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1 **Sodium/iodide symporter is expressed in the majority of seminomas and embryonal**
2 **testicular carcinomas**

3

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21 Short title: NIS expression in testis tumours

22

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24 symporter

25

26 **Abstract**

27 Testicular cancer is the most frequent cancer in young men. The large majority of patients
28 has a good prognosis, but in a small group of tumours the current treatments are not
29 effective. Radioiodine is routinely used in the treatment of thyroid cancer and is currently
30 investigated as a potential therapeutic tool even for extra-thyroid tumours able to
31 concentrate this radioisotope. Expression of Na⁺/I⁻ symporter (NIS), the glycoprotein
32 responsible for iodide transport, has been demonstrated in normal testicular tissue. In this
33 study, we analyzed NIS expression in a large series of testicular carcinomas. Our
34 retrospective series included 107 patients operated for testicular tumours: 98 typical
35 seminomas, 6 embryonal carcinomas, 1 mixed embryonal-choriocarcinoma and 2 Leydig
36 cells tumours. Expression and regulation of *NIS* mRNA and protein levels were also
37 investigated in human embryonal testicular carcinoma cells (NTERA) by real time RT-
38 PCR and western blotting respectively. Immunohistochemical analysis showed presence
39 of NIS in the large majority of seminomas (90/98) and embryonal carcinomas (5/7) of the
40 testis, but not in Leydig cell carcinomas. Expression of NIS protein was significantly
41 associated to the lymphovascular invasion. In NTERA cells treated with the histone
42 deacetylase inhibitors SAHA and valproic acid, a significant increase of *NIS* mRNA
43 (about 60 and 30 fold *vs* control, $p < 0.001$ and $p < 0.01$ respectively) and protein levels,
44 resulting in enhanced ability to uptake radioiodine, was observed. Finally, NIS expression

45 in testicular tumours with the more aggressive behavior is of interest for the potential use
46 of targeting NIS to deliver radioiodine in malignant cells.

47

48 **Introduction**

49 Testicular cancer represents about 1-1.5% of all human neoplasia and is the most frequent
50 malignancy in young adult men between 15 and 40 years, representing the leading cause
51 of cancer-related mortality and morbidity in this age group (Winter & Albers 2011).
52 Although conventional treatments or high-dose chemotherapy are able to treat
53 approximately 80% of these patients, it is highly desirable to identify novel effective
54 therapeutic options provided with minimal side effects (Sonpavide *et al.* 2007; Schrader
55 *et al.* 2009).

56 Radioiodine (I^{131}), used in the treatment of thyroid cancer, has recently been proposed as
57 novel therapeutic tool even for extra-thyroid tumours, if able to concentrate this
58 radioisotope (Riesco-Eizaguirre & Santisteban 2006; Kogai *et al.* 2006). Radioiodine
59 concentration requires the presence and function of the Na⁺/I⁻ symporter (NIS), the
60 glycoprotein responsible for iodide transport across the basal membrane of the thyrocytes
61 (Dohan *et al.* 2003). Thus, stimulation of NIS expression by TSH is adopted in the
62 radioiodine-based treatment of thyroid recurrent and metastatic cancer and defects in its
63 functional expression is a major cause of failure of such a treatment (Arturi *et al.* 2000;
64 Schlumberger *et al.* 2007). Similarly, attempts to induce/enhance NIS expression in
65 extra-thyroid tumour cells, to make them able to concentrate the radioisotope, may offer
66 the opportunity of using the same therapeutic approach adopted for thyroid tumours. NIS
67 expression has been recently demonstrated in normal testicular tissue both at transcript

68 and protein levels (Russo *et al.* 2011a), while only one study, analyzing a small number
69 of samples, has been performed on neoplastic testicular tissues, showing NIS expression
70 in 1 of 11 malignant cores examined (Wapnir *et al.* 2003).

71 In this study, NIS expression was investigated in 98 typical seminomas, 7 embryonal
72 testicular carcinomas (including one mixed embryonal-choriocarcinoma) and 2 Leydig
73 cell tumours. In addition, we attempted to stimulate *in vitro* NIS gene and protein
74 expression and iodide uptake in testicular tumour cells. For this purpose we used an
75 experimental model of embryonal testicular cancer, known for its high aggressiveness,
76 testing the effects of a series of stimulators in NTERA human cells.

