Isolation of patient-derived cancer stem cells from GIST liver metastasis

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Application note

Tumors consist of a heterogeneous mix of multiple interacting cell types organized in a complex hierarchy. Only a small subpopulation of the tumor cells are cancer stem cells (CSCs) capable of driving progression and ultimately the dissemination of the malignancy. The largest proportion of the cells contained in most tumors are non-tumorigenic, differentiated cells and benign cancer-associated cells such as cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs) and stromal cells [1].

Despite the unique cellular features of CSCs – e.g. tumorigenicity, unlimited proliferation potential, self-renewal and resistance to cytotoxic drugs – the complex organization of tumors complicates the purification and characterization of the causative subpopulation of cancer driving cells.

The lack of specific markers as well as selective *in vitro* culture methods add to the difficulties.

Traditional culture systems for cancer cells based on classical media formulations lack specificity for CSCs. These media predominately support the proliferation of benign cells, e.g., stromal cells, or differentiated (non-tumorigenic) cancer cells, thus leading to a gradual loss of the original CSC population. Only cells isolated from the most malignant types of tumors in these media, from which most traditional cancer cell lines have been established, have been successful. These cell lines, however, are model systems with a long history of selection and adaption in various ill-defined *in vitro* culture conditions and do not comprehensively reflect the behavior of primary tumors [2].

Mouse models have been developed for establishing cultures from tumors with a lower degree of malignancy, since direct *in vitro* isolation was not effective. After several rounds of serial *in vivo* transplantation of tumor tissue in severely immune-compromised mice, the cells of the primary tumor eventually develop into a stable tumor cell population. However, only a small fraction of these tumor cells is stable under traditional *in vitro* culture conditions. Most can only be maintained by serial *in vivo* transplantation in the mouse. These techniques are expensive, time-consuming and elaborate, and – most importantly – they induce major changes in the initial primary tumor cells inherent in a serial selection process in rodents. Consequently, direct *in vitro* isolation of patient-specific primary tumor cells in an unbiased defined culture environment is highly preferable [3].

The principle of the Primary Cancer Culture System (PCCS)

This advanced culture system, consisting of the Primary Cancer Cell Medium D-ACF and the NCCD-Reagent, was designed to be the first universally applicable, cost-effective solution for *in vitro* isolation and long-term primary cultures of human CSCs, e.g., from patient tumor samples or patient-derived xenografts (PDX).

The PCCS is a functional medium, designed to support specifically the aberrant metabolic traits of malignant cells. Since on the cellular level CSC-specific traits represent the only selection criterion, the cell diversity of the cancerous subpopulations of the original tumor is preserved.

With regard to cancer cell primary isolation, traditional tumor cell media generally support the growth of benign cells, e.g., tumor stroma, CAFs and TAMs, but do not sufficiently support the cancer cell subpopulations that drive the progression of tumors *in vivo*. The vast majority of these *in vitro* primary cultures are therefore transient and exhibit a gradual loss of the original CSCs that cause the disease.

In contrast, the Primary Cancer Culture System makes it possible to reliably deplete benign cells from the culture due to the selective support of CSCs. This process usually takes a few to several weeks depending on the intrinsic growth rate of the CSC population within the sample.

Since malignancy itself serves as the sole functional selection criterion, the culture system is applicable to all types and entities of CSCs, regardless of their origin and the stage of the tumor. Provisional enrichment techniques, e.g., cell sorting while relying on unproven markers, are therefore obsolete.

The selection process dispenses with the use of cytotoxic agents in a defined and animal-free culture environment. One bottle of medium is typically sufficient for 3–5 primary isolations.

The Primary Cancer Culture System can also be used for other applications, e.g., enriching CSC subpopulation(s) in established cell lines or depleting of stromal cells and other non-cancerous cells from established primary cancer cell cultures to identify CSC biomarkers.

Primary isolation of cancer cells from a gastointestinal stromal tumor (GIST)

Using our PCCS, CSCs can be isolated and cultivated from resected liver metastases of GIST according to the protocol in our application note *Isolation of patient-derived primary cancer cells*.

After homogenization and enzymatic digestion of the tumor tissue, the cells are plated on NCCD-treated tissue culture vessels and allowed to grow for 13 days before passaging. As shown in Figure 1, after 14 days of cultivation (i.e., day 1 after passage), the tumor-derived CSCs switch from suspension growth to adherent growth and appear as proliferating spindle-shaped cells, a morphology typical of stromal tumor-derived cells.

