REPORT

Cell Cycle 13:10, 1530–1539; May 15, 2014; © 2014 Landes Bioscience

CRABP1 provides high malignancy of transformed mesenchymal cells and contributes to the pathogenesis of mesenchymal and neuroendocrine tumors

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Keywords: CRABP1, tumorigenicity, metastasis, pancreatic neuroendocrine tumors, synovial sarcomas

Abbreviations: CRABP1, cellular retinoic acid-binding protein 1; EMA, experimental metastatic activity; pNETs, pancreatic neuroendocrine tumors ; RSV, Rous sarcoma virus ; SMA, spontaneous metastatic activity; RARE, retinoic acid responsive element

CRABP1 (cellular retinoic acid binding protein 1) belongs to the family of fatty acid binding proteins. Retinoic acid binding is the only known functional activity of this protein. The role of CRABP1 in human carcinogenesis remains poorly understood. Here, for the first time we demonstrated pro-metastatic and pro-tumorigenic activity of CRABP1 in mesenchymal tumors. Further functional analysis revealed that the pro-tumorigenic effect of CRABP1 does not depend on retinoic acid binding activity. These results suggest that CRABP1 could have an alternative intracellular functional activity that contributes to the high malignancy of transformed mesenchymal cells. Microarray analysis detected CRABP1-mediated alterations in the expression of about 100 genes, including those encoding key regulatory proteins. CRABP1 is ubiquitously expressed in monophasic synovial sarcomas, while in biphasic synovial sarcomas it is expressed uniquely by the spindle cells of the aggressive mesenchymal component. High level of CRABP1 expression is associated with lymph node metastasis and poor differentiation/high grade of pancreatic neuroendocrine tumors (pNETs). Presented data suggest CRABP1 as a promising biomarker of pNETs' clinical behavior. Our results give the first evidence of pro-tumorigenic and pro-metastatic activity of CRABP1 in mesenchymal and neuroendocrine tumors.

Introduction

Derived from a single cell, a malignant tumor grows in the body, invades the surrounding tissues and blood vessels, spreads by the blood and lymphatic system, and forms the secondary tumors in distant sites, the metastases. This process, known as tumor progression, leads to overwhelming majority of cancer-associated deaths. In clinical practice the extent of neoplasm malignancy is commonly indicated by tumor grade and presence of metastases. In experimental studies, 2 main criteria of cancer cells aggressiveness are used: tumorigenicity in vivo (an ability of transformed cells to initiate tumor being inoculated in experimental animals) and metastatic activity. Here we address the impact of CRABP1 (cellular retinoic acid binding protein 1) on tumorigenic and metastatic potential of transformed cells in vivo as well as its contribution to the high malignancy of human tumors.

CRABP1 is a well-conserved member of the fatty acid binding family of proteins. It localizes predominantly in the cytoplasm and can bind retinoic acid with high affinity.[1] Retinoic acid is the most active derivative of vitamin A. It stimulates differentiation and inhibits proliferation of both normal and tumor cells and plays an important role in carcinogenesis.[2-7] Alterations of genes regulating activity and transport of retinoic acid are commonly found in human tumors.[8-10] Although the binding of retinoic acid is the only known function of CRABP1, the effect of this interaction on the retinoic acid-dependent signaling remains unclear. Some data indirectly attest to CRABP1's ability to activate retinoid signaling,[11] while others evidence its negative role in this process[12,13] or even show the absence of the CRABP1 influence

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Submitted: 01/31/2014; Accepted: 03/08/2014; Published Online: 03/12/2014
http://dx.doi.org/10.4161/cc.28475

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on the intracellular activity of retinoic acid.[1,14] Similarly, little is known about the participation of CRABP1 in human cancer pathology, and the limited data concerning its link to cancer progression are inconclusive. Thus, sporadic screening-based studies reported hypermethylation of CRABP1 promoter or decrease of the CRABP1 mRNA level in some human epithelial tumors (colorectal cancer, hepatocellular, thyroid, and renal cell carcinomas),[15-18] suggesting this protein as a potential tumor suppressor. Similarly, decrease of CRABP1 expression was associated with the presence of distant metastases in esophageal squamous cell carcinoma[19] and with unfavorable prognosis of serous and clear cell ovarian adenocarcinomas.[20] Conversely, overexpression of CRABP1 was found in endometrioid ovarian cancer and highly invasive poorly differentiated endometrial adenocarcinomas.[21,22] Up to date nothing is known about CRABP1 protein expression in mesenchymal and neuroendocrine tumors. Therefore, the actual role of CRABP1 in cancer pathology is still unclear, and its functional implication in cancer progression has not yet been addressed experimentally.

Here we provide novel data that link CRABP1 to the aggressiveness of mesenchymal and neuroendocrine tumors. Using experimental mesenchymal cell models, we demonstrate that CRABP1 contributes to the cell malignancy by promotion of tumor-initiation ability and metastatic activity of transformed mesenchymal cells. IHC analysis of CRABP1 in human tumor samples revealed the significance of this protein for the pathogenesis of synovial sarcomas and pancreatic neuroendocrine tumors (pNETs).

Results

CRABP1 is essential for metastatic ability of transformed fibroblasts

Previously we described the appropriate experimental model system for the in vivo investigation of metastasis. It comprises the well-characterized panel of stable cell lines from the same origin (Rous sarcoma virus [RSV]-transformed Syrian hamster primary fibroblasts). These cell lines differ crucially in their ability to form lung metastases in SMA (spontaneous metastatic activity) assay performed on immunocompetent syngeneic animals.[23-25] SMA assay simulates all the stages of metastatic process, including the formation of a primary subcutaneous tumor, survival, and spreading of cancer cells in the bloodstream, and formation of secondary growth loci under the conditions of antitumor immunity. The panel described above includes originally low-metastatic HET-SR cell line, 2 originally high-metastatic cell lines, HET-SR1 and HET-SR8, and high-metastatic derivative of HET-SR line obtained through in vivo selection—HET-SR-2SC (**Fig. 1A**).