77

78

79 **Materials and Methods**

80

81 **Materials**

82 Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin,
83 streptomycin and amphotericin B were purchased from Lonza (Milan, Italy).
84 Suberoylanilide hydroxamic acid (SAHA), Decitabine, Bortezomib and Rapamycin were
85 obtained from Aurogene (Rome, Italy); Valproic Acid, Forskolin, 5-azacytidine,
86 Mevinolin, Apha compound 8, Hepes, KClO₄, NaI and monoclonal anti β -actin antibody
87 were from Sigma Aldrich s.r.l (Segrate, Milan, Italy). Monoclonal anti-NIS antibody
88 MAB3562 was purchased from Prodotti Gianni (Milan, Italy), anti-human NIS
89 monoclonal antibody N2750 was from United States Biological (Swampscott, MA),
90 PVDF membrane and ECL plus were from VWR (Milan, Italy), Trizol was from

91 Invitrogen (Carlsbad, CA, USA), nuclease-free H₂O was from GIBCO (Milan, Italy) and
92 horseradish peroxidase-conjugated anti-mouse antibody was from Transduction
93 Laboratories (Lexington, KY, USA). The High Capacity cDNA Reverse Transcription
94 kit, TaqMan Fast Universal PCR master mix, FAM dye-labelled probes, Assay-on-
95 Demand Gene Expression Products and β -actin and were obtained from Applied
96 Biosystems (Foster City, CA, USA). Hank's balanced salt solution from EuroClone
97 (Celbio, Pero, Milan, Italy), carrier free NaI from PerkinElmer (Monza, Milan, Italy).

98

99 **Cell culture**

100 NTERA cells, the only commercial available cell line of human embryonal testicular
101 carcinoma, were purchased from LGC Standards (Sesto San Giovanni, Milan, Italy),
102 cultured in DMEM containing FBS 10% (v/v), penicillin (0.1 mg/ml), streptomycin (2.5
103 μ g/ml), amphotericin B (2.5 μ g/ml) and were maintained at 37 °C in a humidified
104 atmosphere (5% CO₂).

105

106 **Tissue samples**

107 A retrospective series of 107 patients operated at the Policlinic of Modena for testicular
108 tumours who underwent inguinal orchifuniclectomy was analyzed: 98 seminomatous
109 tumours (typical seminomas) and 9 non-seminomatous tumours including 6 embryonal
110 carcinomas, 1 mixed embryonal-choriocarcinoma and 2 Leydig cells tumors. Tumours
111 were histologically classified according to World Health Organization criteria (Eble *et al.*
112 2004). The Tumour staging (TNM), that represents the validated standard tool to describe
113 tumour extent and includes prognostic information on the probability of disease control,

114 was assigned using the current guidelines (Edge *et al.* 2009). Specimens' aliquots were
115 fixed in Bouin's fixative overnight for histological studies. Review of patients' charts was
116 carefully performed to collect the clinical features of each case, as described in Table 1.

117

118 **Ethics Statement**

119 All human tissue samples used in the study were collected with full patients' informed
120 written consent and approval from the Policlinic of Modena ethic committee.

121

122 **Immunohistochemistry**

123 The presence of NIS in testicular tumour tissues was analyzed by immunohistochemistry
124 as described previously (Navarra *et al.* 2010). Dewaxed 4- μ m sections were first
125 incubated with 6% H₂O₂ for 10 min at room temperature to block endogenous peroxidase
126 activity. Then, they were immersed in a citrate buffer (pH 6) for 30 min at 98 °C and
127 incubated at room temperature overnight with the monoclonal anti-NIS antibody N2750
128 diluted 1:100. The avidin-biotin complex was applied using an automatic system
129 (Benchmark, Ventana, Tucson, AZ, USA) and staining was visualized using diamino-
130 benzidine chromogen. The sections were lightly counterstained with Carazzi's
131 hematoxylin and dehydrated, before being mounted and examined by two pathologists,
132 who expressed concordant opinions for all the cases examined. A rate >10% of cells
133 staining associated with at least moderate intensity was used to indicate positivity, 10-
134 50% moderate, >50% high.

