SPANDIDOS PUBLICATIONS

Created Gli-1 duplex short-RNA (i-Gli-RNA) eliminates CD44^{Hi} progenitors of taxol-resistant ovarian cancer cells

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Abstract. Notch and Hedgehog activate cell-cycle progression of adult and cancer stem cells. Notch is activated by DLL and Jag presents on neighboring cells. We investigated the effects of density of the Notch-activating ligand, Jag-1, and targeting Gli-1, in activation of division of paclitaxel/taxol-resistant, (PTX^{Res}) ovarian cancer cells SKOV3 (SKOV3). We used the specific γ-presenilin inhibitor, DAPT, to identify the specificity of activating signals for Notch-1 and created 'butterfly-duplex-3548-Gli-1-inhibitory RNA' (i-Gli-1.RNA) to inhibit cell division. To accurately quantify kinetics of division, the expression of CD44 and CD24 was determined in each gated population of divided cells. CD44^{High} proliferated when activated by Jag-1^{Low} and poorly when activated by Jag-1^{High}. DAPT inhibited proliferation of cells activated by Jag-1^{Low}, and increased proliferation of cells activated by Jag-1^{High}. Only 5-10% of cells activated by Jag-1^{High} and Jag-1^{Low} divided fast, polynomial, and symmetric. i-Gli-1.RNA eliminated more than 50% of the small CD44^{High}/CD24^{Neg} cells in divisions 3 and 4. This effect appeared specific compared with cells transfected with negative control siRNA. i-Gli-1.RNA had no effect on large CD44^{High}/CD24^{Neg} cells, but inhibited the population of CD44^{High}/CD24^{Low} cells. Expansion of CD44^{High} inversely correlated with Jag-1 density on activating autologous tumor and fibrosarcoma cells. Created i-RNAs may decrease the resting CSC pool. Notch and Gli-1 signals play an important role in proliferation/division and survival of cancer stem cells. Targeting Notch-1 through its enhancer Gl-1, should be significant for novel treatments to eliminate taxol-resistant cancer stem cells (CSC). i.Gli-1 RNA should be more effective if used together with Taxol.

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Introduction

Most, if not all, cancers cannot be cured once they have metastasized, perhaps owing to the presence of chemotherapy- and radiotherapy-resistant cells. A small percentage of quiescent cells that are resistant to conventional therapies, known as cancer stem cells (CSCs), are responsible for tumor recurrence. Therapies that specifically target CSCs must be designed to improve the life of cancer patients. To expand, CSCs and their intermediate precursors (IPs) must self-renew (1). Adult stem cells (ASCs) re-enter the cell cycle through the early G_1 phase. If CSCs originate from ASCs, transition from the early G_1 to late G_1 phase is activated by one of the morphogens-Notch, Hedgehog (Hh), or Wnt that do not depend on mitogen-activated protein kinase (MAPK) signals. Notch-1 signals to Myc are essential for breast CSC division because of the central role of Myc in the activation of embryonal stem cell modules in CSC (2-4).

CSCs renew by symmetric and asymmetric division. In symmetric division, two identical CSC progenitors are generated in a shortened cell cycle. Asymmetric division generates one cell identical to the mother and one differentiated cell (5,6). Neighboring cells relay Notch-1 signals to the CSCs, i.e., they function as trans-Notch-1 activators. Notch-1 is activated by its ligands Jagged (Jag) and Delta (DLL) (7-9). Hh-pathway signals synergize with Notch-1 signals to promote CSC/IP renewal. Both Notch-1 and Hh signals are suppressed by Numb, which engages both Notch-1-intracellular domain

(NICD) and glioma-associated antigen-1 (Gli-1) and targets them for degradation (10,11).

In epithelial ovarian cancer (EOC) cells, DLL levels are low or DLL absent. Jag levels are high in EOC cells and low in normal fibroblasts and endothelial cells (12). In the absence of DLL, high Jag levels in tumor cells and low Jag levels in stroma should have distinct roles in the renewal of EOC-CSC and in the expansion of populations of EOC with different properties.

