografts in mice. Individual cells were isolated from the tumor mass and mouse cells were excluded using an anti-mouse H-2k antibody. Cells from these cultures formed tumors with a histology representative of the primary tumor when transplanted into nod-scid mice. The cells were plated into multiple culture dishes, and studied in their second passage in culture at which time they were treated with cyclopamine, DAPT, or carrier for 3 days. At the end of the treatment, the cells were analyzed for the level of expression of Hh and Notch target genes, as well as the proportion of cells sorting to the SP. We found that treatment with these agents decreased the level of expression of \textit{GLI1}, \textit{PTCH1}, \textit{HES1}, and \textit{HEY1}, compared to cultures treated with the carrier. The proportion of cells sorting to the SP declined more than 50% with treatment with either cyclopamine or DAPT (Supplemental Fig. 1).

Since the pharmacologic agents we used could have off target effects, we determined if overexpression of the hedgehog activated transcription factor GLI2 would regulate the proportion of cells sorting to the SP and counteract the effect of cyclopamine, as GLI2’s transcriptional activity is downstream of the effect of cyclopamine(30). We found that overexpressing Gli2 increased the proportion of cells sorting to the SP compared to cells transfected with an empty vector or control cell cultures. When cell cultures overexpressing Gli2 were treated with cyclopamine, there was only a minor change in the proportion of cells sorting
to the SP (Supplemental Fig. 2). Thus, these agents will effect target gene expression and the proportion of cells sorting to the SP in primary cell cultures, as well as in xenografts. This supports the notion that Hh and notch signaling has an effect on the tumor cells themselves, suggesting that the effect we observed on the SP is not completely due to a paracrine effect mediated by non-cancerous stromal cells, or to altering the numbers of non-cancerous stromal cells sorting to the SP.

*Undifferentiated pleomorphic sarcoma growth is regulated by Hh and notch signalling.*

To examine if inhibition of Hh or notch pathways had an effect on the overall tumor growth, we weighed tumors that were harvested after three weeks of treatment. Triparanol, cyclopamine, or DAPT treatment significantly reduced the weight of the xenografted tumors (Fig. 3). There was not a significant difference in overall weight or health of the mice treated with the drugs compared to vehicle treated controls.

*Hh and notch signalling regulates undifferentiated pleomorphic sarcoma self renewal.*

To determine if pharmacologic modulation of Hh or notch signalling would inhibit tumor initiating potential in undifferentiated pleomorphic sarcomas, we examined the ability of the
cells from tumors that developed in the mice treated with these agents to form new tumors when serially transplanted into additional immunodeficient mice. Cells were harvested from each treatment group of the five distinct undifferentiated pleomorphic sarcoma xenograft lines and 10,000 cells from each line and treatment group were re-transplanted into ten additional NOD/SCID mice using the identical protocol as used to establish the primary grafted tumors. Mice were observed for gross appearance of tumors each week for three months, and then sacrificed to determine the size and number of tumors that formed. When a tumor reached 1.5 cm in size (the maximum size allowed by our animal care committee) the mice were sacrificed. Cells from tumors that formed in these mice were then transplanted into additional mice and analyzed in an identical manner as in the primary xenografts to determine if a tertiary transplanted tumor would form. All of the mice that were transplanted with tumors treated with carriers formed tumors on secondary transplantation, and all of the mice needed to be scarified before the end of the observation period because the tumor reached 1.5cm in diameter. Tertiary transplantation in these tumors also resulted in tumor development in the recipient mice in every case. In stark contrast, in less than half the cases, secondary transplantsations from tumors grown in mice treated with either Notch or Hh blockade formed tumors. All of the tumors grew to a smaller size than in control mice, and none of the mice needed to be sacrificed before the end of the observation period. We therefore expressed the results of the secondary transplantation using a Kaplan Meier survival curve for the development of a tumor by palpation, and using analysis at autopsy to determine the number of mice with tumors at the time of sacrifice (Fig. 4; Table One). This showed a highly significant difference in the rate of development of tumors between the treatment and control groups. None of the tumors from mice treated with triparanol,
cyclopamine, or DAPT formed tumors upon tertiary transplanted into additional immunodeficient mice. This behaviour is in stark contrast to what was observed in control tumors, and shows that these drugs are effective in depleting the tumour initiating potential of undifferentiated pleomorphic sarcoma cells. This observation is especially striking given the relatively short duration of treatment of the mice with these agents during the primary transplant.