135

136 **Analysis of mRNA levels**

137 Levels of *NIS* mRNA were determined with real-time quantitative RT-PCR, as previously
138 described (Sponziello *et al.* 2010). Briefly, total RNA was extracted from cells treated
139 with various compounds at various incubation times using the Trizol method, according
140 to the manufacturer's instructions. Two micrograms of total RNA were reverse
141 transcribed in a 20 μ l reaction volume using the High Capacity cDNA Reverse
142 Transcription kit following the instructions of the manufacturer. After 1:5 dilution, the
143 cDNAs were amplified using an Applied Biosystems 7900HT Fast Real-Time PCR
144 Sequence Detection System and fast quantitative PCR thermal cycler parameters. Each
145 tube contained, in a total of 20 μ l, 2 μ l of cDNA, 10 μ l of TaqMan Fast Universal PCR
146 master mix, and 1.0 μ l of a pre-developed primer/probe mixture for each gene to be
147 measured. All values were normalized to β -actin as endogenous control, with similar
148 results. The experiments were repeated at least three times. Reactions, results
149 determination and expression and normalization were performed as previously reported
150 (Sponziello *et al.* 2010).

151

152 **Analysis of protein levels**

153 Extraction of total proteins was performed as previously described (Celano *et al.* 2008).
154 Briefly, fifteen μ g of proteins were run on a 7.5% SDS-PAGE gel and transferred to
155 PVDF membrane with the Mini Trans Blot system (Bio-Rad Laboratories S.r.l, Milan,
156 Italy) (2 h at 225 mA). Membranes were blocked with TTBS/milk (TBS, 1% Tween 20
157 and 5% non-fat dry milk) for 1 h at room temperature and incubated overnight with the
158 affinity-purified anti-NIS monoclonal antibody MAB3562 diluted 1:250. The membranes
159 were washed once for 15-min and twice for 5-min in TTBS, and incubated with

160 horseradish peroxidase-conjugated anti-mouse antibody diluted 1:10000 in TTBS/milk.
161 After one 15-min and two 5-min washes in TTBS, the protein was visualized by
162 chemiluminescence using the Western blot detection system ECL Plus. Monoclonal
163 mouse β -actin antibody was used as an internal control.

164

165 **Iodide uptake**

166 Iodide uptake by NTERA cells was measured as previously described (Weiss *et al.* 1984).
167 Briefly, cells were seeded into 12-well plates and treated with SAHA 3 μ M and valproic
168 acid 3 mM for 48h. Then, the culture medium was aspirated and cells washed twice with 1
169 ml Hank's balanced salt solution (HBSS) supplemented with Hepes (10 mM, pH 7.3).
170 125 I-uptake was initiated by adding to each well 500 μ l of HBSS containing 0.1 μ Ci/ml
171 carrier free labeled NaI and 10 μ M NaI. In half of the wells, used as control for specific
172 uptake, this buffer also contained 100 μ M KClO₄, a NIS inhibitor. After 30-40 min at 37
173 $^{\circ}$ C in a humid atmosphere, the radioactive medium was aspirated and cells were washed
174 twice with 1 ml of ice-cold HBSS. The amount of iodide uptake was determined after
175 incubation with 1 ml of 95% ethanol to each well for 20 min and transfer into vials for
176 counting with a γ -counter. The NIS-specific radio-iodine uptake was normalized using
177 data of cell viability measured with MTT assay (data not shown). Each experiment was
178 carried out in triplicate.

179

180 **Statistical analysis**

181 The results are expressed as means \pm SD, and the one-way ANOVA followed by the
182 Tukey-Kramer multiple comparisons test was adopted to determine the significance of

183 differences using the GrafPAD Software for Science (San Diego, CA, USA). Patients
184 were all uniformly followed-up at our Institution. The association between protein NIS
185 expression and clinico-pathological parameters was calculated using contingency table
186 methods and tested for significance using the Pearson's chi-square test. A probability (p)
187 value <0.05 was considered statistically significant.

188

189

190 **Results**

191

192 **Clinical and pathological features**

193 A total of 107 testicular tumour tissues were evaluated: histological types included 98
194 seminomatous tumours (90 fixed in formalin specimens and 8 fresh/not fixed in formalin
195 tissue), 7 embryonal carcinomas (all fresh/not fixed in formalin tissue) and 2 sex
196 cord/gonadal stromal tumours (Leydig cell tumours, both fresh/not fixed in formalin
197 tissue). Eighty-eight tumours (82.3%) are classified as Stage I, 10 (9.3%) as Stage II and
198 9 (8.4%) as Stage III. There was a complete accordance of the two pathologist in
199 attributing the grading of each sample. In 18 cases (17%) we detected lymphovascular
200 invasion. The clinical and pathological findings of the patients are listed in Table 1.