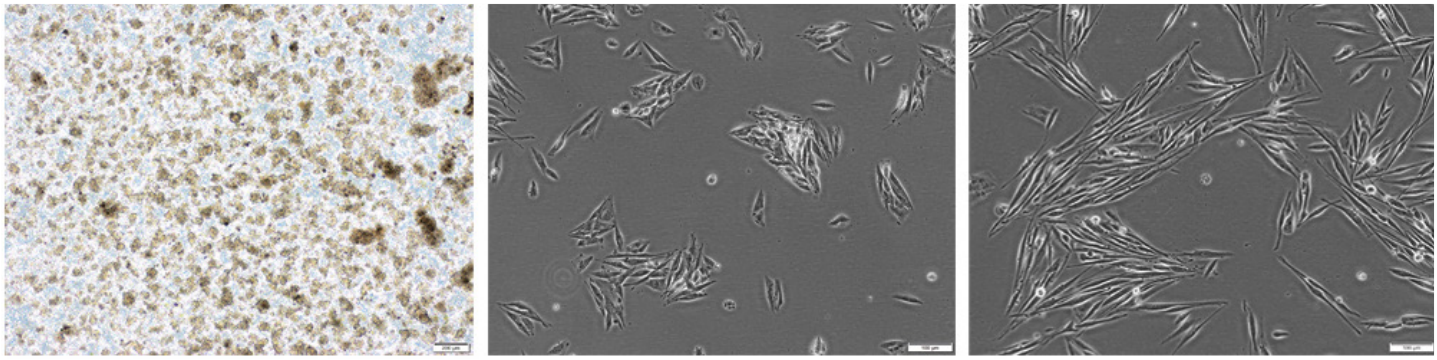

Fig. 1: CSC isolation from GIST liver metastasis using PCCS (C-28081). Left: *Floating multicellular cell aggregates on day 4 (passage 0) after isolation and initial plating (40x magnification).* ***Center:*** *On day 14 (passage 1) after isolation using Primary Cancer Cell Medium D-ACF, primary GIST cells appear as adherently growing cell clusters.* ***Right:*** *After 17 days (passage 1) of cultivation, GIST-derived cancer cells proliferate and exhibit a spindle-shaped morphology (100x magnification).*

Long-term cultivation of the GIST primary isolate

Cancer cells isolated from liver metastases form a nearly confluent 2D monolayer 49 days after isolation and cultivation (Figure 2).

