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# Properties of B16-F10 murine melanoma cells subjected to metabolic stress conditions*

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**Neoplastic cells which co-form tumors are usually subjected to various stress factors, mainly hypoxia and shortage of nutrient factors. Such cells employ different strategies that permit their survival under such conditions. Experiments *in vitro* are usually carried out in the presence of 21% oxygen and medium supplemented with 10% FBS. Altering these parameters can approximate the *in vivo* conditions found within tumor mass. The present paper reports certain properties (especially ability to metastasize) of B16-F10 cells able to grow upon exposure to altered growth conditions (medium supplemented with 0.06% FBS or presence of 1% oxygen for 24 or 72 hours). These properties were compared with those of control cells cultured in the presence of 21% oxygen and in medium supplemented with 10% FBS. Some properties of the cells exposed to medium supplemented with 0.06% FBS differ from those of cells cultured under low oxygenation conditions (ability to form metastases, to migrate, or to express various proteins). Only the partial deprivation of oxygen did increase both the number of migrating cells and the number of metastases formed. Serum deficiency enhanced only the cell ability to metastasize, but not to migrate. It appears that cultured B16-F10 cells employ different adaptation strategies under conditions of oxygen shortage and those of serum deficiency. Under oxygen deprivation, such cells most likely undergo an epithelial-mesenchymal transition, whereas serum deficiency ("starvation"), while increasing the tumorigenicity of B16-F10 cells, does not induce the epithelial-mesenchymal transition.**

**Key words:** Metabolic stress, hypoxia, epithelial-mesenchymal transition

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## INTRODUCTION

Neoplastic cells forming the inner mass of tumors must adapt to unfavorable conditions, including insufficiency of nutrients and oxygen. The latter trigger adaptation mechanisms, but a major fraction of cells exposed to a greater stress, or unable to cope with it, die (Vaupel, 2010; Choi, 2012).

Cell lines are a valuable tool in examining signaling pathways or genetic drifts. Numerous data pertaining to cancer cell biology have been generated by *in vitro* studies based on the use of cell cultures. Cancer cells propagated *in vitro* partially retain the hallmarks of cancer — such as immortality, resistance to apoptosis, etc. Less obvious are the features that manifest themselves only under *in vivo* conditions, such as the ability to escape from immune surveillance or invasiveness (van Staveren *et al.*, 2009).

Long-term cultures of cancer cells suffer from phenotype alterations due to a lack of interactions normally present between such cells and the extracellular matrix, as well as microenvironmental cells. Another disadvantage of cell culture is the discrepancy between culture conditions *in vitro* and those *in vivo* (medium supplemented with 10% FBS and 21% oxygen content in the atmosphere). As a result cultured cells do differ from cells fund in their natura environment (Gazdar *et al.*, 2010; van Staveren *et al.*, 2009).

In our study we examined changes in some properties of cultured B16-F10 murine melanoma cells exposed to non-standard growth conditions (reduced partial pressure of oxygen or reduced concentration of serum). Such culture conditions may reflect, to some degree, the conditions prevailing in growing tumors where, due to a lack of adequate blood supply, some cells are deprived of adequate oxygen tension and nutrient supply. Cancer cells employ defense mechanisms so their ability to survive results in increased aggressiveness. Only those cells survive that are capable of adapting to metabolic stress conditions. This leads to the appearance of a population of more aggressive cells refractive to treatment and capable of metastasizing (Gillies & Gatenby, 2007; Vaupel & Harrison, 2004). In our study we examined whether a similar effect can be obtained *in vitro* by manipulating cell culture conditions.

## MATERIALS AND METHODS

***In vitro* studies**. **Cell Culture.** B16-F10 murine melanoma cell line (ATCC, No. CRL-6475) was cultured in RPMI 1640 medium supplement with 10% GOLD serum (ICN), at 37°C and under 5% $CO_2$. Micrographs were taken with a Nicon Eclipse 80i microscope equipped with a Nikon Digital Sight DS-5Mc camera.