201

202 **Expression of NIS in human testicular cancer tissues**

203 Expression of *NIS* mRNA was evaluated in the available samples of fresh frozen
204 testicular tumours. We observed detectable levels of *NIS* mRNA in 5 of 8 seminomas, in
205 5 of 7 embryonal carcinomas while in Leydig cell tumours *NIS* resulted absent (Fig.1).

206 All tumours were analyzed by immunohistochemistry to evaluate the expression of NIS
207 protein. NIS protein staining was detected in the cell plasma membrane in the majority of
208 the cases with intense staining (Fig.2). As shown in fig.2, in 64 seminomas and 5
209 embryonal carcinomas we observed more than 50% of cell stained. Twenty-six
210 seminomas presented moderate to weak staining, while 8 seminomas, 2 embryonal
211 carcinomas and both Leydig cell tumours were negative (Fig.3). Interestingly, NIS
212 protein expression was significantly associated to the lymphovascular invasion ($p < 0.005$)
213 but not with the other clinical and pathological parameters, as reported in Table 2. In the
214 samples in which both RNA levels and tissue slice could be examined, concordance in
215 the positivity of NIS mRNA and protein was observed, except for one seminoma positive
216 for NIS mRNA expression and only weak staining of the protein.

217

218 **Stimulation of NIS expression in testicular embryonal carcinoma cells**

219 Expression of *NIS* mRNA was then evaluated in the embryonal human testicular
220 carcinoma cells NTERA. A series of molecules, including Suberoylanilide hydroxamic
221 acid (SAHA), Decitabine, Bortezomib, Rapamycin, Valproic Acid, Forskolin, 5-
222 azacytidine, Mevinolin and Apha compound 8, known to stimulate NIS expression in
223 thyroid cells (Frölich *et al.* 2008), were tested at various doses and incubation times
224 (Table 3). The strongest stimulating effect was observed with the histone deacetylase
225 inhibitors (HDACi) SAHA and valproic acid. Subsequently, we conducted dose-response
226 and time-course analysis of selected dosages of SAHA and valproic acid: the greatest
227 increment of the levels of *NIS* mRNA was observed after 24 h treatment with SAHA 3
228 μM and valproic acid 3 mM, about 60 fold and 30 fold over control, respectively (Fig.4).

229 We next examined the expression of NIS protein in NTERA cells exposed to the same
230 HDAC inhibitors. As shown in fig.5, a specific band of approximately 90 kDa,
231 corresponding to human NIS protein was detected in the total protein extracts of NTERA
232 cells in basal condition and after treatment with SAHA 3 μ M or valproic acid 3 mM, with
233 the strongest effect observed in NTERA after 48 h of incubation (Fig.5).

234

235 **Radioiodine uptake in NTERA cells**

236 In order to test whether stimulation of NIS protein by HDACi determined an increase of
237 its function, radioiodide uptake experiments were performed in the cells treated with
238 SAHA and valproic acid (3 μ M and 3 mM, respectively). After 48 h of treatment, we
239 observed a significant increase of the uptake with both compounds (Fig.6).

240

241

242 **Discussion**

243 Testicular cancer, the most common malignancy occurring in young males, is a highly
244 curable tumour even in patients with metastatic disease. Indeed, seminomas, the most
245 frequent histotype, have a high radiosensitivity, so that combination of orchiectomy and
246 adjuvant radiotherapy on the para-aortic and ipsilateral iliac lymph nodes, the standard
247 therapy adopted in the last 60 years, has reduced the risk of relapse to 1-3%, resulting in a
248 global survival rate close to 100% (Warde *et al.* 2002). In the less radiosensitive
249 nonseminomatous tumours, including embryonal cell carcinomas, yolk sac tumor,
250 choriocarcinoma and teratoma, the chemotherapy, mainly based on 3 to 4 cycles of PEB
251 (cisplatin, etoposide, bleomycin), is the alternative choice. However, resistance to such a

252 treatment often arises (Krege *et al.* 2001; Castillo-Avila *et al.* 2009). Recently, it was
253 reported the description of some molecular mechanisms potentially involved in the
254 pharmacological resistance and developed by the more aggressive tumours of the testis
255 (Looijenga *et al.* 2011). Novel therapeutic strategies are therefore urgently required for
256 those tumours resistant to the current treatment.