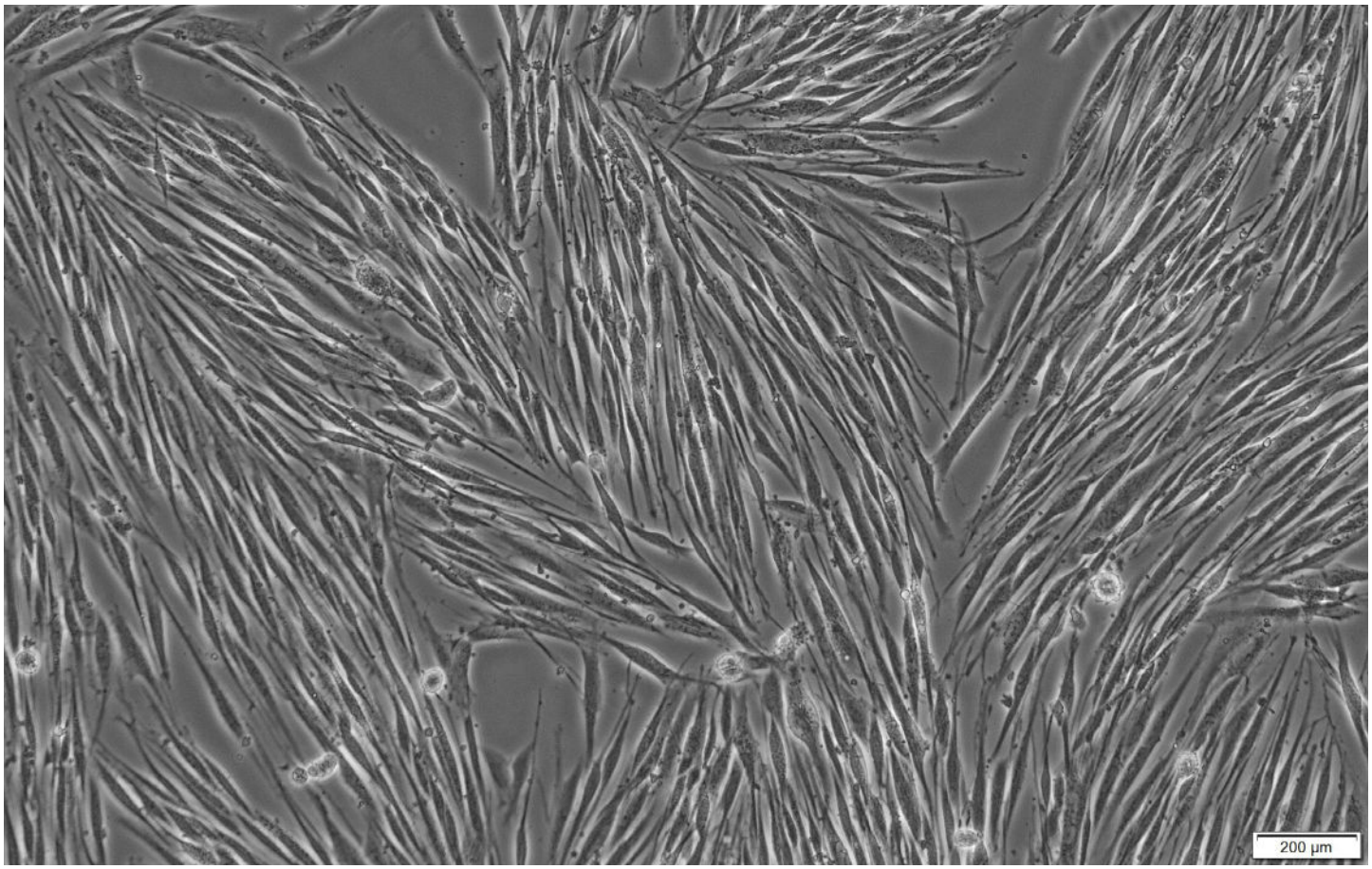

Fig. 2: Expansion of the GIST primary isolate using the PCCS (C-28081). *Following establishment of a stable primary culture, the GIST-derived cancer cells could be expanded and serially passaged using the PCCS. The population doubling time (PDT) averaged approx. 200 hours. 49 days after isolation (passage 2) the cells maintain a spindle-shaped morphology and form a 2D monolayer (100x magnification).*

Characterization of the primary cancer cell isolate

The isolate derived from GIST liver metastasis was subjected to immunostaining for the transcription factors and stem cell markers SOX2 and Oct4. Double-stained cells were analyzed using flow cytometry in passage 3.