Earlier in search of the proteins responsible for high-metastatic phenotype we performed comparison of proteomic profiles of high- and low-metastatic cells (HET-SR1 and HET-SR) using 2-dimensional gel electrophoresis (2D-PAGE). This analysis revealed the qualitative difference in CRABP1 expression: high level of CRABP1 in HET-SR1 cells and the absence of this protein in HET-SR cells (**Fig. S1**). Here, we compared the expression of CRABP1 in the whole panel of cell lines described above using western blot analysis. All high-metastatic lines demonstrated high level of CRABP1 expression, while in low-metastatic cells, expression of this protein has not been detected (**Fig. 1A**). Importantly, HET-SR-2SC (the high-metastatic subclone isolated from lymph node metastasis of the low-metastatic HET-SR line) was also positive for CRABP1 (**Fig. 1A**, western blot). Such differential expression of CRABP1 suggests the involvement of this protein in acquisition of high-malignancy of mesenchymal tumor cells.

To elucidate the contribution of CRABP1 to the metastatic ability of studied cells we downregulated endogenous CRABP1 in highly aggressive HET-SR1 cells using 2 different shRNAs (shGFP was used as a negative control) (**Fig. 1B**, western blot). Analysis of spontaneous metastatic activity (SMA assay) of cells with depleted CRABP1 revealed the significant reduction of the number of lung metastatic nodules in comparison to the control (**Fig. 1B**). This effect was proportional to the degree of CRABP1 dowregulation by shRNA (see the western blot on **Fig. 1B**). Moreover, CRABP1 depletion resulted in marked decrease of the size of lung metastases formed after intravenous inoculation of cells (EMA assay), suggesting poor capacity of such cells to grow in host tissues. Thus, we observed more than 4-fold decrease in number of macrometastases formed by HET-SR1 shCRABP1 cells, while the total amount of metastatic nodules was similar in the experimental and control groups of animals. (**Fig. 1C**, bars).

These data demonstrate the significant role of CRABP1 in the maintenance of high metastatic phenotype of transformed fibroblasts.

CRABP1 promotes tumorigenicity of transformed mesenchymal cells, and this effect is independent from the retinoic acid binding ability

Tumorigenicity (tumor-initiation ability) along with metastatic activity is the other essential experimental criteria of malignancy of transformed cells. To test the effect of CRABP1 on tumorigenic activity, HET-SR1 shCRABP1 cells were injected subcutaneously into syngeneic hosts at different doses, and the frequency of tumor formation as well as tumor growth dynamics were quantified. Downregulation of endogenous CRABP1 resulted in significant reduction of the tumorigenicity (cell dose-dependent frequency of subcutaneous tumor formation) of HET-SR1 cells in comparison to control cells (**Fig. 2D**). We then overexpressed exogenous hamster Crabp1 within the retroviral vector (pLXSN) in HET-SR cells that originally did not express CRABP1 (**Fig. 2A**). This overexpression resulted in a significant increase of the tumorigenicity of HET-SR CRABP1 cells in comparison to the control (**Fig. 2D**). Noteworthy, we did not found any CRABP1-dependent changes in growth dynamics and size of subcutaneous tumors as well as in proliferation rate of investigated cells (data not shown). To validate the contribution of CRABP1 to the high malignant properties of human mesenchymal tumor cells, we used HT-1080 fibrosarcoma line that did not express endogenous CRABP1. We cloned human *CRABP1* in pLXSN vector and overexpressed the protein in HT-1080 cells (**Fig. 2B**). CRABP1 overexpression in human fibrosarcoma cells

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resulted in a significant increase of tumorigenicity in nude mice (**Fig. 2D**) similar to the effect observed on hamster model.

Taken together, these results show that CRABP1 could contribute to the acquisition of high malignant cell phenotype, promoting both tumor-initiating ability and metastatic activity.

As the interaction with retinoic acid is the only known function of CRABP1, we next studied the significance of retinoic acid binding for the pro-tumorigenic activity of CRABP1. For this purpose we used a previously described mutant form of CRABP1 (CRABP1 R131A) carrying arginine–alanine substitution in the protein ligand-binding site. This mutation blocks retinoic acid-binding activity of CRABP1.[26] The mutant sequence was also overexpressed within the pLXSN vector in HET-SR and HT-1080 cells. We found that overexpression of CRABP1 R131A mutant, similarly to the wild-type protein, significantly increased tumorigenicity of both studied cell lines (**Fig. 2D**). This effect could be achieved through 2 different scenarios. First, the CRABP1-dependent modulations in retinoic acid metabolism and downstream signaling could be insignificant for the described pro-tumorigenic activity of this protein. Second, CRABP1 does not influence retinoic acid-dependent signaling at all. To clarify this issue we performed luciferase reporter assay using luciferase reporter with retinoic acid-responsive element (RARE) from promoter of human RARβ (retinoic acid receptor, β).[27] We found that modulation of CRABP1 expression gives no valid effect on retinoic acid reporter activation in all investigated cells (HET-SR, HET-SR1, and HT-1080) (**Fig. 2C**). These results suggest that CRABP1-retinoic acid interaction has no effect on retinoic acid dependent signaling. Correspondingly, retinoic acid binding is not essential for CRABP1 pro-tumorigenic activity.

Searching for the additional mechanisms that could contribute to the pro-tumorigenic activity of CRABP1, we studied CRABP1-associated changes in gene expression patterns using microarray analysis. We compared the transcriptome profiles of HT-1080 CRABP1 cells and the control HT-1080 pLXSN cells and found statistically significant alterations in expression of about 100 genes in CRABP1-overexpressing cells (**Table S1**; **Fig. S2**). Significant alterations were defined as $P < 0.05$ and an absolute fold change >1.3. This list consists of a wide range of genes attributed to the regulation of numerous cellular activities and involved in various signaling pathways. Interestingly, gene ontology (GO) terms enrichment analysis of obtained genes list revealed overrepresenting in