257 Expression of the NIS, the protein which actively transport the iodide into the thyrocytes,
258 in extra-thyroidal tumour tissues has been exploited for its potential use to target
259 radioiodine in malignant cells for diagnosis and/or treatment of the disease (Riesco-
260 Eizaguirre & Santisteban 2006; Kogai *et al.* 2006). Therefore, induction of NIS
261 expression in cancer cells to deliver radioiodine is currently being explored for many
262 types of extra-thyroid neoplasia (Hingorani *et al.* 2010). While encouraging results have
263 been obtained in some preclinical models, unresolved issues are still present about the
264 feasibility of a gene therapy-based approach on humans (Haberkorn *et al.* 2003). Equally
265 promising are the attempts to stimulate endogenous NIS expression in those tumour cells,
266 from thyroid and non-thyroid cancers, with detectable levels of *NIS* mRNA (Kogai *et al.*
267 2006). The feasibility of such an approach has been addressed in various tumours,
268 including prostate cancer. In a previous report, expression of the NIS has been detected in
269 the more aggressive forms of prostate tumours, suggesting a potential use as target for a
270 therapy with radioiodine as well as biomarker for identifying individuals with
271 biologically active disease (Navarra *et al.* 2010). Interestingly, even in breast cancer NIS
272 expression was detected in the more aggressive ‘triple-negative’ samples (Renier *et al.*
273 2009), at variance with thyroid cancer, in which lymph node metastatic tissues have
274 usually reduced or lost NIS expression (Arturi *et al.* 2000). In the only other study

275 addressing this issue in testicular tumours, no information was provided according to the
276 histotype, the clinical characteristics of the patient(s) and the localization of the NIS in
277 the specimen examined (Wapnir *et al.* 2003).

278 In this study we demonstrate that NIS is expressed in the plasma membrane of the large
279 majority of seminomas and embryonal carcinomas of human testis, while is absent in 2
280 Leydig cell cancer. Our data also demonstrate a significant association of the expression
281 of NIS protein with the lymphovascular invasion, a well-known marker of
282 aggressiveness. We believe that the association between NIS expression in the tumour
283 cells and the lymphovascular invasion may reflect the different biological aggressiveness
284 of testis tumours suggesting the presence of the NIS as an unfavorable prognostic factor.

285 Thus, the majority of the aggressive seminomas and embryonal carcinomas express the
286 NIS protein so that may be considered, in case of refractoriness to the standard treatment,
287 potential candidate to an alternative radioiodine-based therapeutic strategy. Since the
288 embryonal carcinomas, for their refractoriness to the current treatment, represent
289 potential candidates for such a novel therapeutic approach, we chose the NTERA cells,
290 the only available commercial human embryonal carcinoma cell line, to attempt to
291 stimulate NIS expression in tumour cells. Our present findings reveal that NIS expression
292 may be enhanced in vitro by HDAC inhibitors. Histone acetylation is a known epigenetic
293 mechanism of regulation of gene expression and its alteration has been reported in many
294 human cancers (Chi *et al.* 2010). In many cell lines of thyroid and non-thyroid cancer,
295 HDAC inhibitors have been successfully tested to induce radioiodine uptake due to
296 increased NIS expression (Puppini *et al.* 2005; Russo *et al.* 2011b; Liu & Xing 2012). The
297 same result was obtained in the NTERA cells in the present study, showing that at least *in*

298 *in vitro* embryonal testicular tumour cell susceptibility to radioiodine administration may
299 occur and suggesting the possibility to use the radioiodine after pharmacological
300 induction of NIS expression even in this rare tumour histotype. It is noteworthy that these
301 drugs are being tested in clinical trials at doses compatible with those effective *in vitro*
302 (www.clinicaltrials.gov).

303 In conclusion the present data demonstrate that NIS is expressed in the large majority of
304 seminomas and embryonal carcinomas of human testis, including those with a more
305 aggressive phenotype (i.e. with lymphovascular invasion). Its presence in the
306 plasmamembrane compartment of the tumour cells suggests that it may serve as potential
307 carrier of radioiodine for an ablative treatment of cancer tissue.

308

309

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311 The authors declare that there is no conflict of interest that could be perceived as
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314

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430 www.clinicaltrials.gov

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432 **Figure legends**

433 ***Fig. 1 Expression of NIS mRNA in testicular tumours***

434 NIS mRNA levels assayed by RT-PCR in seminomas, embryonal testicular carcinomas
435 and in Leydig cell fresh frozen tumours.