In breast cancer, cells that are epithelial specific, antigens ESA^{High}, $CD44^{High}$, and $CD24^{Neg/Low}$ are defined as CSC or CSC/IP (13). A population with this same phenotype is also present in EOC. In a recent study, the number of $CD44^{High}$, $CD24^{Neg/Low}$, and $CD133^{+}$ cells was higher in PTX^{R} population than in PTX^{S} population (14). EOC-CSC express stem cell factor receptor CD117 (c-kit) (15,16). Most $c\text{-}kit^{+}$ cells are $CD24^{Neg}$, and expression of CD24 is associated with lineage commitment in other cells (17).

The purpose of our study was to determine whether: i) EOC-bound Jag-1^{High} or sarcoma-bound Jag-1^{Low} preferentially expand cells with CSC and non-CSC phenotypes from PTX^{R} resistant cells, ii) the Jag-1^{High}- or the Jag-1^{Low}-induced proliferation is inhibited or enhanced by DAPT, and iii) PTX^{R}-EOC cells with the CSC phenotype can be eliminated by i-Gli-1.RNA.

Materials and methods

Cells and reagents. Ovarian (SKOV3) and fibrosarcoma (A204 and HT1080) cell lines were purchased from ATCC (Manassas, VA). All cell lines were cultured in RPMI-1640 medium with 10% FCS and antibiotics. PTX was from Bristol-Myers Squibb, Princeton, NJ, and recombinant human DLL4 rhDLL4 from R&D Systems Inc., Tusin, CA.

mAbs were: FITC-anti-ESA (Biomeda, Foster City, CA), PE-anti-CD24 mAb (Abcam Inc., Cambridge, MA) and isotype control antibodies; FITC-, PE-, and APC-conjugated were obtained from BD Pharmingen, San Diego, CA, for flow cytometry. Anti-Notch-1 mAb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal anti-activated Notch-1/NICD, anti-Numb, anti-Gli-1, mouse anti-Bax and ß-actin (Abcam) were used in immunoblotting.

Generation of PTX^{R} SKOV3. Cells ($50x10^{6}$) were cultured with 9.3 nM (2-fold IC_{50}) PTX for 3 days. PTX concentration was reduced to 3.1 nM (0.7 x IC_{50}) for the following 4 days. Reduction of PTX concentration to 0.7 x IC_{50} aimed to resemble conditions *in vivo* when PTX decays and surviving cells start dividing.

Activation of division. PTX^{R}-SKOV3 were co-cultured in medium containing 10% FCS, in 24-well plates at a 1:1 ratio with irradiated SKOV3 and HT1080 cells, soluble rhDLL4 or medium containing 10% FCS (as cytokine growth factor activator only). HT1080 express low levels of Jag-1 compared with SKOV3. SKOV3 are Notch-1^{Normal}, Notch-2^{Low}, 3^{Low}, 4^{Low}, Jag-1^{High}, Jag-2^{High}, DLL-$1^{Low/Neg}$, $2^{Low/Neg}$, $3^{Low/Neg}$, $4^{Low/Neg}$.

HT1080 are Notch-1^{Normal}, Notch-2^{Low}, 3^{Low}, 4^{Low}, Jag-1^{Low}, Jag-2^{Neg}, and DLL-1^{Neg}, 2^{Neg}, 3^{Neg}, 4^{Neg24}. The levels of Jag-1 in HT1080 are similar to those in normal human fibroblasts. HT1080 has low levels of Jag-1 compared with SKOV3. We designated HT1080 as Jag-1^{Low}; SKOV3 as Jag-1^{High}. Because activators and responders have normal levels of Notch-1 and low levels of Notch-2, 3 and 4, we refer to cells as Notch-1-positive.

Activators were irradiated with 10,000 rads (100 Gy) to inhibit division. The γ-presenilin inhibitor, DAPT, was used at 10 μM (18). Division of SKOV3 was determined 3 and 6 days later in carboxyfluorescein-succinimidyl ester (CFSE)-labeled cells. PTX^{R}-SKOV3 were stimulated with 62.5 ng/ml rhDLL4 for 24 h in serum-free medium (7). Cultures were continued for 6 days. Cells were quantified as described (19).

Inhibition of division of SKOV3 by i-Gli-1.RNA. PTX^{R}-SKOV3 were labeled with CFSE and allowed to attach to plastic in T-75 flasks. Following 24-h incubation at 37°C, i-Gli-1.RNA, and negative control siRNA (Dharmacon) were transfected into these cells in the same flasks (20). Irradiated activators were added 24 h after transfection. The number of cells of each phenotype was determined in gated populations for CFSE levels.