Metabolic stress (starvation) Subpopulations of B16-F10 cells capable of growth in serum-free medium were

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**Abbreviations**: bFGF, fibroblast growth factor – basic; CSC, cancer stem cells; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; PE, phycoerythrin

obtained by consecutive passages using media containing gradually reduced concentrations of serum (10% FBS, then 5% FBS, 2.5% FBS, etc.). Medium containing 0.06% FBS was supplemented with 20 μg/ml bFGF (BD-Biosciences) and 20 μg/ml EGF (BD-Biosciences), following a strategy employed by Dou *et al.* (2007).

**Hypoxia conditions.** Growing cell cultures were exposed to lowered oxygen tension conditions (1% $O_2$, 5% $CO_2$, 94% $N_2$) for either 24 or 72 h.

**FACS analysis.** Cell surface markers were analyzed by flow cytometry (FACSCanto, Becton Dickinson). FITC-conjugated anti-CD133 (eBioscience) and PE-conjugated anti-Notch1 (BD Pharmingen) antibodies were used for CD133 and Notch1 detection. For Oct4 marker identification, as primary antibody we used goat polyclonal anti-Oct4 (Abcam), and PE-conjugated donkey polyclonal anti-goat IgG (Abcam) served as the secondary antibody. The results present average from 3 independent experiments.

**Western blotting.** Cell lysates were subjected to SDS polyacrylamide gel electrophoresis. The presence of N- and E-cadherin, as well as beta-actin, was confirmed by Western blot using nitrocellulose membranes (Schleicher & Schuell). The proteins were detected with rabbit monoclonal anti-mouse N-cadherin antibody (Abcam), rabbit polyclonal anti-mouse E-cadherin antibody (Abcam) and rabbit anti-mouse beta-actin (Cell Signaling). Goat anti-rabbit IgG antibody conjugated with HRP was used as the secondary antibody (Vector). 3,3'-diaminobenzidine tetrahydrochloride (Sigma) was used as a substrate in peroxidase reaction for protein visualization.

**Clonogenic assay.** Aliquots of $2\times10^2$ cells were plated into 3-cm dishes, in medium supplemented with 10% FBS. After 8 days, the cultures were fixed using methanol, stained with Giemsa, and the colonies formed were counted. The experiment was conducted twice.

**Migration assay.** Motility of cells was studied using 24-well plates (BD Falcon) that contained cell culture inserts (8.0-μm pores). Briefly, 800 μl of RPMI or RPMI with 10% FBS was added to the lower chambers. Next, $3\times10^4$ B16-F10 cells (either control cells or cells subjected to starvation/hypoxia) were suspended in 300 μl of RPMI + 0.5% BSA medium and loaded into the upper chambers. The cultures were then incubated for 4 h at 37°C, following which the cells in the lower chamber were fixed using methanol, stained with 5% Giemsa and counted. The experiment was conducted three times.

***In vivo* studies. Animals.** Six- to eight-week-old C57BL/6 mice were bred and maintained at the on-site animal facility. The mice were injected iv. with $2\times10^5$ B16-F10 cells (either control cells or cells subjected to starvation/hypoxia). After 19–21 days, the animals were sacrificed, their lungs removed and metastatic lesions counted.

## RESULTS AND DISCUSSION

In our laboratory practice we have often met with the problem of repeatability of experimental metastasis formation in C57Bl6 mice following iv. tail injection of B16-F10 murine melanoma cells. The results of such experiments may depend on the animals' age or day of lung tissue collection. We have noted decreased numbers of such metastases when older passages of a given culture batch were used. This may reflect a decreased tumorigenicity of long-term cultured cells.

Here we investigated whether it was possible to restore the ability of such cells to form metastases by changing conditions of cell culture. We first studied such possibility by culturing cells under hypoxic conditions. Cells from different passages (7th, 12th or 17th) were transferred from standard culture conditions into an incubator with a decreased supply of oxygen (1% $O_2$, 72 hours). It was found that underoxygenation indeed restored the ability of older passages to form metastases (Fig. 1).