Treatment of xenograft undifferentiated pleomorphic sarcomas with conventional chemotherapy does not reduce their serial transplantation potential

To determine if the effects of Hh or notch blockade are a consequence of targeting the tumor initiating potential, or related to a more general effect from an inhibition of tumor cell viability, we examined the ability of the tumors from the mice treated with convention chemotherapies to form tumors when serially transplanted into immunodeficient mice. Mice with established undifferentiated pleomorphic sarcoma xenografts were treated in an identical manner as used for Hh or notch blocking agents, but treated instead with doxorubicin or cisplatinum. After three weeks of treatment, the tumor size was significantly reduced compared to control. However, there was a significant increase in the proportion of SP in tumors in mice treated with traditional chemotherapy compared the control (Fig.5). 10,000 cells from tumors that developed in mice following both treatments were re-transplanted into additional NOD/SCID mice. Mice were observed for gross appearance of tumors each week for 3 months, and then sacrificed to determine the number of tumors that ultimately formed. In contrast to tumors that formed in mice
treated with hedgehog or notch blocking agents, secondary transplantation of the tumors from these mice resulted in tumor growth in all the cases. These conventional chemotherapeutic agents did not inhibit the ability to form tumors upon serial transplantation, suggesting that the effect observed from hedgehog and notch blockade is related to their ability to specifically target these pathways in the SP cell fraction.

*SP cells from undifferentiated pleomorphic sarcomas have a high proliferation rate, and Hh and notch signalling preferentially inhibits SP cell proliferation.*

Since the SP represents a small subpopulation of undifferentiated pleomorphic sarcoma, it is possible that the difference in bulk tumor size is due to a difference in proliferation or apoptosis rate in the bulk tumor itself. Thus, we examined if these agents had an effect on the apoptosis or proliferation rate using TUNEL staining or Ki-67 staining in histologic sections from the xenografted tumor. We did not observe any difference in apoptosis rates, although we did observe a modest decrease in Ki-67 staining with the use of the various chemotherapeutic agents. To determine how Hh and notch blockade alters SP cell behavior, we then analyzed the SP and non-SP populations. BrdU incorporation and Annexin V staining were examined in sorted SP cells, and sorted non-SP cells, and the total cell population of cells from xenografted undifferentiated pleomorphic sarcomas. Mice were treated with BrdU six hours before sacrifice. Surprisingly, we observed a much higher level of BrdU uptake in the SP population compared to the non-SP population. Treatment with either triparanol or DAPT substantially reduced the BrdU uptake in the SP fraction, but not in the non-SP fraction. In contrast, treatment with
conventional chemotherapeutic agents substantially reduced the BrdU incorporation in the total and non-SP populations, but not in the non-SP population (Fig. 6). Treatment with either triparanol or DAPT also increased the proportion of cells in the SP stained for Annexin V from 4.1 percent to 8.3 or 7.3 percent (p <0.05) respectively. Annexin V staining was not performed for tumors treated with chemotherapeutic agents.

Discussion

Here we show that SP cells from undifferentiated pleomorphic sarcomas exhibit an elevated level of Hh and Notch signalling activation compared to non-SP cells. Pharmacologic blockade of these pathways in human undifferentiated pleomorphic sarcoma tumors engrafted into immunodeficient mice substantially decreases the size of the tumors that developed, and inhibited their ability to form tumors on serial transplantation. Thus, targeting a pathway that is activated in the SP fraction of tumor cells has the potential to inhibit the growth of bulk tumors. This data is consistent with the notion that SP cells are responsible for maintaining self renewal in undifferentiated pleomorphic sarcoma. However, it is also possible that other cells could play a role in self renewal. Indeed, both the SP and non-SP fractions are effected by pharmacologic blockade of Hh or Notch signalling, and as such, an effect on cells in the non-SP fraction altering tumor self-renewal capacity.

Tumor initiating cells demonstrate characteristics of stem cells, in that they are able to self renew as well as differentiate to produce a tumor with the cellular heterogeneity characteristic of the
primary neoplasm upon serial transplantation into immunodeficient mice (40). Undifferentiated pleomorphic sarcomas most likely arise from a mesenchymal precursor cell (41), and as such, signalling pathways which regulate mesenchymal precursor behaviour may also regulate cell behaviour in this sarcoma. The Hh and notch signalling pathways inhibit MSC differentiation and maintain stem cells in an undifferentiated state (22, 23). These signalling pathways could play a similar role in undifferentiated pleomorphic sarcoma tumor initiating cells. Inhibiting these pathways could cause the tumor initiating cell population to attain a more differentiated cellular phenotype with a limited capacity for self renewal. Notch and Hh signalling also play roles maintaining self renewal or tumor initiating populations in other tumor types (42-45). Our data suggests that the Hh and notch signalling pathways play a similar role in undifferentiated pleomorphic sarcoma, maintaining tumor initiating cells in a less differentiated state.