436

437 ***Fig. 2 Expression of NIS in testicular carcinoma tissues***

438 Immunohistochemistry of NIS in testicular tumors. In seminomas and embryonal
439 carcinomas NIS staining is detected in both cytosol and plasma membrane of cancer
440 cells. Thyroid hyperfunctioning adenoma is used as positive control; one Leydigoma,
441 one embryonal carcinoma and one seminoma negative for NIS mRNA expression are
442 shown as negative controls. Experiments were performed using a primary monoclonal
443 anti-human NIS antibody diluted 1:100 as described in Methods.

444

445 ***Fig.3 Immunohistochemical results of NIS intensity in seminoma and embryonal***
446 ***testicular carcinomas***

447 Bars represent the percentage of total seminomas or embryonal carcinomas with absent,
448 moderate or high intensity staining, evaluated in immunohistochemical experiments as
449 indicated in Methods.

450

451 ***Fig.4 Expression of NIS mRNA in NTERA cells***

452 NIS mRNA levels assayed by RT-PCR in NTERA cells exposed for 4-8-24 h to SAHA
453 0.3 and 3 μ M (**p<0.001 vs control), valproic acid (VPA) 0.3 and 3 mM (**p<0.01 vs

454 control). Data are means \pm SD of 3 experiments using ANOVA followed by the Tukey-
455 Kramer multiple comparisons test.

456

457 ***Fig.5 Expression of NIS protein in NTERA cells***

458 Western blot analysis was performed under reducing conditions using a monoclonal anti-
459 NIS antibody and a monoclonal anti-human β -actin antibody. A representative of three
460 separated experiments is shown. A specific band of approximately 90 kDa, corresponding
461 to human NIS protein, was detected in the total protein extracts of carcinoma testicular
462 cells and increased after treatment with HDAC inhibitors.

463

464 ***Fig.6 SAHA and valproic acid increase radio-iodine uptake in NTERA cells.***

465 NTERA cells were treated with SAHA 3 μ M and valproic acid 3 mM for 48 h. Radio-
466 iodine uptake was evaluated as described in Materials and Methods section. Each bar
467 represents the mean value (\pm SD) of three different experiments. ***, statistical
468 significance compared to untreated cells ($p < 0.001$).

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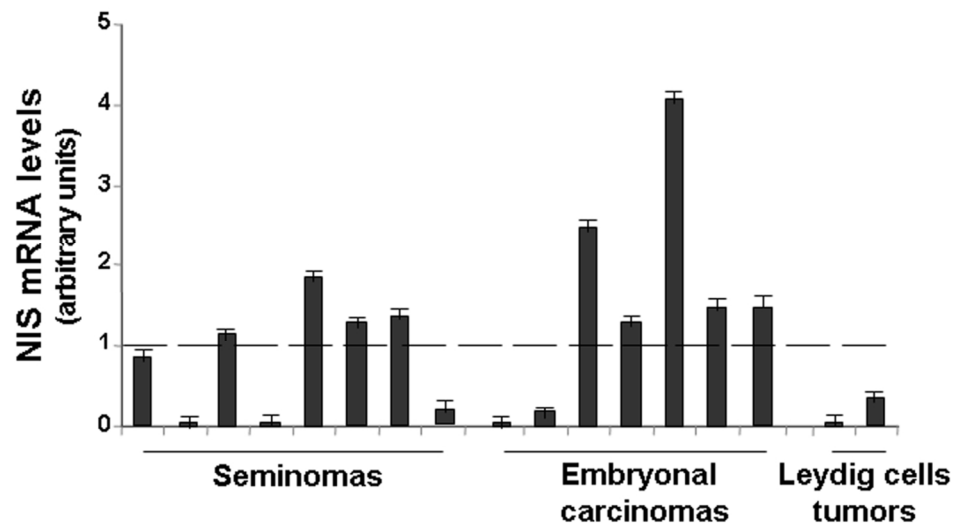


Figure 1

Fig. 1 Expression of NIS mRNA in testicular tumours
NIS mRNA levels assayed by RT-PCR in seminomas, embryonal testicular carcinomas and in Leydig cell fresh frozen tumours.