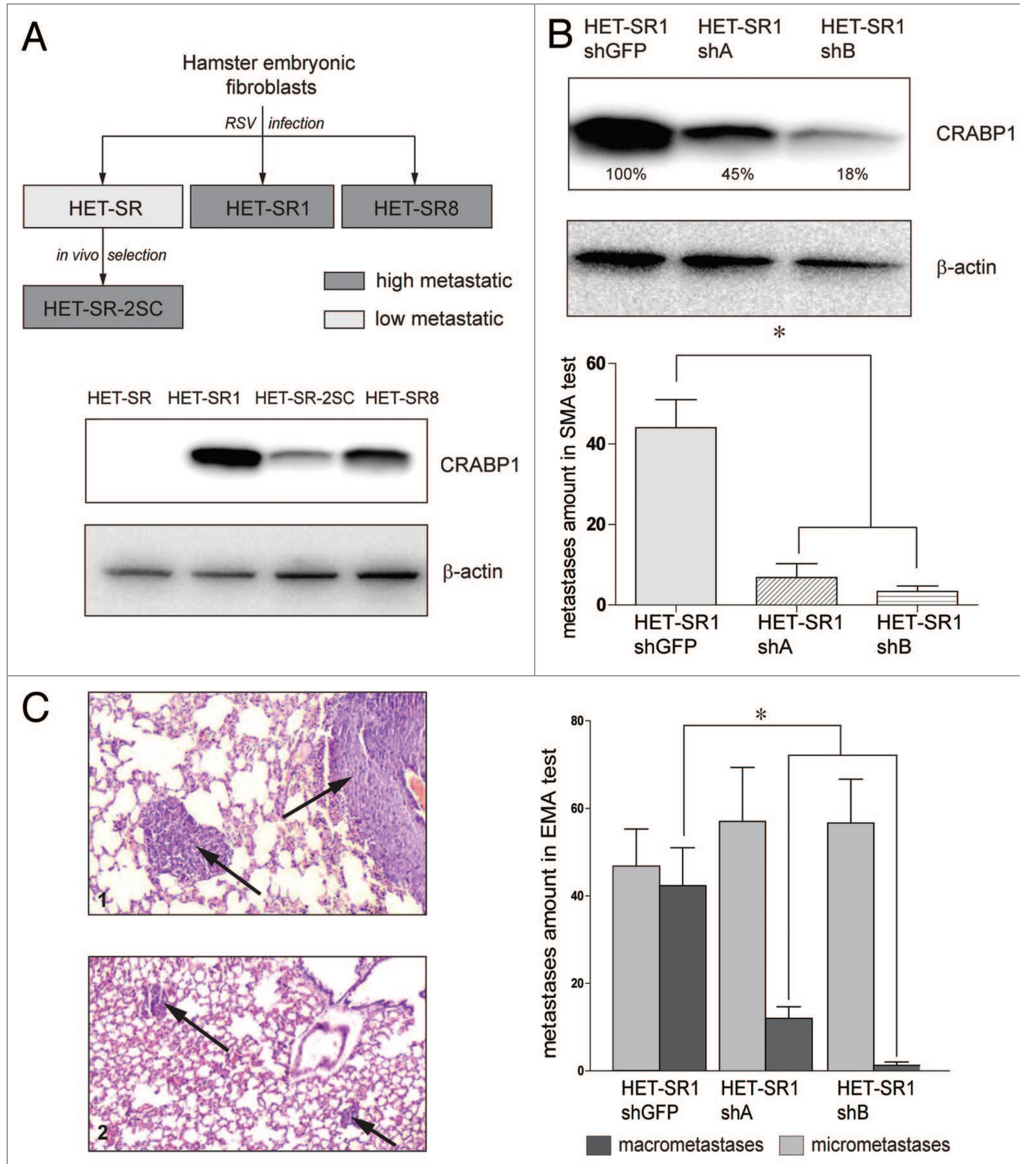

Figure 1. CRABP1 contributes to the metastatic ability of transformed hamster fibroblasts. (**A**) Endogeneous CRABP1 expression correlates with the level of spontaneous metastatic activity (SMA) of RSV-transformed hamster fibroblasts; (**B**) depletion of endogeneous CRABP1 in high-metastatic HET-SR1 cells reduces total amount of lung metastases in SMA test. Bars represent the amount of histologically verified lung metastases formed after subcutaneous injection of investigated cells; (**C**) depletion of CRABP1 decreases the amount of macrometastases in EMA test. (**C1**) example of macrometastasis (×200), (**C2**) example of micrometastasis (×200). Bars represent the amount of lung macro- and micrometastases formed after intravenous injection of investigated cells; *$P < 0.05$

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genes that participate in activation of transcription, subcellular localization of proteins, SUMO-binding, and tRNA methyltransferase activity (**Table 1**).