Flow cytometry. Cells were incubated first with 20 μg of purified human IgG (Sigma Chemical Co.) for 1 h on ice, to inhibit non-specific binding of Ab during staining, followed by specific mAb. Analysis was performed using a Becton-Dickinson FACSCalibur with CellQuest software. Expression of CD24 and CD44 was quantified in divided cells as we described (19).

Western blot analysis. Cytoplasm was isolated from live PTX^{R}-SKOV3. Blotting and quantification of Notch-1 NICD, Gli-1, Numb-Long and -Short isoforms (Numb-L and Numb-S), Bax, and ß-actin were performed as we described (21,22).

Results

PTX^{R}-SKOV3 have higher levels of Gli-1 and lower levels of Bax than PTX^{S}-SKOV3. PTX^{R} cells had significantly higher levels of cytoplasmic Gli-1 (2.11-fold) and NICD/TMIC (2.2-fold) and lower levels of Bax than $PTX^{Sensitive}$ cells. TMIC is the precursor of NICD linked by disulfide bonds to NECD. TMIC is membrane-bound and cannot signal. It is unclear if the upper strong band represent the TMIC only and only a small amount of NICD is released. The lower levels of the Bax suggested these cells were more resistant to apoptosis. (Fig. 1).

Jag-1 activates division of $CD24^{Neg,\ Low,\ and\ High}$ cells. Small size cells (small cells) PTX^{R}-SKOV3 (mean FS = 200) accounted for 70% of total. Small cells had divided as shown by CFSE distribution. Cells of large-size (large cells) (mean FS= 600) were 30% of total and similar in size to non-PTX-treated SKOV3 (not shown). Most PTX^{R} cells are resting in G_1-phase (20,22). Small-size tumor cells are considered metabolically less active than large-size tumor cells (25). Cells which rest in G_1 or cannot use nutrients are smaller in size than cells in S-phase.

Western analysis cannot distinguish differences in four different populations. Further, cells surviving PTX were 3-4%

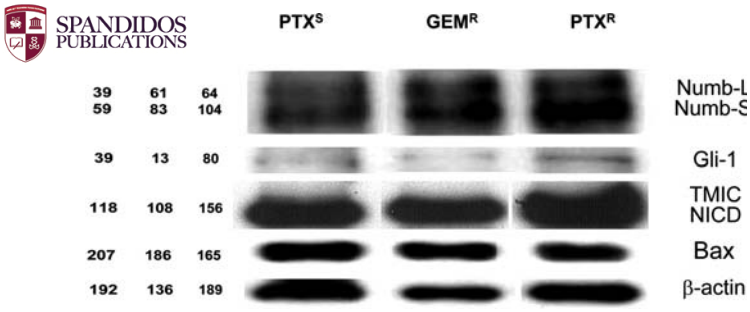

Figure 1. Expression of Numb-L, Numb-S, Gli-1, TMIC/NICD, Bax, and in PTX^{Res} SKOV3 cells.

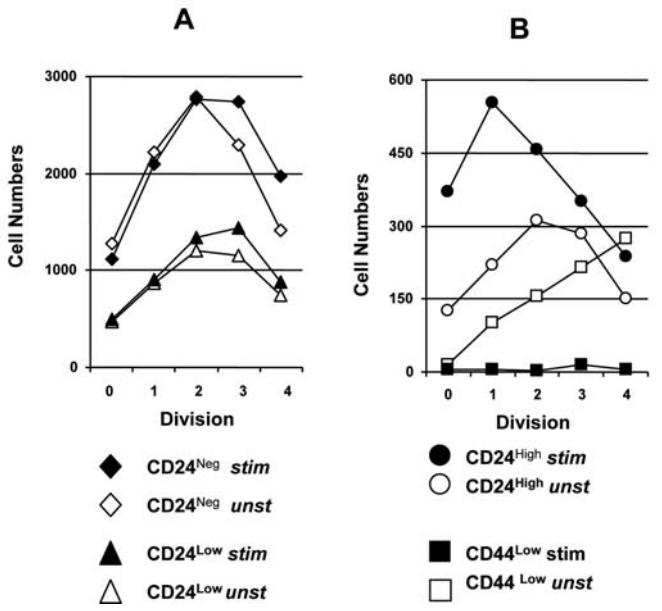

Figure 2. (A and B) Activation of PTX^R SKOV3 with Jag-1^{Low} increases the number of CD24^{Neg} and decreases the number of CD24^{High} cells (see also Table I for details).

of initial culture. To separate populations by sorting we must have started with at least 10^9 cells. Thus, we analyzed cells by multicolor flow cytometry. We activated PTX^R-SKOV3 with irradiated Jag-1^{High} and Jag-1^{Low} cells.