126x86mm (300 x 300 DPI)

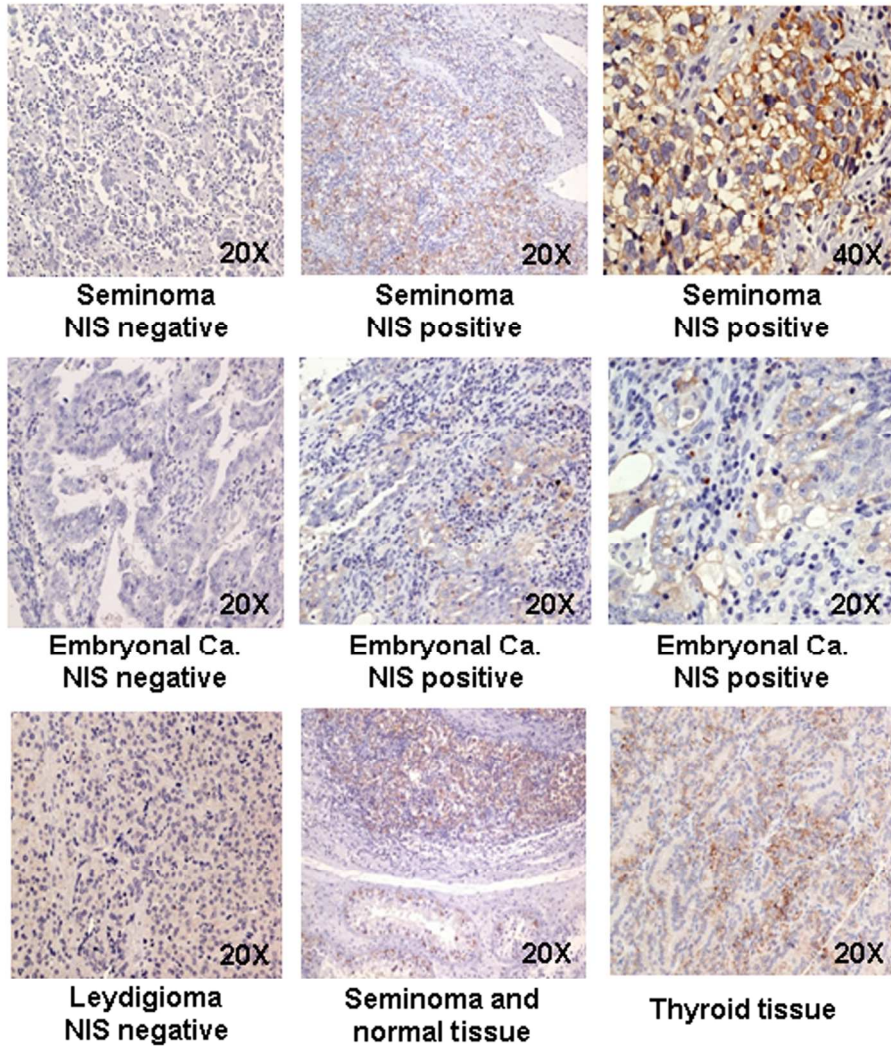


Figure 2

Fig. 2 Expression of NIS in testicular carcinoma tissues
Immunohistochemistry of NIS in testicular tumors. In seminomas and embryonal carcinomas NIS staining is detected in both cytosol and plasma membrane of cancer cells. Thyroid hyperfunctioning adenoma is used as positive control; one Leydigoma, one embryonal carcinoma and one seminoma negative for NIS mRNA expression are shown as negative controls. Experiments were performed using a primary monoclonal anti-human NIS antibody diluted 1:100 as described in Methods.

148x185mm (300 x 300 DPI)