CRABP1 is ubiquitously expressed in human synovial sarcomas

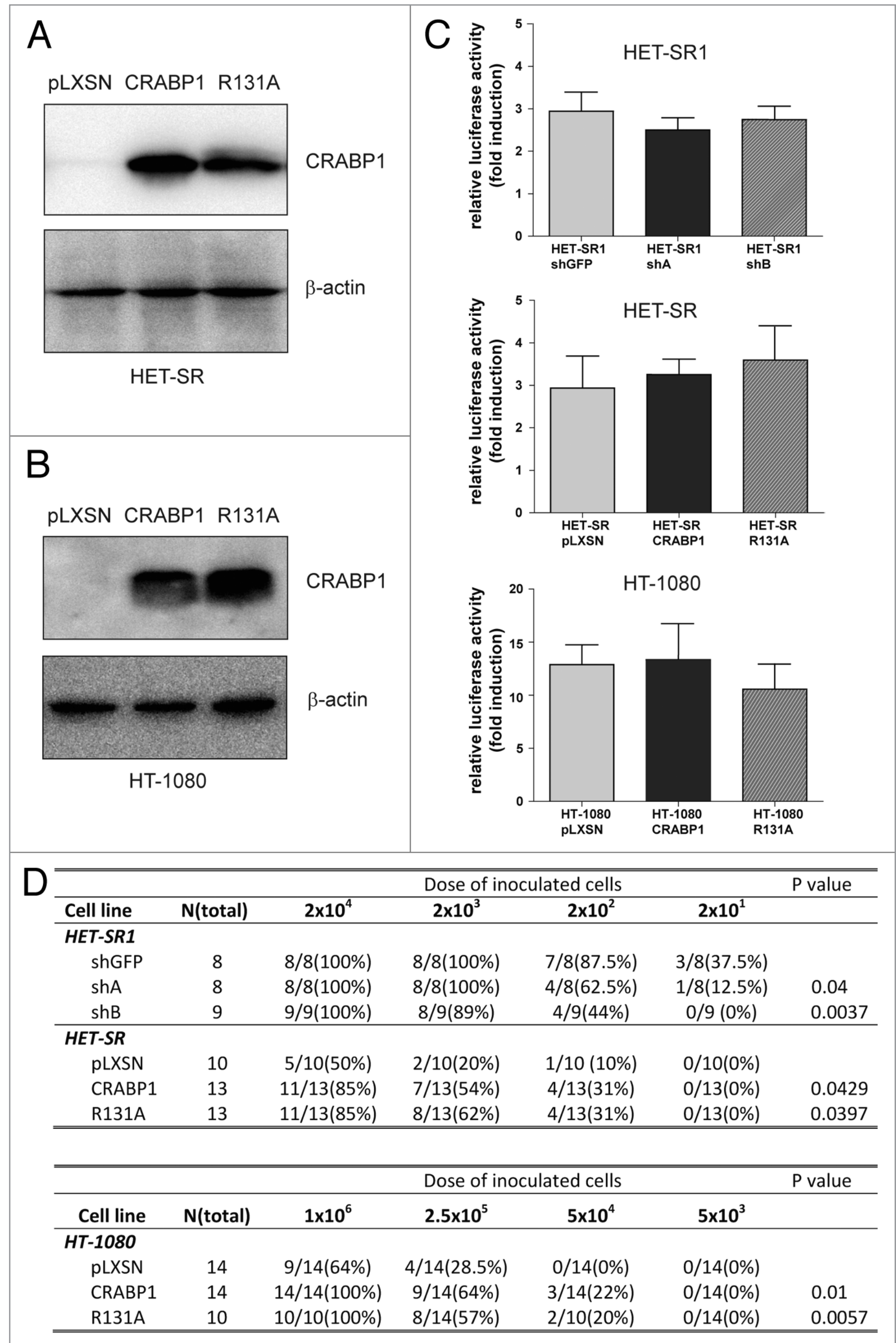
To elucidate the role of CRABP1 in pathogenesis of human mesenchymal tumors we performed the IHC analysis of synovial sarcomas. Synovial sarcoma is predominantly spindle cell mesenchymal tumor that represents up to 10% of all soft tissue sarcomas. Histologically, synovial sarcomas are divided into biphasic and monophasic types depending on the presence of glandular epithelial component. Synovial sarcomas belong to the highly aggressive neoplasms characterized by the absence of G1 grade according to FNCLCC classification. Since there are currently no data concerning CRABP1 protein expression in soft tissue sarcomas, we analyzed CRABP1 in 40 synovial sarcomas, including 28 monophasic and 12 biphasic. Studied sampling was rather heterogeneous with respect to the localization, size, and grade of primary tumor (G2/G3), and patients' age. Along with primary tumors (32), sampling included relapsed tumors (6), and lung metastases (2).

All studied tumors showed positive CRABP1 staining. The level of CRABP1 in tumor cells varied from moderate (37.5% samples) to strong (62.5% samples), while adjacent tissues were CRABP1-negative (**Fig. 3A**, 1–4). Such uniform pattern of CRABP1 expression together with high aggressiveness attributable to synovial sarcomas complicates the further search for correlations with clinicopathological characteristics of tumors. Importantly, in biphasic synovial sarcomas, only spindle cells of mesenchymal component were positive for CRABP1 while no CRABP1 staining was observed in glandular epithelial component (**Fig. 3A**, 4).

These results suggest the involvement of CRABP1 in the pathogenesis of synovial sarcomas in a general manner.

High level of CRABP1 expression is indicative of aggressiveness of human pancreatic neuroendocrine tumors

Pancreatic neuroendocrine tumors (pNETs) represent one of the most



Cell line	N(total)	Dose of inoculated cells: $2x10^4$	$2x10^3$	$2x10^2$	$2x10^1$	P value
HET-SR1						
shGFP	8	8/8(100%)	8/8(100%)	7/8(87.5%)	3/8(37.5%)	
shA	8	8/8(100%)	8/8(100%)	4/8(62.5%)	1/8(12.5%)	0.04
shB	9	9/9(100%)	8/9(89%)	4/9(44%)	0/9 (0%)	0.0037
HET-SR						
pLXSN	10	5/10(50%)	2/10(20%)	1/10 (10%)	0/10(0%)	
CRABP1	13	11/13(85%)	7/13(54%)	4/13(31%)	0/13(0%)	0.0429
R131A	13	11/13(85%)	8/13(62%)	4/13(31%)	0/13(0%)	0.0397

Cell line	N(total)	Dose of inoculated cells: $1x10^6$	$2.5x10^5$	$5x10^4$	$5x10^3$	P value
HT-1080						
pLXSN	14	9/14(64%)	4/14(28.5%)	0/14(0%)	0/14(0%)	
CRABP1	14	14/14(100%)	9/14(64%)	3/14(22%)	0/14(0%)	0.01
R131A	10	10/10(100%)	8/14(57%)	2/10(20%)	0/14(0%)	0.0057

Figure 2. CRABP1 promotes tumorigenicity of transformed mesenchymal cells independently from retinoic acid binding ability. (**A**) Expression of exogenous wild-type CRABP1 and R131A mutant (unable to interact with retinoic acid) in HET-SR cells determined by western blot; (**B**) expression of exogenous wild-type CRABP1 and R131A mutant in HT-1080 cells determined by western blot; (**C**) CRABP1 expression has no influence on retinoic acid-mediated activation of RARE-based luciferase reporter. Bars represent fold-activation of RARE-based luciferase reporter after stimulation with retinoic acid in comparison to non-stimulated cells. For the details see "Materials and Methods"; (**D**) depletion of CRABP1 in HET-SR1 cells reduces tumorigenicity (dose-dependent frequency of primary tumors formation after subcutaneous inoculation of cells); wild-type CRABP1 and R131A mutant similarly promote tumorigenicity of both HET-SR and HT-1080 cells. (For investigation of tumorigenicity, cells were subcutaneously injected to experimental animals in serial dilutions [dose] and the occurrence of tumors was examined. The total number of animals and the number of tumors derived from each dose are presented in the table. Statistical significance was defined as $P < 0.05$).