For high stringency, we divided PTX^R cells into CD44^{High} and CD44^{Neg}. We divided CD44^{High} cells into 3 sub-populations: CD24^{Neg}, CD24^{Low}, and CD24^{High}. We counted cells in each population after activation with Jag-1^{High} or Jag-1^{Low} in both small and large, adherent PTX^R cells, over 5 clearly distinguished divisions. To identify whether the activation by Jag-1 was Notch-1 specific, we used the Notch cleavage inhibitor, DAPT. DAPT does not interfere with Gli-1-signals (18). Jag-1^{Low} expanded 2 times more CD24^{Neg} cells and CD24^{High} cells than medium alone (Fig. 2, Table I). DAPT inhibited expansion of CD24^{Neg} cells activated by Jag-1^{Low} (Fig. 2, Table I).

In contrast, DAPT increased the numbers of CD24^{Neg} and CD24^{Low} cells expanded by Jag-1^{High} (Table I). Surprisingly, Jag-1^{High} + DAPT and Jag-1^{Low} alone expanded similar number of cells. DLL4, as the control, had a weak effect on cell expansion (data not shown). Therefore, DAPT inhibited proliferation of cells activated by Jag-1^{Low} but protected from death cells activated by Jag-1^{High}. DAPT may not be useful to inhibit Jag-1^{High} EOC interacting with each other. Ablation of effects by a specific Notch-1 cleavage inhibitor; indicated the outcome is the result of Notch-1 activation (Table I).

To identify effects of high and low concentrations of Jag-1/per cell on responder expansion we quantified large-size cells 6 days after activation by Jag-1^{High} or Jag-1^{Low} in the presence or absence of DAPT. Only CD44^{Neg} cells increased in number in response to both Jag-1^{High} and Jag-1^{Low}. CD44^{High}/CD24^{Neg} and CD44^{High}/CD24^{Low} cells activated by Jag-1^{High} in presence of DAPT increased; cells activated by Jag-1^{Low} decreased. Density of activator, Jag-1, determined the effects of DAPT on proliferation of PTX^R-SKOV3. The effects were mediated by a specific Notch-1 activator and ablated by a specific Notch-1 cleavage inhibitor; thus we conclude the outcome is the result of Notch-1 activation (Table I).

Some but not all CD44^{High} (CD24^{Neg}, CD24^{Low}, and CD24^{High}) PTX^R SKOV3 divide symmetrically. To identify the kinetics of division, we fitted divided live cell numbers in different equations. The best fit (R^2>0.95) of increase in cell number after division is with polynomial kinetics of division for cells stimulated either by Jag-1^{High} or by Jag-1^{Low} (Tables II and III).

We determined the number of cells in one division needed to double to reach the numbers counted in the next division as follows: to obtain the 293 CD24^{Neg} cells seen in 5th division, we need 147 cells from 4th division to divide symmetrically (and double), plus the 1029 cells undivided (Table I, line 1, columns 5 and 4). The total number of CD24^{Neg} cells in the 4th division, would therefore be 147+1029=1176. CD24^{Neg} cells (588-one half of 1176) should be present in the 3rd division and divide symmetrically, to reach 1176 cells. We counted, in the 3rd division, 1045 cells without DAPT and 1788 cells with DAPT. Total expected cells equal 1633 (no DAPT) and 2320 (+DAPT). Therefore, we found alive 64% and 77% of expected cells, respectively.