SP cells exclude Hoechst dye based on the ability to exclude the dye through ABC transporters, and the dye’s ability to bind in the nucleus. These transporters can also exclude other agents from the cell, including cytotoxic agents. SP cells can be enhanced for their ability to exclude chemotherapeutic or other toxic agents, and this property could make them resistant to certain therapies (46-48). SP cells could become resistant clones of tumor cells that are characteristic of neoplastic cells found in recurrent disease (49). Such a process could be independent of the presence of stem cell like properties in SP cells. Our data suggests that SP cells are resistant to conventional chemotherapies and as such targeting unique molecular properties could be developed into a useful therapeutic approach, regardless of the responsible mechanism.
Surprisingly, we found that SP cells have a high proliferation rate. This is a mitotically active cell population, a property not always associated with stem cells which are thought to be relatively quiescent(50). Inhibition of hedgehog or notch signalling inhibits the SP cell proliferation rate. Thus, in this tumor type, SP cells may act as tumor initiating cell because they have a high proliferation rate, which might allow a small number of cells to more easily form a tumor when grafted into immunodeficient mice.

Like other malignancies, bulk sarcomas contain non-cancerous stromal cells. Indeed, normal mesenchymal stromal cells have been isolated from sarcomas (38). Furthermore, Hh and Notch signaling both can act on non-cancerous stromal cells to alter tumor cell behavior, and could express ligands activating these pathways (39). Thus, it is possible that effects on normal stromal cells could be causing the changes we observed in the neoplastic phenotype in our study. While we cannot exclude this possibility, given that in our xenograft experiments the stromal cells are likely derived from the mouse, and we depleted mouse cells in our analysis of these cells as well as in the serial transplantation studies; and that we found similar results in tumor cell cultures as in xenografts in mice, it is less likely that paracrine effects alone are responsible for our observed results. Never the less, even if the changes we observe are due to effects on stromal cells, our data still demonstrates that the Hh and Notch pathways are activated in a population of cells enhanced for tumor initiating potential, even if this population includes non-cancerous stromal cells; and that the pharmacologic agents we tested do inhibit tumor growth and tumor self renewal capacity.
Undifferentiated pleomorphic sarcomas, like most other solid tumors, are composed of cytologically heterogeneous populations of cells. Here we examined one such cell population, the SP, that is highly proliferative and confers tumor initiating potential. Targeting signaling pathways activated in this small subpopulation of cells had a dramatic effect reducing tumor self renewal. Identifying unique properties of this subpopulation of tumor cells has the potential to be developed into a novel approach to cancer therapy.
References


Table One

The proportion of immunodeficient mice developing tumors after 10,000 undifferentiated pleomorphic sarcoma cells are transplanted into the mice. For each treatment, a tumor cells were implanted into a single mouse, and the number of tumors transplanted into a mouse is the denominator, and the number that formed tumors is the numerator. All of the primary transplantations developed tumors, but the size of the tumors varied depending on the treatment (see figure three). Data is shown for the secondary and tertiary transplantations from the tumors from mice treated with the various agents only in the primary transplantation.