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Table 1. Enrichment in Gene Ontology terms in the list of CRABP1-regulated genes in HT-1080 cells

Description	*P* value	Enrichment score	Genes (fold change)
Regulation of transcription	$5.69 \times 10^{E-04}$	3.17	SATB1 (1.47), WNT10B (1.31), CHEK2 (1.44), RGMB (1.32), RNF111 (1.37), HSF2 (1.39), PHF8 (1.35), INSIG2 (1.33), KLF12 (1.34), RNF20 (1.31), RARA (1.36), NACA (1.39), ADORA2B (1.35)
Cellular protein localization	$8.98 \times 10^{E-04}$	6.68	AP1AR (1.34), HOOK3 (1.31), CELSR1 (1.31), PAQR3 (1.34), ATP6V1D (1.32)
SUMO binding	$7.02 \times 10^{E-04}$	50.12	RNF111 (1.37), SIMC1 (1.33)
tRNA methyltransferase activity	$4.36 \times 10^{E-04}$	63.89	TRMU (0.75), TRMT61A (0.74)

common groups of neuroendocrine tumors with unclear pathogenesis, variable malignant properties, and clinical diversity. Up to date the diagnosis and clinical prognosis of certain neuroendocrine tumors are restricted due to the high heterogeneity in etiology and malignant potential. The absence of high specific and high sensitive biomarkers further complicates the diagnostics. Grading of pNETs is based exclusively on proliferative rate (with Ki-67 index as the main criteria). According to the 2010 WHO (World Health Organization) classification all neuroendocrine tumors (including pNETs) are separated into 2 categories: well-differentiated tumors (traditionally referred to as carcinoids and characterized by grade G1–G2, Ki-67 index <20%) and poorly differentiated tumors (referred to as high-grade [G3] neuroendocrine carcinomas, Ki-67 index >20%). Expression of CRABP1 has never been tested in pNETs, although some indirect data obtained on transgenic mice model point to the potential role of CRABP1 in the carcinogenesis of neuroendocrine tumors.[28] Here we performed the IHC analysis of CRABP1 on a panel of 71 primary pNETs from patients who had undergone surgical resection at N.N. Blokhin Russian Cancer Research Center.

We observed positive cytoplasmic CRABP1 immunostaining of tumor cells in the majority of studied samples. At the same time, native ductal and acinar structures demonstrated complete absence of CRABP1 expression, excluding the single cells of Langhergans islets. Representative pictures of immunostaining variants are shown in **Figure 3B**, 1–4.

We analyzed the correlation between the expression level of CRABP1 (strong vs. weak/moderate) and clinicopathologic characteristics listed in the **Table 2**. The levels of CRABP1 expression were classified as weak (composite score as 0–3), moderate (composite score as 4–6), and strong (composite score as 7–9). A composite score was calculated by multiplying staining intensity and area scores. We found that high CRABP1 expression was significantly associated with the presence of lymph node metastases ($P = 0.011$). We also detected statistically significant association ($P = 0.009$) of high CRABP1 expression and poor differentiation of pNETs (G3 grade, Ki-67 index > 20%). These data provide

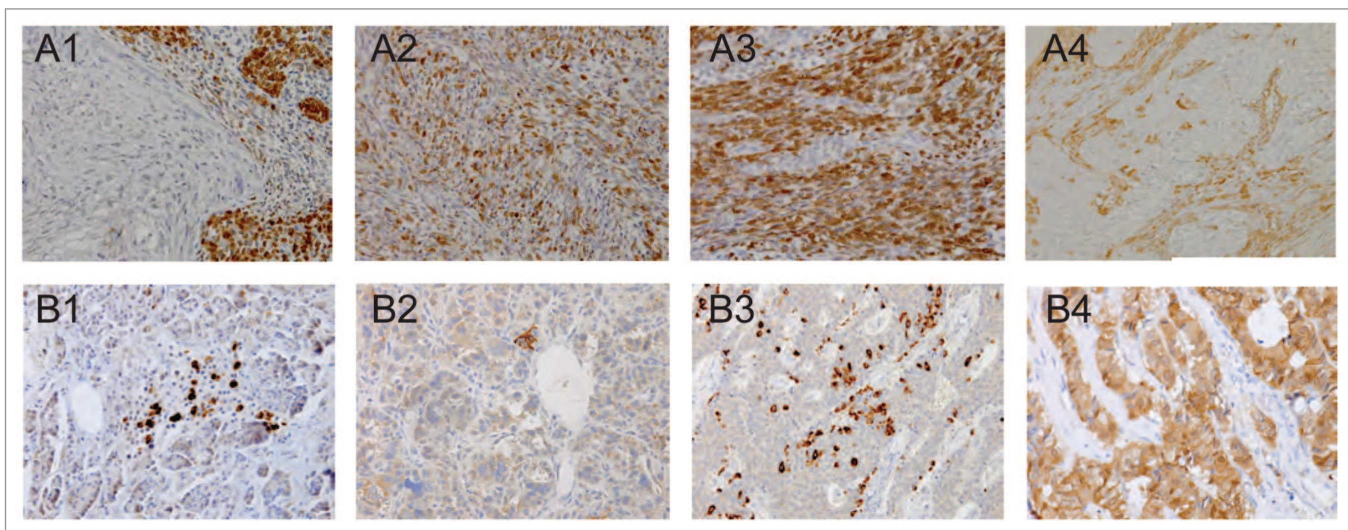

Figure 3. Immunohistochemical analysis of CRABP1 expression in synovial sarcomas (SS) and pancreatic neuroendocrine tumors (pNETs). (**A1–4**) Examples of CRABP1 immunostaining in synovial sarcomas: (**A1**) CRABP1-positive monophasic SS cells and CRABP1-negative surrounding tissues (×400); (**A2**) example of moderate expression in monophasic SS (×400); (**A3**) example of strong expression in monophasic SS (×400); (**A4**) example of expression of CRABP1 in mesenchymal component of biphasic SS (×400). (**B1–4**) Examples of CRABP1 immunostaining in pNETs: (**B1**) example of normal pancreatic islet tissue (×400); (**B2**) example of weak expression (×400); (**B3**) example of moderate expression (×400); (**B4**) example of strong expression (x600).

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the first evidence that high CRABP1 expression is associated with aggressiveness of pNETs and suggest this protein as a candidate biomarker for this type of tumors.