Alternatively, to obtain 293 CD24^{Neg} cells by asymmetric division, we need 293 cells from the 4th division to generate 293 CD24^{Neg} mothers and 293 CD24 ^{Low} daughters. The number of cells in the 4th division, should be 293+1029=1322. We counted 56 CD24^{Low} daughters in the 5th division (Table I, line 5, column 5). We counted 33 CD24^{High} daughters (legend). Total 89 cells or 30% of cells expected. Thus, symmetric division dominates.

Activation by Jag-1^{Low} increased the levels of CD44. Jag-1^{Low} increased the level of CD44 in PTX^R cells more than 2.5 times compared with FCS only, containing medium (growth factor only) of PTX^R cells. The levels of CD44 decreased with division but remained higher in Jag-1^{Low}-activated, than in medium-activated cells (Table IV).

CD44^{Neg} and CD44^{High} cells divide with different kinetics in response to Jag-1^{Low}. To characterize the kinetics of expansion of PTX^R CD44^{Neg} and PTX^R CD44^{High} cells, we compared the numbers of Jag-1^{Low}-expanded cells and medium-expanded cells, over 5 divisions in 3 days. Expansion of all populations followed polynomial kinetics, except the CD44^{Neg}/CD24^{Neg}

Table I. DAPT protects from death $CD24^{Neg}$ and $CD24^{Low}$ cells activated by Jag^{High}.

	Division number/ Cells per 10,000 live SKOV3							
	1	2	3	4	5	Sum (Σ)	DAPT effect	SI
$CD44^{High}$ $CD24^{Neg}$ (MFI=3-5)								
Activator: $Jag\text{-}1^{High}$								
No inhibitor	53	517	1045	1029	293	2937	Protects	2.0
+DAPT	156	1234	2320	1708	389	5807		
Activator: $Jag\text{-}1^{Low}$								
No inhibitor	110	868	1549	2057	450	5034	Inhibits	0.61
+DAPT	50	390	899	1288	436	3063		
$CD44^{High}$ $CD24^{Low}$ (MFI=15)								
Activator: $Jag\text{-}1^{High}$								
No DAPT	12	93	219	187	56	567	Protects	2.29
+DAPT	48	224	447	441	135	1295		
Activator: $Jag\text{-}1^{Low}$								
No DAPT	52	441	1184	1648	395	3745	Inhibits	0.19
+DAPT	12	67	188	329	118	714		

We started the culture with 300,000 cells for 6 days. We extrapolated the $CD24^{Neg}$ (CSC-like) and $CD24^{Low}$ (CSC-like-1) to 10,000 cells in each division. $CD44^{High}$ $CD24^{High}$ (MFI >100) stimulated by $Jag\text{-}1^{High}$ in each division were: 35, 100, 139, 110, and 33. Total 421. $CD44^{High}$ $CD24^{High}$ expanded by $Jag\text{-}1^{Low}$ were: 65, 155, 242, 162, and 96. Total 725.

Table II. Polynomial kinetics of expansion of $CD24^{Neg}$, $CD24^{Low}$ and $CD24^{High}$ cells.

$CD24^{Neg+Low}$	$T^{Neg+Low} = x_1\,(0.035) + x_2\,(0.069) + x_3\,(0.230) + x_4\,(0.354) + x_5\,(0.260) + x_6\,(0.069)$
$CD24^{High}$	$T^{High} = x_1\,(0.070) + x_2\,(0.137) + x_3\,(0.274) + x_4\,(0.313) + x_5\,(0.205) + x_6\,(0.053)$

More $CD24^{High}$ cells than $CD24^{Neg\ and\ Low}$ cells are present in divisions 1 and 2. The total (T) numbers of $CD44^{High}$ $CD24^{Neg+Low}$ and $CD44^{High}$ $CD24^{High}$ cells depends on the value of x_1 (cells which divided once) and of his predecessor x_0 (undivided quiescent cells).

cells, which did not divide. $Jag\text{-}1^{Low}$ expanded more cells than medium alone in divisions 3, 4 and 5 (Fig. 2A and B): i) Linear: $CD44^{Neg}/CD24^{Neg}$ cells activated by medium followed a linear kinetics of expansion. $Jag\text{-}1^{Low}$ inhibited proliferation. This finding confirms that DAPT rescues $CD44^{Neg}/CD24^{Neg}$ cells in the presence of $Jag\text{-}1^{Low}$. ii) Linear to exponential: $CD44^{High}/CD24^{Neg}$ cells divided almost exponentially during the first 2 divisions, regardless of activator. Medium-activated cells decayed rapidly; the number of $Jag\text{-}1^{Low}$-activated cells was 30% higher. iii) Slow linear: $CD44^{High}/CD24^{Low}$ cells followed a slower kinetics than $CD44^{High}/CD24^{Neg}$ cells (see the small angle of the slope). iv) Polynomial. Most $CD44^{High}/CD24^{High}$ cells divided once at activation with $Jag\text{-}1^{Low}$. Afterwards only a small fraction progressed to divisions 2 to 5.