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Figure legends

Figure One: SP cells from undifferentiated pleomorphic sarcoma cells express Hh and notch target genes. A) Real time RT-PCR data for the relative level of expression of Hh target genes for individual tumor relative to the level of expression in non-SP cells (arbitrarily defined as “1” for each sample). Each data point represents the average for that individual tumor, and the error bars are 95% confidence intervals. An asterisk above the bar signifies a statistically significant difference in level of expression compared to the level in non-SP cells. B) Real time RT-PCR data for the relative level of expression of notch target genes for individual undifferentiated pleomorphic sarcomas relative to the level of expression in non-SP cells (arbitrarily defined as “1” for each sample). C) Immunohistochemistry using an antibody to GLI1 showing expression of in a subpopulation of cells (a positively stained cell is labelled by an arrow from the letter P), while the majority of cells do not exhibit staining (a negatively stained cell is labelled by an arrow from the letter N). D) Immunohistochemistry using an antibody to HES1 showing expression of in a subpopulation of cells (a positively stained cell is labelled by an arrow from the letter P), while the majority of cells do not exhibit staining (a negatively stained cell is labelled by an arrow from the letter N). This data show that there is a subpopulation of cells in which Hh or notch signaling is activated in undifferentiated pleomorphic sarcomas.
Figure Two. Inhibition of Hh or notch signalling reduces the proportion of SP cells in undifferentiated pleomorphic sarcoma tumors. A and B) Treating mice with agents that block Hh or notch signaling results in downregulation of the expression of Hh and notch target genes in undifferentiated pleomorphic sarcoma xenografts respectively. Each data point represents the average for that individual tumor in multiple mice, and the error bars are 95% confidence intervals. An asterisk above the bar signifies a statistically significant difference in level of expression compared to the tumors in mice treated with a carrier. The expression level in the mice treated with a carrier is arbitrarily defined as “1”. C) Representative flow cytometry plots showing Hoechst dye exclusion from a subpopulation of cells in undifferentiated pleomorphic sarcomas. The SP is labelled with a box in the lower left quadrant, and the percentage of cells sorting to the region is given in each figure. Treating the cells with verapamil inhibits Hoechst dye exclusion, and the graphs in the lower part of the figure show that this treatment substantially reduces the number of cells which sort to the SP region. D) A graphical representation of data showing how these agents inhibit the percentage of SP cells in the xenografts. Data from multiple mice from each tumor is given as a single data point. The SP percentage of mice treated with controls is defined as 100, and the relative proportion of SP cells for each tumor is given. The error bars are 95% confidence intervals, and an asterisk above the bar signifies a statistically significant difference in level of expression compared to the tumors in mice treated with a carrier.
Figure Three. Inhibition of Hh or notch signalling reduces the size of undifferentiated pleomorphic sarcomas when established as xenografts in immunodeficient mice. A graphical representation of the average weight of tumors from each individual undifferentiated pleomorphic sarcomas when established as xenografts in multiple mice. Data from multiple mice grafted with cells from a single tumor is given as a single data point (and treated as a single “n”). The weight of the tumors in mice treated with controls is defined as 1, and the relative proportion average weight for each treated tumor in multiple mice is given as a data point. The error bars are 95% confidence intervals, and an asterisk above a bar signifies a statistically significant difference in weight compared to the tumors in mice treated with a carrier. For cyclopamine, the mean for each of the two samples is given as a single data point. Since there are only two samples, 95% confidence intervals are not included.

Figure Four. Inhibition of Hh and notch signalling reduces the serial transplantation potential of undifferentiated pleomorphic sarcoma xenografts. A Kaplan Meier survival curve for the development of a tumor following serial transplantation after treatment with a Hh or notch blocking drug or a carrier. Mice were sacrificed when a 1.5cm diameter tumor developed. All of the mice in which tumors from control treated mice were sacrificed during the observation period due to the size of the tumor that developed. In contrast, most of the mice which received grafts from mice treated with Hh or notch blocking dugs did not develop
identifiable tumors, even at autopsy. Data is given as 95% confidence interval for each data point on the survival curve. There was a statistically significant difference at each time point.

**Figure Five. Treatment of undifferentiated pleomorphic sarcoma xenografts with conventional chemotherapy does not reduce the proportion of SP cells.** Treatment of the mice with doxorubicin or cisplatinum resulted in a smaller relative weight of the xenografted tumors, but a higher proportion of cells sorting to the SP. The weight of the tumors in carrier treated mice is defined as 1, and the relative proportion average weight for the average of each treated tumor treated with each agent in multiple mice is given as a single data point for that tumor. The error bars are 95% confidence intervals, and an asterisk above the bar signifies a statistically significant difference in weight compared to the tumors in mice treated with a carrier. For the percentage of cells sorting to the SP, the relative value for mice treated with a carrier is arbitrarily defined as 100, and the average relative change in SP percentage for each tumor treated in multiple mice is given as a single data point. The error bars are 95% confidence intervals, and an asterisk above the bar signifies a statistically significant difference in weight compared to the tumors in mice treated with a carrier.

**Figure Six. Hh and notch signalling regulates undifferentiated pleomorphic sarcoma SP cell proliferation.** The SP cells from undifferentiated pleomorphic sarcoma xenografts have a high proliferation rate, as measured by short term BrdU uptake. Treatment of the mice with Hh or notch signalling inhibitors reduced short term BrdU uptake in the SP of xenografted
undifferentiated pleomorphic sarcomas, while treatment with conventional chemotherapies did not have a significant effect on the SP proliferation rate.
Fig. 3

Relative tumor weight compared to control

- triparanol
- DAPT
- cyclopamine

* Indicates significant difference compared to control.
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Cancer Res  Published OnlineFirst January 9, 2012.