Discussion

To date, the actual role of CRABP1 in carcinogenesis and tumor progression remains unclear. A few previous studies have provided contradictory data concerning the association of CRABP1 expression with the prognosis of several epithelial cancers. It seems likely that the prognostic value of CRABP1 is tumor context-dependent. The involvement of CRABP1 in pathogenesis of mesenchymal and neuroendocrine malignancies has not yet been addressed. Here, for the first time, we demonstrated that CRABP1 has the significant impact on the formation of tumor cells malignant phenotype affecting metastatic ability and tumorigenicity. Our data also first link CRABP1 to carcinogenesis and aggressiveness of 2 types of human neoplasms from different origins: synovial sarcomas and pancreatic neuroendocrine tumors (pNETs).

We showed that CRABP1 is ubiquitously expressed in primary synovial sarcoma tumors, relapsed tumors, and metastases. These data are in concordance with 2 previous reports that have demonstrated an elevated level of *CRABP1* mRNA in human synovial sarcomas in contrast to other soft tissue sarcomas.[29,30] Synovial sarcomas are highly aggressive tumors (characterized by the absence of G1 grade according to FNCLCC classification) that are usually diagnosed at advanced stages. We found that all synovial sarcomas were CRABP1 positive and demonstrated predominantly strong (62.5%) or moderate (37.5%) expression of CRABP1 with no samples expressing low level of CRABP1. Moreover, in biphasic synovial sarcomas, CRABP1 was detected exclusively in the aggressive mesenchymal component. These data suggest that CRABP1 is involved in the pathogenesis of synovial sarcomas in a general manner. We did not find any significant associations between expression of CRABP1 and clinicopathologic characteristics of tumors (such as tumor grade and stage of disease) due to the high heterogeneity of sampling on the one hand and homogeneous character of CRABP1 expression on the other.

The involvement of CRABP1 in progression of mesenchymal tumors was further supported by our experiments with mesenchymal cancer cell models (human fibrosarcoma cell line and RSV-transformed primary fibroblasts of Syrian hamster). We demonstrated that CRABP1 participates in the formation of malignant phenotype of mesenchymal tumor cells, promoting tumorigenicity and metastatic activity. Particularly, we found that CRABP1 is expressed exclusively in high-metastatic cell lines, including the derivative selected in vivo (obtained from lymph node metastasis formed after subcutaneous inoculation of initially low-metastatic CRABP1-negative cells). We next showed that downregulation of CRABP1 in highly tumorigenic and high-metastatic cell line resulted in a decrease of both potentials in syngeneic hosts. Besides, the capacity of cells to grow in the lung microenvironment (to form macrometastases) after direct intravenous injection was also suppressed as a result of CRABP1 downregulation. Furthermore, overexpression of CRABP1 in low-metastatic hamster cell line as well as in human fibrosarcoma HT-1080 cells significantly promotes tumorigenicity (defined as cell dose-dependent frequency of subcutaneous tumors formation). These results suggest that

Table 2. Association of CRABP1 expression with clinicopathological characteristics of the human pancreatic neuroendocrine tumors (pNETs)

		CRABP1 expression		
		Weak + moderate	**Strong**	***P* value**
Variables	**n**	**n (%)**	**n (%)**	
Sex				0.045
Male	35	24(43%)	11(73%)	
Female	36	32(57%)	4(27%)	
Age				0.725
<40	13	11(20%)	2(13%)	
>40	56	45(80%)	13(87%)	
Grade				**0.025**
1	6	5(9%)	1(7%)	
2	57	48(86%)	9(60%)	
3	8	3(5%)	5(33%)	
pT				0.7
1	4	3(5%)	1(6.3%)	
2	25	21(37.5%)	4(26.3%)	
3	32	25(45%)	7(46.3%)	
4	10	7(12.5%)	3(20%)	
pN				**0.011**
0	49	43(77%)	6(40%)	
1	22	13(23%)	9(60%)	
M				0.385
0	41	34(61%)	7(47%)	
1	30	22(39%)	8(53%)	
Differentiation				**0.009**
Well (Ki-67 index < 20%)	63	53(95%)	10(66.5%)	
Poor (Ki-67 index > 20%)	8	3(5%)	5(33.5%)	

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CRABP1 contributes to the primary tumor initiation and secondary colonization by conferring to cancer cells a higher capacity to survive and grow in different microenvironments. CRABP1 does not seem to be involved in proliferation per se, because we did not observe any significant changes in growth rate or size of experimental primary tumors. Additionally, experiments in vitro did not reveal significant differences in proliferation rate of cells with modulated CRABP1 expression (data not shown).

Despite that the binding of retinoic acid is the only known function of CRABP1, our data suggested that this ability is not essential for the pro-tumorigenic activity of CRABP1. Thus, we didn't establish any changes in activity of retinoic acid signaling after modulation of CRABP1 expression in all investigated cell lines. Besides, the CRABP1 R131A mutant that binds retinoic acid with very low affinity[26] promoted tumorigenicity similarly to the wild-type protein in both HET-SR and HT-1080 cell lines. It seems like CRABP1 has no influence on retinoic acid metabolism and downstream retinoic acid-dependent signaling in described experimental models. These results are in concordance with a previously published study, where the similar absence of the effect of CRABP1 on retinoic acid signaling was demonstrated on COS-7 and CV-1 cells.[1,14] Taken together, these results allow to assume the existence of some additional intracellular functions of CRABP1 that are not connected with retinoic acid binding. Different evidences of atypical mitochondria localization of this protein,[31] perinuclear localization of CRABP1 mRNA and protein,[32] and phosphorylation of this protein during the cell cycle[33] indirectly support our hypothesis.

The mechanism by which CRABP1 promotes tumor formation needs further attention. Our data suggest that CRABP1 helps tumor cells to survive and grow in tissues, i.e., it promotes tumor formation at primary and secondary sites. The described pro-tumorigenic activity of CRABP1 might be explained by promotion of cancer cells' "stemness", since tumorigenicity is considered to be the key property of cancer stem cells.[34-36] Data obtained by microarray analysis indirectly support this hypothesis. In particular, overexpression of CRABP1 in the HT-1080 cells enhances expression of *SATB1* gene that promotes stem cells quiescence, represses differentiation, and participates in induction of stem-cell phenotype in both normal and transformed cells.[37-40] We also observed the modulations in expression of a list of transcription activators/repressors, such as *KLF12*, *RFX1*, and *HOXC6*, that play an important role in differentiation of wide range of cells.[41-44] Interestingly, microarray analysis did not reveal significant changes in expression of genes from classic retinoid signaling pathway (except *RAR*α) or well-known retinoid responsive genes. This finding also implies the existence of "non-classical" functional activity of CRABP1. Further investigations are needed for better understanding of the precise molecular mechanisms that enable CRABP1 to play an important role in progression of mesenchymal tumors.