$Jag\text{-}1^{Low}$ increased the number of $CD44^{High}/CD24^{Neg}$ cells by 9 times while medium alone by 7 times. $CD44^{High}/CD24^{Low}$ cells increased in number similarly when activated by $Jag\text{-}1^{Low}$ (9.77 times) and medium (8.84 times). In contrast $CD44^{High}/CD24^{High}$ cells expanded less with $Jag\text{-}1^{Low}$ (5.52 times) than with medium (8.26 times) (data not shown).

i-Gli-1.RNA eliminates small-size $CD44^{High}/CD24^{Neg}$ cells and inhibites expansion of large-size $CD44^{High}/CD24^{Low}$ and $CD44^{High}/CD24^{High}$ cells. Elimination of PTX^{R} SKOV3, which survive DAPT, requires a novel approach. We attempted to inhibit Gli-1, using Gli-1-micro-RNA because Gli-1 activates the effector Bim-1 (10). The strongest inhibitory effects of i-Gli1.RNA were seen in cells divided 3 times after taxol was removed. Cells divided 3 and 4 times are the majority (50% of all cells).

i-Gli-1.RNA eliminated small $CD44^{High}/CD24^{Low}$ cells, while control siRNA less cells. The decrease in number of cells was stable over 5 divisions during 6 days (Fig. 3A, and not shown). i-Gli-1.RNA inhibited division of $CD44^{High}/CD24^{High}$ cells. However, a part of this inhibition (33%) was due to 'off target' effects. i-Gli-1.RNA did not inhibit expansion of $CD44^{High}/CD24^{Low}$ cells, because 100% of inhibition was due