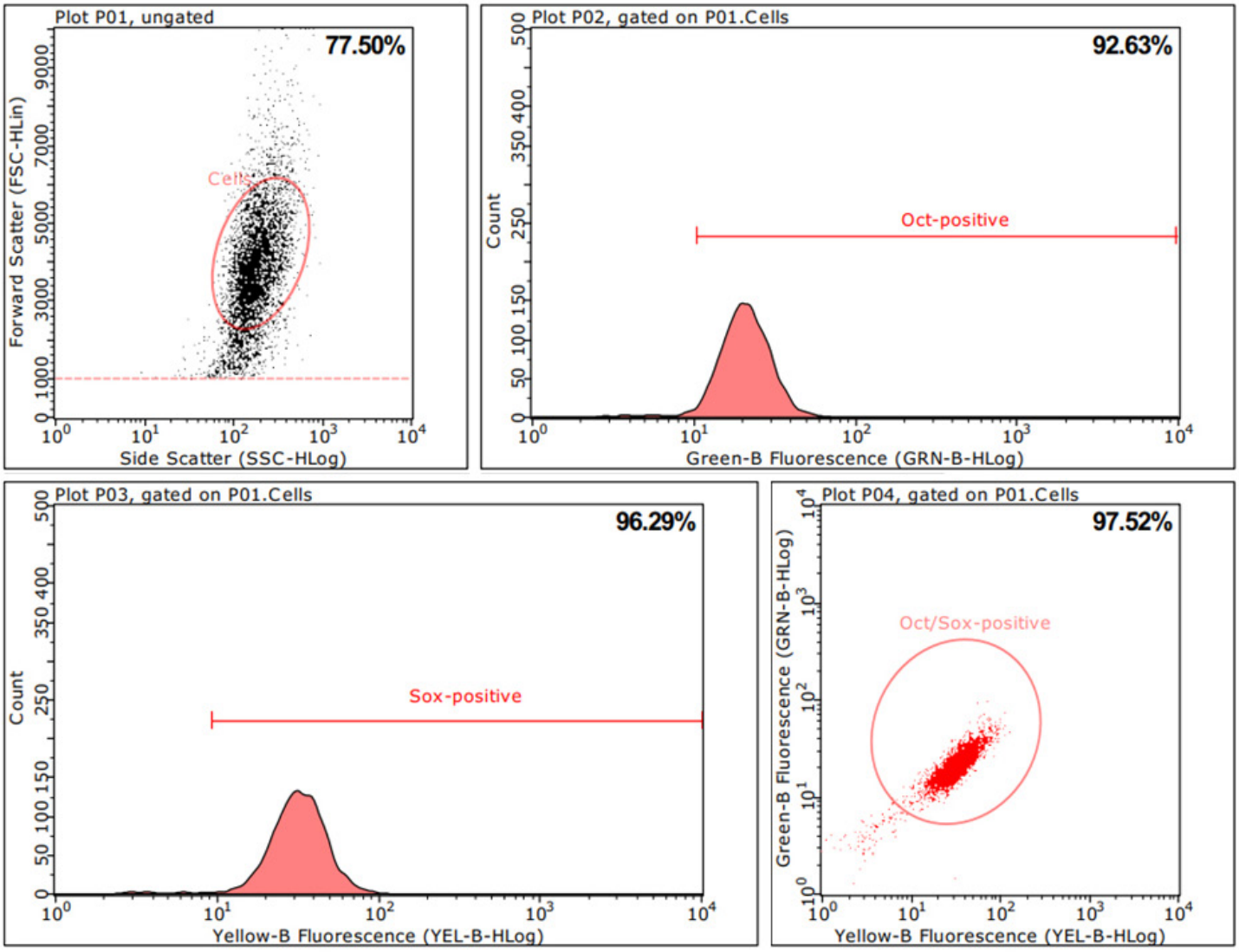

Fig. 3: Flow cytometric analysis of cancer cells isolated from GIST liver metastases. *The GIST primary isolate was cultivated until passage 3 using the PCCS. Cells were double-stained using antibodies specific for the stem cell markers Oct4 and SOX2. Flow cytometric analysis revealed that 97.5% of the cells were positive for both markers, indicating a homogenous cell population with stem cell properties.*

Oct4, also known as POU5F1, belongs to a family of transcription factors that regulate stem cell pluripotency. SOX2 is a stem cell marker that regulates organ development. In CSCs, the functional interaction of SOX2 and Oct4 regulates various stem cell characteristics. Therefore, the co-expression of Oct4 and SOX2 represents an excellent and reliable marker of stem cells.

The double-positive staining of >= 97% of the cells for Oct4/Sox2 suggests that the primary isolate consists of a pure population of cancer cells with distinct stem cell properties and self-renewal potential indicative for cancer stem cells (CSC).

Next-generation sequencing analysis revealed the presence of a heterozygous mutation in the KIT proto-oncogene that is commonly found in GIST. The KIT gene encodes a receptor tyrosine kinase, the mutation of which is associated with GIST. The heterozygous mutation in KIT occurred in 98% of the analyzed cells in passage 3.