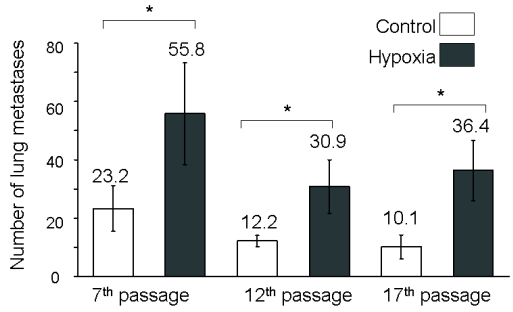


**Figure 1. Metastasis-forming ability of B16-F10 cells at various passages.**
C57Bl6 mice were injected iv. with $2\times10^5$ B16-F10 cells (various passages) cultured in 21% or 1% $O_2$ atmosphere for 72 h. After 21 days mice were sacrificed, their lungs removed and metastatic lesions counted. Each data point represents average ±S.D. from 9 or 10 animals (*$p<0.05$, Mann-Whitney test).

Another factor that could increase the aggressiveness of cells used for tumor inoculation is nutrient deprivation. To verify this, we switched culture medium from a regular one (containing 10% serum) to a medium with a reduced concentration of serum. B16-F10 cells cultured under these conditions soon ceased to proliferate. We thus decided to try to gradually adapt the investigated cultures to "starvation" conditions: at every next passage we decreased the amount of serum in the culture medium by half. After approximately 6 weeks we derived a population of cells capable of growth in a medium with a minimum amount of serum. Initially we did not observe any changes in morphology or in the behavior of such cells. When the concentration of serum was decreased to

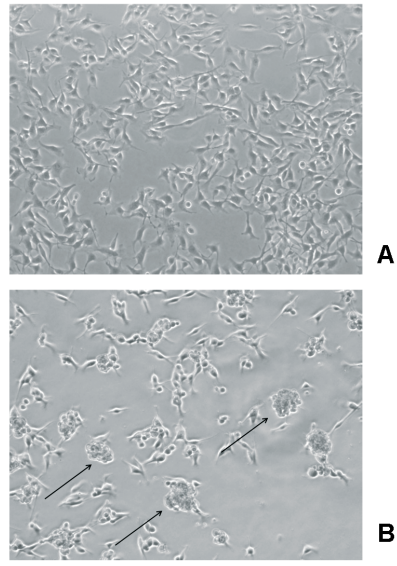


**Figure 2. Micrographs of cells adapted to starvation conditions**
Adherent and monolayer-forming starting-pool cells **(A)**, microspheres-forming cells (arrows) adapted to medium containing 0.06% FBS **(B)**.

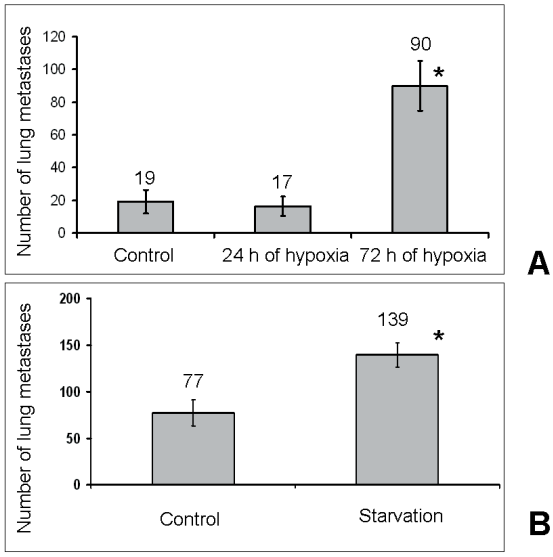


**Figure 3. Metastasis-forming ability of B16-F10 cells cultured under different conditions**
C57Bl6 mice were injected iv. with $2\times10^5$ B16-F10 cells (**(A)** cells cultured under hypoxic conditions; **(B)** cells cultured under starvation conditions, i.e. in medium with 0.06% FBS). After 19 days **(A)** or 21 days **(B)** the mice were sacrificed, their lungs removed and metastatic lesions counted. Each data point represents average ±S.D. from 6 animals (*$p<0.05$, Mann-Whitney test).

below 1%, the proliferative ability of the cells declined. At 0.06% FBS and addition of growth factors the cells underwent an abrupt switch: besides adherent cells we observed specific cell clusters, i.e. microspheres (Fig 2).