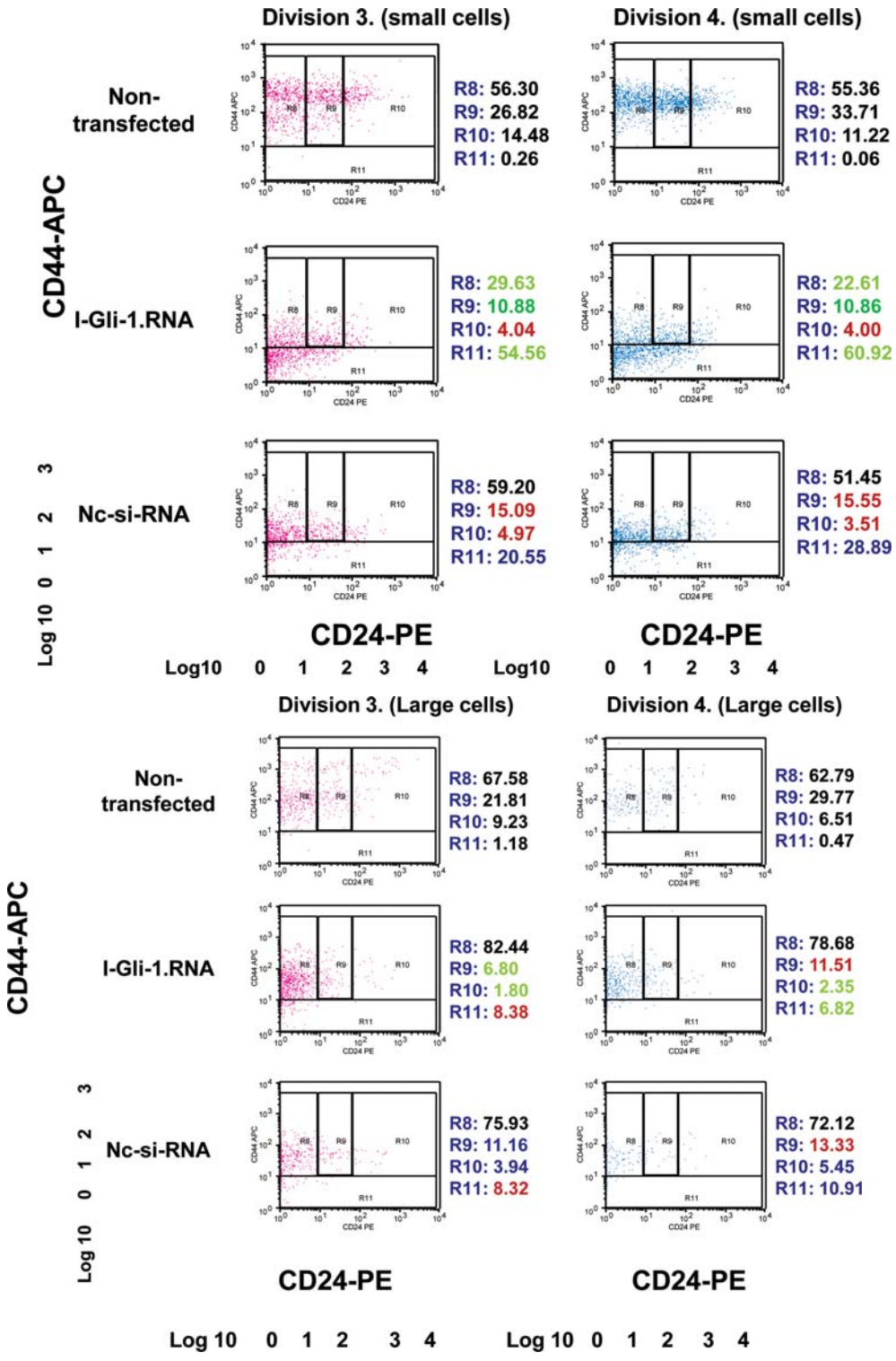

Figure 3. i-Gli-1 inhibition of division of CD44 CD24 populations depends on cell size and phenotype. (A) Small cells: i-Gli-1.RNA inhibited specifically division of $CD44^{High}$ $CD24^{Neg}$ and non-specifically division of $CD44^{High}$ $CD24^{Low}$ and $CD44^{High}$ $CD24^{Hi}$ cells. (B). Large cells: i-Gli-1 inhibited specifically division of $CD44^{High}$ $CD24^{High}$ and non-specifically division of $CD44^{High}$ $CD24^{Low}$ cells. All cells were treated with PTX, transfected with short RNAs, labeled with CFSE and activated with Jag-1^{Low}. Analysis performed 3 days later.

to 'off-target' effects. Both i-Gli-1.RNA and its control eliminated cells with high levels of $CD44^{High}$.

i-Gli-1.RNA did not inhibit division of large-$CD44^{High}$/$CD24^{Neg}$ cells but did decrease the number of large-$CD44^{High}$/$CD24^{Low}$ and $CD44^{High}$/$CD24^{High}$ cells. However, 50% of inhibition by i-Gli-1.RNA was due to 'off target' effects, because negative control, si-RNA, also inhibited expansion of these cells (Fig. 3B). If only CD44 cells with y^2 (MFI>200) are scored as $CD44^{High}$, as recently described (26) one will prematurely conclude that i-Gli-1.RNA eliminated all $CD44^{High}$ (MFI>200) and all CSC.

Therefore, i-Gli-1.RNA has cell-dependent 'on-target' (decrease of number of small and large $CD44^{High}$/$CD24^{Neg}$ cells and increase of $CD44^{Neg}$/$CD24^{Neg}$ cells) and 'off-target'

Table III. $CD24^{Neg}$ and $CD24^{High}$ cells divide with similar kinetics when activated by Jag-1^{High} or Jag-1^{Low}.

Responder	Activator	Total cells divided 5 times
$CD24^{Neg}$ and $CD24^{Low}$	Jag-1^{High}	T_5 = A (2x3.35x1.54x0.72x0.25)
$CD24^{Neg}$ and $CD24^{Low}$	Jag-1^{Low}	T_5 = B (2x3.39x1.76x0.91x0.21)
$CD24^{High}$	Jag-1^{High} or Jag-1^{Low}	T_5 = C (2x1.97x1.16x0.66x0.36)

Kinetics of renewal of PTX^R SKOV3. Numbers represent fold-increase in one division compared with the previous division. $CD24^{High}$ cells expand less than $CD24^{Neg}$ cells. The formulas show the distribution of CSC-like cells in each division. The MFI-CFSE of cells was between 200-5000. The number of cells which divided 5 times (T_5) can be obtained from the total number of cells counted (A, B and C) multiplied with the factor of increase in each division.