Cancer cell isolation from tumor tissue samples

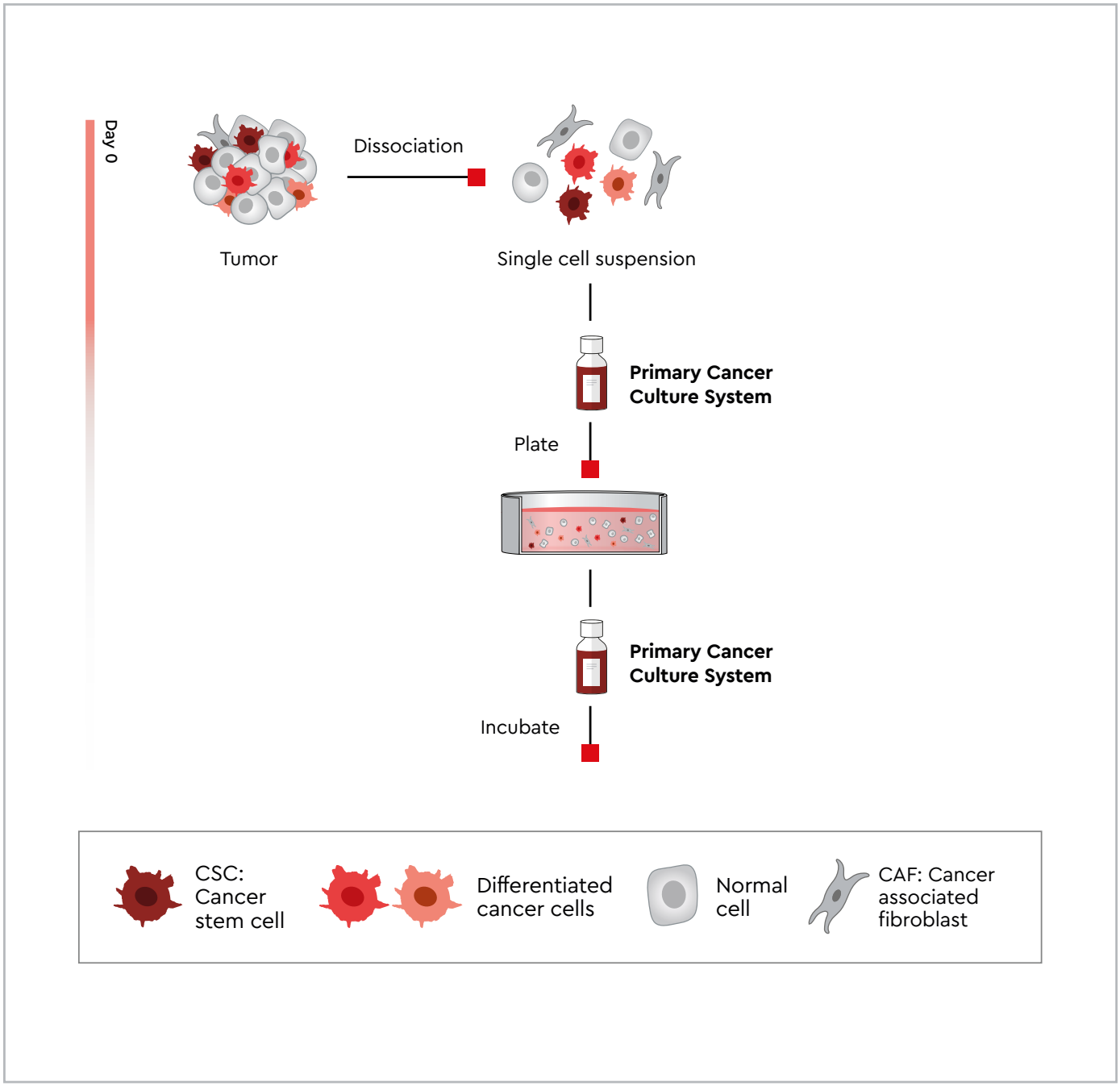

Fig. 4: Tumor cell isolation from biopsy using PCCS (C-28081).