Both cells cultured using medium with 0.06% FBS and those cultured for 72 hours under hypoxic conditions were capable of forming substantially greater numbers of metastases, compared to cells cultured in the presence of 10% FBS and 21% of oxygen. In both cases the tumorigenicity of the challenged cells was higher than that of corresponding controls (Fig. 3). Increased was also their ability to form colonies (Table 1). Cells exposed to hypoxic conditions for 24 hours did not undergo such changes.

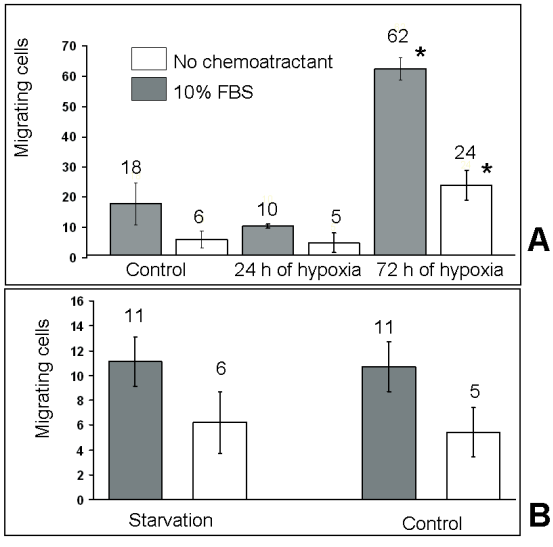


**Figure 4. Cell motility**
Boyden chamber assay was used to check cell motility (**(A)** cells cultured under hypoxic conditions; **(B)** cells cultured under starvation conditions, i.e. in the medium containing 0.06% FBS). B16-F10 cells ($3\times10^4$) were added to the upper chamber of transwells. After 4 hours, cells that migrated were stained with Giemsa and counted. Each data point represents average ±S.D. from 3 chambers; 5 or 6 counting fields in each (*$p<0.05$, Mann-Whitney test).

**Table 1. Colony-forming ability of cells growing under metabolic stress conditions**
Aliquots of $2\times10^2$ cells were plated into 3-cm dishes, in medium supplemented with 10% FBS. After 8 days the cells were fixed using methanol, stained with Giemsa and colonies formed were counted. Each data point represents average ±S.D. from 4 plates.

| | Number of colonies |
|---|---|
| Control | 37±4 |
| 24 h of hypoxia | 16±2 |
| 72 h of hypoxia | 136±21* |
| Starvation | 63±8* |

Underoxygenation can induce endothelial-mesenchymal transition (EMT) (Cannito *et al.*, 2008; Mani *et al.*, 2008). This is a process involving reprogramming of certain cell functions such as cell adhesion, polarity and motility. Expression of N-cadherin (responsible for interactions with extracellular matrix) rises, whereas that of E-cadherin (responsible for cell-cell interactions) becomes lower. Induction of EMT causes cancer cells to acquire the ability to metastasize, as well as certain features characteristic of cancer stem cells (CSC) (Polyak & Weinberger, 2009).

A migration test performed with Boyden chambers confirmed that cells cultured for 72 hours under hypoxic conditions could relocate (Fig. 4A). Western blotting demonstrated, in turn, that hypoxic conditions increase expression of N-cadherin, whereas that of E-cadherin becomes lower (Fig. 5). Starvation of cells did not induce greater cell motility (Fig. 4B), but resulted in lower expression of both types of cadherins (Fig. 5).