Interestingly, it seems that CRABP1 could also stimulate the aggressiveness of tumor cells from non-mesenchymal origin. It was previously shown that overexpression of CRABP1 in transgenic mice resulted in the formation of spontaneous pancreatic neuroendocrine neoplasms.[28] This observation led us to assume that CRABP1 could be involved in pathogenesis of human pancreatic neuroendocrine tumors (pNETs). pNETs are characterized by high diversity in pathogenesis and clinical course of disease. The last WHO classification subdivided pNETs into well-differentiated tumors (commonly referred to as carcinoids) and poorly differentiated high-grade carcinomas. Noteworthy, these 2 groups are strongly distinct in biological behavior. They could be even conditionally considered as different pathologies, since the transformation from well-differentiated tumors into poorly differentiated carcinomas has only rarely been reported.[45] The separation of poorly differentiated carcinomas (G3) is based solely on high proliferative rate that corresponds to Ki-67 index >20%. The prognosis and clinical outcome of pNETs remains also difficult, because the few available markers are not enough specific and sensitive for a given tumor. IHC analysis performed in this study revealed a strong association between the intensity of CRABP1 staining and the presence of lymph node metastases as well as the poor differentiation/high grade of pNETs. These results point on the high contribution of CRABP1 to the progression of pNETs and correspond well with our data obtained on mesenchymal tumor models.

In sum, we provide the first evidence of CRABP1 involvement in the progression of mesenchymal and neuroendocrine tumors and substantiate its contribution to the tumorigenicity and metastatic activity of transformed mesenchymal cells. Obtained results provide the basis for further investigation of intracellular functions and activities of CRABP1 in the context of carcinogenesis. Additionally, presented data open the perspectives for application of CRABP1 as a biomarker of clinical behavior and prognosis of pNETs.

Materials and Methods

Cell cultures and production of stable cell lines

Human fibrosarcoma cell line HT-1080 (ATCC# CCL-121) and Syrian hamster embryonic transformed fibroblasts (HET-SR, HET-SR1, HET-SR8, HET-SR-2SC) were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (PAA laboratories) with penicillin and streptomycin. All cell lines were maintained in standard conditions (37 °C, 5% CO_2).

For stable cell line production packaging cell lines (GP-293/293FT) (Clontech/Sigma-Aldrich) were co-transfected with retroviral/lentiviral vectors (pLXSN neo/pLKO.1 puro) (Clontech/Sigma-Aldrich) and additional plasmids (pVSVG or pVSVG/pCMVdeltaR8.2 mix) (Clontech/Sigma-Aldrich) using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Cell-free and virus-contained medium (24, 48, and 72 h after infection) from packaging cells was added to investigate cells in the presence of 8 μg/ml Polybrene (Sigma-Aldrich). Infected cells were selected on G-418 (14 d, Calbiochem) or puromycin (7 d, Sigma-Aldrich) depending on the vector type.

Vectors construction

Coding sequence of hamster *Crabp1* was amplified from HET-SR1 cDNA and cloned into pLXSN neo

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using following primers (restriction sites are underlined): TATCTCGAGACCATGCCCAACTTC (forward) and TAAGGATCCG GCCACCTTTA CTCC (reverse). To establish the coding sequence of hamster *Crabp1* gene amplification and cloning procedures were provided independently in duplicate. This newly identified sequence was submitted to GenBank (GQ139546.1). Human *CRABP1* coding sequence was obtained from cDNA of human synovial sarcoma sample and cloned into pLXSN neo using primers: ATTCTCGAGC CACCATGCCC AACTTC (forward) and ACAGGATCCC TGCCTTCACTCTCGG (reverse). The oligonucleotides used for site-directed mutagenesis were: GCCGGATCCG GCCACCTTTA CTCCCGGACA TAAATTGCTG TGCACA (for hamster CRABP1 R131A) and GCCGGATCCC TGCCTTCACT CTCGGACATA AATTGCGGTG CAGAC (for human CRABP1 R131A, mutant codons are underlined). For knockdown of *Crabp1* 2 non-overlapping precursors of shRNA were cloned in pLKO.1 puro vector: CCGGCGGACG CAAGTGCAGG AGTTTCTCGA GAAACTCCTG CACTTGCGTC CGTTTTTG and CCGGCACAAG AATTTATGTC CGGGACTCGA GTCCCGGACA TAAATTCTTG TGTTTTTG (anti-sense sequences are underlined). shRNA to GFP was used as a negative control. To obtain a retinoic luciferase reporter dimer of 2 minimal promoter regions of the human RARβ (from –59 to +1 nucleotide position contained direct-5 retinoic responsive element [DR5-RARE]) was cloned in pGL3 basic vector (Promega). All constructs were verified by sequencing.

Western blot analysis

Cells were lysed in buffer contained 50 mM TRIS-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM EDTA, 0.1% SDS and Complete Protease Inhibitor Cocktail (Roche). The protein concentration of lysates was determined using Bradford Assay Kit (Bio-Rad). Twenty micrograms of total protein from each lysate were denaturated by boiling, separated in 12–15% SDS-PAGE gel, and transferred to Immobilon transfer membrane (Millipore). The membranes were blocked with 5% non-fat dry milk in standard TBS buffer with 0.05% Tween-20 and incubated (overnight, +4 °C) with anti-CRABP1 (Abcam, ab2816, dilution 1:2000–5000) antibodies and anti-β actin (Abcam, ab8227, dilution 1:5000) antibodies. After washing and incubation of blots with HRP-conjugated secondary anti-rabbit (Jackson ImmunoResearch Europe Ltd, 111-035-045) and anti-mouse (Abcam, ab2571) antibodies, respectively (1 h, room temperature), bands were detected using Immobilon detection system (Millipore). Images of blots were captured using Kodak GelLogic 2200 Imaging system and processed using Carestream Molecular Imaging Software SE ver. 5.3.3.