Separation of adherent cancer cells from primary aggregates

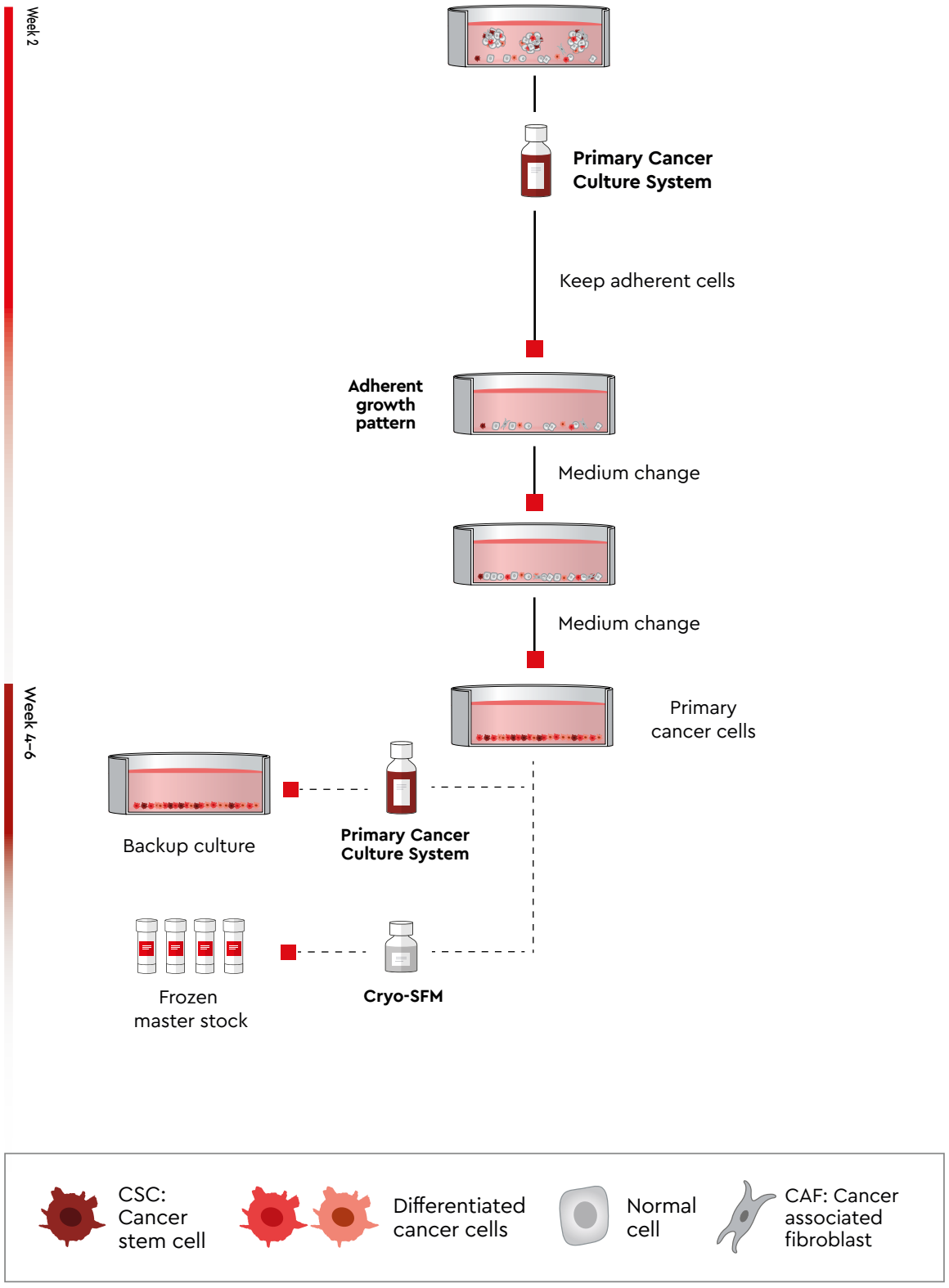

Fig. 5: Cancer cell culture expansion.

Protocol for establishment of primary cancer cell cultures

Depending on the tissue quality, type and malignancy stage of the tumor sample, obtaining a homogeneous primary culture may require 4–8 weeks.

I. Cancer cell isolation from tumor tissue samples

Materials

- Fresh tumor tissue (0.2–3 grams; ≥ 1 gram is optimal)
- Hanks Balanced Salt Solution (HBSS) with Ca^{2+}/Mg^{2+} without Phenol Red
- Primary Cancer Culture System (C-28081)*
  *consists of the Primary Cancer Cell Medium D-ACF and 2 ml of NCCD-Reagent (C-43080; also available separately)
- RPMI, MEMalpha or a comparable standard TC Basal Medium
- Gentamicin (50 mg/ml stock)
- Phosphate buffered saline (PBS) without Ca^{2+}/Mg^{2+} (C-40232)
- Accumax (e.g. Sigma #A7089) – tissue digestion/isolation
- Accutase (C-41310) – passage/subcultivation of established culture
- Scalpel/forceps/scissors
- Cell strainers of descending size down to 40 µm (e.g. 400/100/40 µm)
- Tilt-roll-shaker, rotary mixer or comparable
- Tissue culture flasks and dishes

Use aseptic techniques and a laminar flow bench.

1

NCCD treatment of the plasticware (day 0 or earlier)

The use of the NCCD-Reagent provided with the Primary Cancer Culture system is indispensable for successful isolation and maintenance of cancer cells. Dilute the thawed NCCD-Reagent stock solution 1:20 with PBS. Use 100 µl/cm^2 of culture surface to treat the tissue culture vessel with the diluted NCCD-Reagent and leave the closed vessel for at least one hour at room temperature. Make sure that the NCDD covers the entire vessel surface. Aspirate the NCCD solution just before seeding the cells

Note: Unless used immediately, the sealed vessel containing the NCCD-Reagent may be stored for up to three months at 2–8°C for later use. Diluted NCCD-Reagent solution may be stored for up to four weeks at 2–8°C protected from light.

2

Wash and weigh the tumor tissue

Remove visible residues of healthy tissue from the tumor. Place the tumor sample in a tube and wash twice with a generous amount of PBS and vigorous shaking. Then weigh the tumor tissue in a pre-tared sterile petri dish.

Note: The tumor tissue should be as fresh as possible and stored in HBSS at 2–8 °C immediately after surgical removal. Tissue up to six hours old is optimal for isolation purposes. However, successful isolations have been accomplished from tumor samples as old as 24 hours. Keep in mind that recently applied chemical or radiation therapy may severely affect the isolation results.