Table IV. Interaction PTX^R SKOV3: HT1080 increased the levels of CD44 on PTX^R cells.

PTX^R SKOV3	Division number/Geometric MFI					
	0	1	2	3	4	5
1. Medium cytokines only	219	118	89	74	96	93
2. Jag-1^{Low} activated	**742**	**360**	**395**	**294**	**266**	**256**
3. i-Gli-1.RNA then Jag-1^{Low} activated	134	140	97	78	87	110
4. Nc-si-RNA then Jag-1^{Low} activated	45	55	32	33	39	69

Cells surviving i-Gli.RNA and Nc-si-RNA have lower levels of CD44 than untransfected cells.

inhibitory effects. The $CD44^{Neg}/CD24^{Neg}$ cells require further study to determine whether they are a metabolically quiescent population, ongoing differentiation or dying.

Discussion

We present novel and unpublished evidence on function of Jagged-1 on cancer cells which lack DLL. Jag-1^{Low} expanded more $CD44^{High}$ cells than Jag-1^{High}. Notch-1-inhibitor, DAPT, enhanced expansion of $CD44^{High}/CD24^{Neg}$ by Jag-1^{High} (the presumed stem cells). We found a Notch-1 inhibitor which can expand CSC-cells. We found, using extremely stringent conditions of analysis, that cancer cells, which survive PTX, can exit the G_0-G_1 phase when activated by Jag-1.

We used conditions closer to the physiological situation, i.e. cell:cell activation and not artificial (i.e. isolated Jag-1). Therefore, our results are representative of responses of whole population of cancer cells at the time when PTX treatment stops. We analyzed in the same sample small, large, adherent, non-adherent cells $CD24^{Neg}$ and $CD24^{Low}$ cells. Such studies were not attempted before.

PTX^R differed from PTX^{Sens} by significant (>2.00) fold-increase in amount of TMIC/NICD and Gli-1 and decrease in Bax. Ongoing studies will address which population has more NICD and less Bax than others. Numb-L, did not increase in PTX^R compared with Gem Res cells. Numb-S increased less than Gli-1 in PTX^R compared with Gem^R cells. This result suggests some of 4 PTX^R-populations are 'readier', or 'less inhibited' to respond to small numbers of Notch-1 and Hh ligands.

An important finding is differential kinetics of division of each population studied. The real kinetic is polynomial, because a part of population die and not divide. This finding is significant for development of functional prediction methods of imminent metastasis from small biopsies and therapeutic intervention.

Jag-1^{Low}-induced proliferation indicate PTX^R cells expand more in sites containing fibroblasts and less in sites containing tumor. CSC up-regulate CD44 when interact with stroma; this change helps them to rapidly invade other sites. Activation of CD44 was recently proposed to increase stemness by inducing Nanog (27).

When the number of cancer cells increases, they interact with each other in a Jag-1^{High} dependent fashion. Some die, others stop dividing. Therefore, undetected metastases of few cells will expand fast. The sensitivity to inhibition of $CD44^{High}/CD24^{Neg}$ cells differed in small and large cells: either Gli-1 is not functional in the latter, or division of large-size CSC is not under control of Gli-1, or more i-Gli-1.RNA is needed to inhibit these cells. Simultaneous increase in $CD24^{Low+High}$ cells and in levels of CD44, indicates that Jag-1^{Low} increases the number of metastasis-prone CSC and other cancer cells. Our findings suggest that inhibition of PTX^R cells will be more effective if i-Gli-1 RNA is delivered locally before taxol is removed and survivors start dividing. Our findings show novel differences between populations of PTX^R cells. Our results are novel for PTX^R human cancers. Our findings have significant implications for prevention of metastases by cancer cells, which depend on Notch and Gli-1 signals for entry in cell cycle (28,29).

SPANDIDOS PUBLICATIONS **edgements**

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