Both tested culture conditions involving challenge resulted in the appearance of cells that show features of CSC. The ability to grow in serum-deprived medium and to form microspheres is thought to represent one of the basic features of stem cells. Use of a medium containing 0.06% FBS could thus be regarded as a tool allowing appearance of cells displaying CSC features (Lobo *et al.*, 2007). In order to confirm that challenged cells do possess some features of stemness, chosen markers can

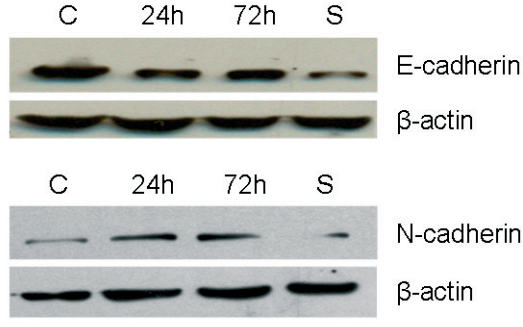


**Figure 5. Altered expression of EMT markers**
Western blot analysis of E-cadherin and N-cadherin expression. As loading control beta-actin was used (C — control; 24 h, 72 h — cells cultured under hypoxic conditions, S — cells cultured under starvation conditions, i.e. in the medium containing 0.06% FBS).

**Table 2. CSC markers in cells cultured under metabolic stress conditions**
Percentage of cells in a given population that express stem cell markers is shown. Cell surface markers were analyzed using FACS (C — control; 24 h, 72 h — cells cultured under hypoxic conditions, S — cells cultured under starvation conditions, i.e. in medium containing 0.06% FBS).

| | C | 24h | 72h | S |
|---|---|---|---|---|
| CD133 | 0.8±0.1% | 0.9±0.07% | 3.3±0.14%* | 2.4±0.7%* |
| Notch1 | 0% | 0.27±0.1% | 5±0.2%* | 3.17±0.46%* |
| Oct4 | 0.57±0.23% | 0.96±0.20% | 1.93±0.06%* | 0.93%±0.3% |

be determined (Hill *et al*., 2009). Based on our results, we can conclude that stress conditions indeed increased the number of cells expressing stem cell markers. Underoxygenation and serum deprivation increased expression of CD133 and Notch1. In response to hypoxia also the percentage of cells expressing Oct4 rose (Table 2).

A paucity of nutrients reaching cells triggers autophagy, an adaptation mechanism. Cells then form specific organelles, autophagosomes, which, upon fusing with lysosomes, become autophagolysosomes. In the latter, degradation of proteins and organelles takes place which permits cells to gain energy (Bursch *et al*., 2008).

Autophagy could not be confirmed in any of the cell cultures examined. Western blotting did not demonstrate conversion of LC3-I protein to its isoform LC3-II, which is thought to be an autophagy marker. Nor could the presence of autophagolysosomes be confirmed using flow cytometry with LysoTracker™ (Molecular Probes) (data not shown). Most likely, B16-F10 melanoma cells are resistant to autophagy, since conversion of LC3-I to LC3-II and autophagolysosome formation does occur to a minimum degree, even upon an abrupt switch to a serum-deprived culture medium. As a positive control, we used A172 human glioma cells. In that case culture medium substitution resulted in an instantaneous conversion of LC3-I to the LC3-II isoform and formation of autophagolysosomes. This confirms that autophagy does not play a role in the adaptation of B16-F10 melanoma cells to stress-inducing culture conditions.

To conclude, by altering cell culture conditions one can influence the cell phenotype. Both examined manipulations in culture conditions brought about an increased tumorigenicity and clonogenicity of the challenged cells. This may be elicited by cell adaptation – under stress conditions only the strongest, or the most aggressive clones survive. Triggering various adaptive mechanisms can also be a direct cause of the phenotype changes. Although the final effect appears identical in both cases, the underlying mechanism of alteration is different. Other examined features, such as expression of certain proteins or cell motility, change in a different manner. This testifies to the great plasticity of B16-F10 melanoma cells, capable of adaptation to different conditions in their microenvironment.

## Acknowledgements

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