3

Homogenize the tumor tissue

Place the washed tumor sample on the lid of a petri dish. Add a small volume (1–2 ml) of Primary Cancer Cell Medium D-ACF to the tumor tissue and dissect it into small pieces of approximately 1 mm^3 using a scalpel. Avoid attrition of the tissue.

4

Wash the homogenized tumor tissue

Transfer the homogenized tumor tissue to a 50 ml tube using forceps. Add 10x the volume (w/v) of PBS and then invert the tube 5–10 times to remove residual blood and debris associated with the pieces of tumor tissue. Let the tissue pieces settle for two minutes and then aspirate the supernatant. Repeat if there is still a lot of blood/debris observable. Remove as much as possible of the PBS without loosing the tissue.

Note: If there is floating homogenized tissue, use a suitable sieve or cell strainer, for separating the washed, homogenized tissue from the washing buffer.

5

Perform the enzymatic digest of the tumor tissue

Resuspend the tissue pellet in Accumax solution at a concentration of 20 ml per gram of tumor tissue. Incubate at room temperature (20-25°C) with gentle but constant mixing, e.g. by a tilt-roll mixer at 40-50 rpm. Addionally invert the tube manually every 5 minutes. Digest until the solution becomes distinctly turbid. Depending on the type of tissue, this is typically the case after approximately 30–45 minutes. A 35-minute incubation is a good starting point.

Note: Do not digest the tissue longer than necessary and never digest for longer than 45 minutes since this may significantly compromise cell viability. Always perform the digestion reaction at room temperature and consult the Accumax manual for instructions on proper storage and handling.

6

Remove tissue residues from the sample

Let the remaining tissue pieces settle down for 2 minutes. In order to obtain a single-cell suspension, progressively filter the turbid supernatant using cell strainers of descending pore size down to 40 µm, e.g. 400 µm → 100 µm → 40 µm.

Note: Discard the remaining tissue pieces.

7

Dilute the sample with medium

Dilute the single-cell suspension at least 1:4 with RPMI or a comparable Basal Medium. Use a higher dilution ratio if the solution is still viscous.

8

Obtain the isolated single cells

Pellet the cell suspension for 10 minutes at 240 x g at room temperature and carefully aspirate the supernatant without disturbing the cell pellet.

9

Determine the number of viable nucleated cells

Gently resuspend the cell pellet in 5 ml of Primary Cancer Cell Medium D-ACF. Combine all cell pellets in 5 ml of medium in case your sample was divided into several tubes during the dilution step (7). Determine the number of viable nucleated cells using an appropriate method.

Note: In case of cell clumps, which cannot be resuspended, filter the cell suspension once more through a 40 µm cell strainer before counting. The expected yield is 1–3 million viable nucleated cells per gram of tumor tissue.

If it is not possible for any reason to determine the viable nucleated cell count in the primary isolate, continue with step 10 and refer to the Note in step 11. Keep in mind that omitting cell counting may lead to suboptimal seeding densities which may strongly impede the isolation efficiency.

10

Wash the cells

Pellet the cell suspension for 10 minutes at 240 x g at room temperature and carefully aspirate the supernatant without disturbing the cell pellet. Finally, resuspend the cell pellet in 1 ml of Primary Cancer Cell Medium D-ACF.

11

Plate the cells

Plate 100.000 to 300.000 viable nucleated cells per cm^2 in the prepared NCCD- treated tissue culture vessel(s). Use approximately 300 µl of medium per cm^2 for vessels ≤ 25 cm^2 of culture surface and approximately 130 µl medium per cm^2 for > 25 cm^2. Add 50 µg/ml of Gentamicin to the final volume and incubate at 37°C with 5% CO_2.

Example: Plate 1–3 million nucleated viable cells per well of a 6-well plate using 3 ml of medium. Plate 2.5–7.5 million nucleated viable cells per T-25 flask using 5 ml of medium.

Note: If the viable nucleated cell count was not determined in step 9, then plate the primary isolate from up to 2 grams of tumor tissue in 1–2 wells of a 6-well plate using 3 ml of Primary Cancer Cell Medium D-ACF per well.

II. CSC selection and expansion

12

Initiate of the primary tumor cell culture (day 0)

Incubate the culture for a total of 10–14 days to let the primary tumor cell culture begin but proceed with step two on day six after plating (see step 13).

Note: Typically, adherent and non-adherent cells as well as formation of multicellular primary suspension aggregates can be observed during the first two weeks of culture.