Luciferase-reporter assay

Investigated cells (1,5–5 × 10^4 per well) were seeded on 24-well plates and transfected with 500 ng of plasmid mix (450 ng retinoic reporter/ 50 ng pRL-TK Renilla luciferase vector [Promega] as internal control) using Plus/Lipofectamine transfection system (Invitrogen). All-trans retinoic acid (ATRA) dissolved in DMSO was added to 10^{-8}–10^{-9} M final concentration after 6–8 h following transfection. After 24 h cells were lysed and relative luc-activity was measured using Dual-Luciferase Reporter Assay System (Promega). Induction of retinoic reporter activity was calculated as ratio of relative luc-activity in presence of ATRA to the control (normal medium). All experiments were performed in triplicate.

Analysis of tumorigenicity

All animal experimental protocols were approved by the Committee for Ethics of Animal Experimentation; the experiments were conducted in accordance with the Guidelines for Animal Experiments in N.N. Blokhin Russian Cancer Research Center.

For investigation of tumorigenicity hamster cell lines HET-SR, HET-SR1, and their derivatives were subcutaneously injected to adult (10-wk-old) Syrian hamsters (Mesocricetus auratus) in 4 serial dilutions (from 2 × 10^4 to 2 × 10^1 cells). The occurrence of tumors was examined after 8 wk. In case of human HT-1080 cell line derivatives, cells were subcutaneously injected in 4 serial dilutions (from 1 × 10^6 to 5 × 10^3) to adult D2 × J nude mice.[46] The occurrence of xenografts was examined after 3 wk.

Spontaneous metastatic activity (SMA) assay and experimental metastatic activity (EMA) assay

For spontaneous metastasis assay 2 × 10^4 cells in 0.5 ml of serum-free media were subcutaneously injected to 10 adult Syrian hamsters. After 8 wk, animals were sacrificed, and lungs were collected. Lungs were fixed in alcoholic formalin (10% of formalin and 63% of ethanol). Paraffin-embedded tissues were step-sectioned and stained with hematoxylin-eosin (18 slides per each lung lobe for each hamster in group). Metastatic tumor nodules in the lungs were counted microscopically. All metastases were divided by size into 2 subgroups: micrometastases (<20 cells) and macrometastases (>20 cells).

For analysis of experimental metastatic activity (EMA) 1 × 10^5 cells in 0.1 ml of serum-free media were injected in eye vein of 10 adult Syrian hamsters. Animals were sacrificed and lungs were collected 4 wk after injection. Number and size of metastases were counted as described above. SMA and EMA tests for each cell line were performed in triplicate.

Patient samples

Tissue samples of 71 human pNETs (36 female and 35 male patients; median age of 53.0 y with a range of 19–76 y) and 40 human synovial sarcomas (18 female and 22 male patients; median age of 34.0 y with a range of 5–62 y) were obtained from patients treated with resection surgery at the N.N. Blokhin Russian Cancer Research Center. Written informed consent was obtained from each patient prior to participation. The study and all protocols were approved by the Institutional Review Board of the N.N. Blokhin Russian Cancer Research Center according to the legal regulations. All tumors were staged on the basis of the pathologic tumor-node-metastasis classification of the American Joint Committee on Cancer. Grade was determined according to FNCLCC classification systems (for synovial sarcomas) and WHO Classification of Tumors of the Digestive System (for pNETs).

Immunohistochemical analysis

Samples of tumor and adjacent tissues were fixed in buffered formalin for 24 h. Paraffin-embedded tissues were step-sectioned and stained with hematoxylin-eosin. Endogenous

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peroxidase activity was quenched with 3% hydrogen peroxidase for 10 min. Heat-induced epitope unmasking was provided in 10 mmol/L citrate buffer (pH 6.0) in water bath at 95 °C for 40 min. Sections were incubated with primary antibodies at room temperature: anti-CRABP1 (Sigma-Aldrich, HPA017203) and anti-Ki-67 (Dako, Clone MIB-1, No. M724001). Super Sensitive Polymer-HRP System (BioGenex) was used for the detection. The area and intensity of the staining were evaluated by 2 independent observers. The intensity of immunostaining was scored as no staining (0), light positive staining (1), medium positive staining (2), and strong positive staining (3). Immunostaining area was scored as: <5% of positive cells (0), 5–30% of positive cells (1), 30–60% of positive cell (2), and >60% of positive cells (3). A composite score was calculated by multiplying intensity and area scores. The composite scores of 0–3 were defined as "weak", scores of 4–6 were defined as "moderate", and score of 7–9 as "strong" CRABP1 expression.

cDNA microarray analysis

Nimblegen Roche expression array HG18_12x135k (Roche NimbleGen, Inc) was used to examine the CRABP1-related changes in gene expression profiles of investigated cell lines. All cell lines were tested in 4 replicates. RNA was isolated using Trizol reagent (Invitrogen) according to manufacturer's protocol. cDNA were constructed with SuperScript® Double-Stranded cDNA Synthesis Kit (Invitrogen) and Cy5-labeled. After hybridization microarrays were scanned with the Axon GenePix 4400A High-Resolution Microarray Scanner (Molecular Devices). Relative expression was calculated using Subio Platform ver.1.16 by Subio Inc. Significantly modulated genes were defined as those with $P < 0.05$ and an absolute fold change >1.3. These genes are then mapped to relevant biologic processes and pathways and analyzed using the special tools for identifying and visualizing enriched GO terms in ranked lists of genes, GOrilla (as described in cited papers).[47,48] The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus[49] and are accessible through GEO Series accession number GSE48673 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE48673).

Statistical analysis

Statistical analyses were run on the IBM SPSS Statistics ver. 21 by IBM and GraphPad Prizm ver. 5.02 by GraphPad Software. CRABP1 immunoreactivity was assessed for association with several clinicopathologic variables such as gender, age, stage, differentiation, presence of metastases, and others by using the Fisher exact test. The comparison of tumorigenicity, SMA, EMA, and luciferase activity within the different groups were calculated using Mann–Whitney U statistics (2-tailed and 1-tailed significance test). Statistical significance was defined as $P < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

This study was supported by Russian Foundation of Basic Research (grant 14-04-31744 MOL_A_2014), PROTEK charity fund (grant Nº12d), Russian Ministry of Education and Science (grant Nº 8066, code 2012-1.1-12-000-1002-064).

Acknowledgments

We would like to thank Boris Kopnin for granting nude mice, Pavel Kopnin, Natalya Khromova, and Sophia Malakho for the technical assistance and Michael Durando for the editing of manuscript.

Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/cc/article/28475

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