13

Add fresh medium (day 6)

On days 5–7, add an additional volume of the initial culture volume of fresh medium (without antibiotics) to the cells. Do not change the medium; simply add more fresh medium. Continue incubation until the culture reaches the stage described in step 14.

Example: : For an existing culture with a volume of 5 ml of medium, add another 5 ml of fresh medium. The resulting total culture volume is then 10 ml.

Note: If the medium turns orange-yellow due to high metabolic activity of the isolated cells before day six, the fresh medium should be added sooner. A slightly orange color is noncritical, however. If significant media exhaustion is still observed before the culture is ready for step 14, increasing the total culture volume by adding fresh medium is recommended.

14

Change medium during cancer cell selection (every 10–14 days)

Completely replace the medium of all samples every 10–14 days as described in step 14a. Adherent cultures remain in the same culture vessel until the first passage. Always keep flasks with adherent cells for at least four weeks or until you are absolutely sure they do not contain cells of interest.

Note: Make sure to prevent extensive medium exhaustion (indicated by an orange-yellow color; a slightly orange hue is still acceptable). Isolations in which no viable primary culture has become successfully established within six weeks after initial plating are not promising and can be discarded.

14a

Change medium for adherent cultures

Aspirate the used medium of adherent cells, wash the culture twice with PBS and add an appropriate amount of fresh medium (see step 15) to the cells.

15

Passage the tumor cell primary culture

Passaging the cells before they proliferate to a high confluence level is not recommended. Until they do, continue changing the medium as described for step 4.

Prepare new NCCD-treated culture vessels (see step 1). Depending on the overall confluence, perform a 1:1 or 1:2 split of the culture using Accutase (not Accumax). Wash the culture twice with ambient tempered PBS without Ca^{2+}/Mg^{2+} and then incubate the cells for 5–10 minutes with 100 µl/cm^2 Accutase at 37°C. After the first 5 minutes of incubation, monitor the detachment process visually. When the cells start to detach, facilitate their complete dislodgement by tapping the flask. Add the same volume of Primary Cancer Cell Medium D-ACF to the detached cells and spin down for 3 minutes at 300 x g at room temperature. Carefully aspirate the supernatant and gently resuspend the cell pellet in an appropriate amount of fresh Primary Cancer Cell Medium D-ACF (see below). Seed the cells into new NCCD-treated vessels and incubate them further at 37°C and 5% CO_2.

Recommended media volume: Use approximately 300 µl of medium per cm^2 for vessels with ≤ 25 cm^2 of culture surface and approximately 130 µl medium per cm^2 for > 25 cm^2. Continue incubation of the cultures at 37°C and 5% CO_2.

III. CSCs selection and expansion

16

Add fresh medium/perform a medium change

Add another volume of fresh medium or perform a complete medium change using the alternative culture medium as required and continue incubating at 37°C and 5% CO_2.

17

Expand and passage the cells

Expand and passage the cells using the PCCS. In case an alternative expansion medium is to be used, make sure keeping always a backup culture using the PCCS.

IV. Cryopreservation

For cryopreservation, harvest the isolated cells or cell aggregates as described in section III depending on the growth pattern of the culture. After aspiration of the used culture medium supernatant, gently resuspend the cell pellet or cell aggregates in an appropriate amount of cell-freezing medium and swiftly perform the cryopreservation according to your established standard procedures. For best results, the use of CryoSFM (C-29910) is recommended. Strictly avoid using serum-containing freezing-media.

Products

Media	Size	Catalog number
Primary Cancer Culture System consists of		C-28081
Primary Cancer Cell Medium D-ACF	250 ml	C-28080*
Primary Cancer Cell Medium D-ACF SupplementMix	for 250 ml	C-39880*
NCCD-Reagent	2 ml	C-43080
Cryo-SFM	30 ml	C-29910
Accutase Solution	100 ml	C-41310
Dulbecco's PBS, without Ca^{++}/Mg^{++}	500 ml	C-40232

**not available as single item*

References

1. Arneth, B. Tumor Microenvironment. *Medicina*, 2020. 56(1), 15.

2. Mitra, A., Mishra, L. and Li S. Technologies for deriving primary tumor cells for use in personalized cancer therapy. *Trends Biotechnol*, 2013. 31(6): p. 347–54.

3. Dey, N., Sun Y., leyland-jones, B. and De P. Evolution of Tumor Model: From Animal Model of Tumor to Tumor Model in Animal. *Journal of Cancer Therapy*, 2013. 04(09):1411–1425.

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