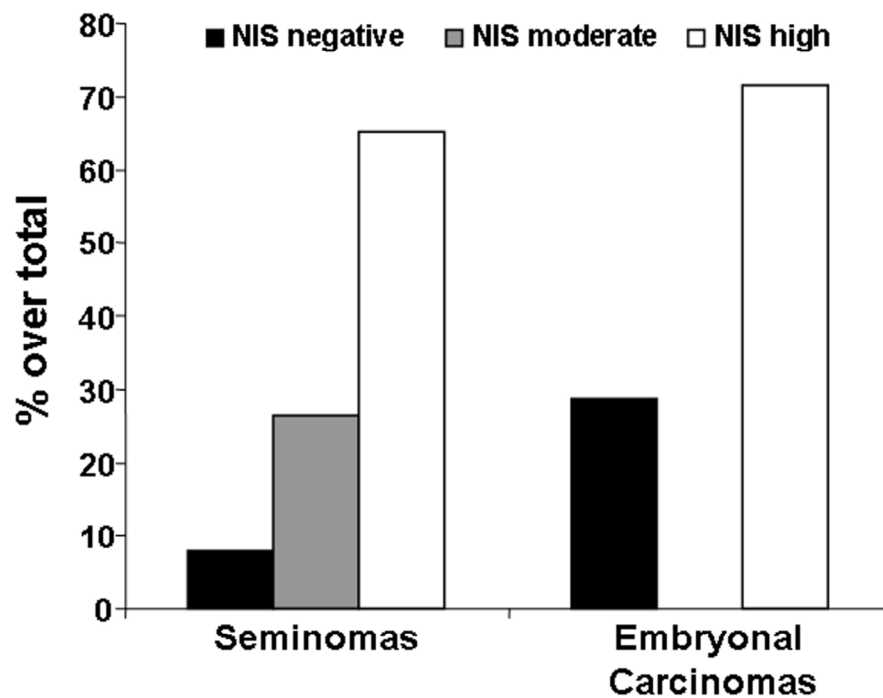


Figure 3

Fig.3 Immunohistochemical results of NIS intensity in seminoma and embryonal testicular carcinomas. Bars represent the percentage of total seminomas or embryonal carcinomas with absent, moderate or high intensity staining, evaluated in immunohistochemical experiments as indicated in Methods.

151x132mm (300 x 300 DPI)

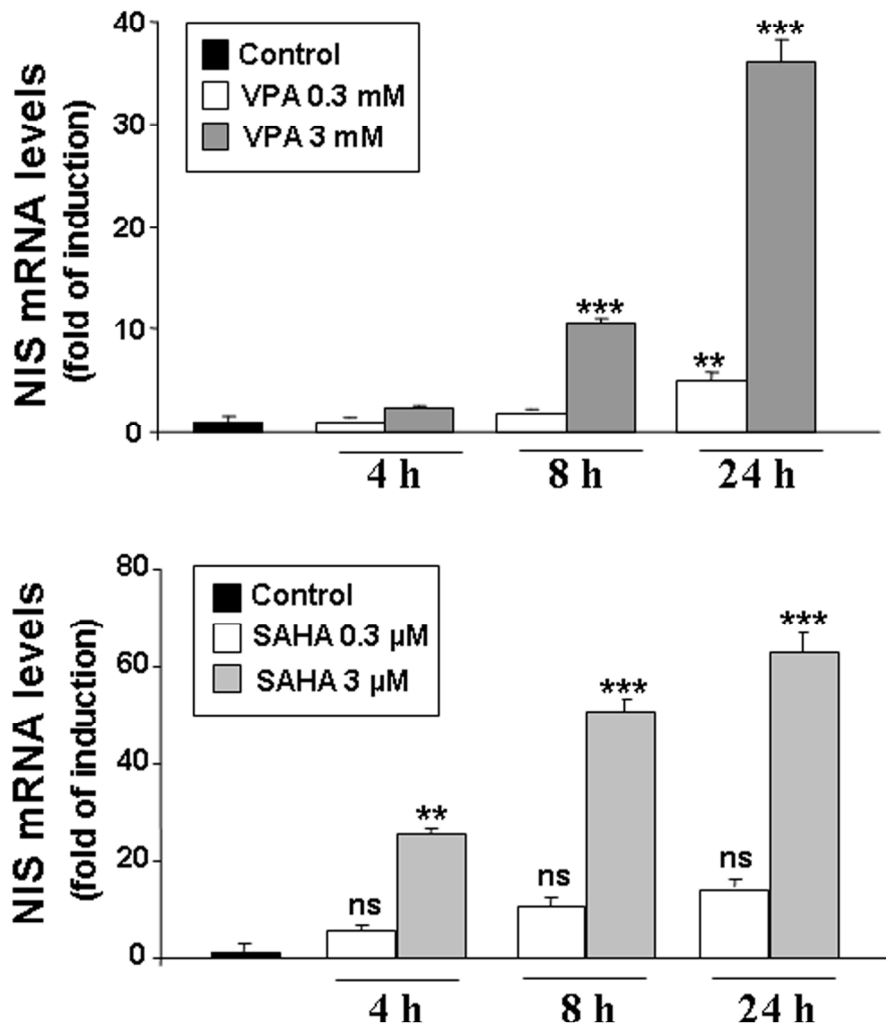


Figure 4

Fig.4 Expression of NIS mRNA in NTERA cells
 NIS mRNA levels assayed by RT-PCR in NTERA cells exposed for 4-8-24 h to SAHA 0.3 and 3 μM (***) $p < 0.001$ vs control), valproic acid (VPA) 0.3 and 3 mM (**) $p < 0.01$ vs control). Data are means \pm SD of 3 experiments using ANOVA followed by the Tukey-Kramer multiple comparisons test.

175x208mm (300 x 300 DPI)

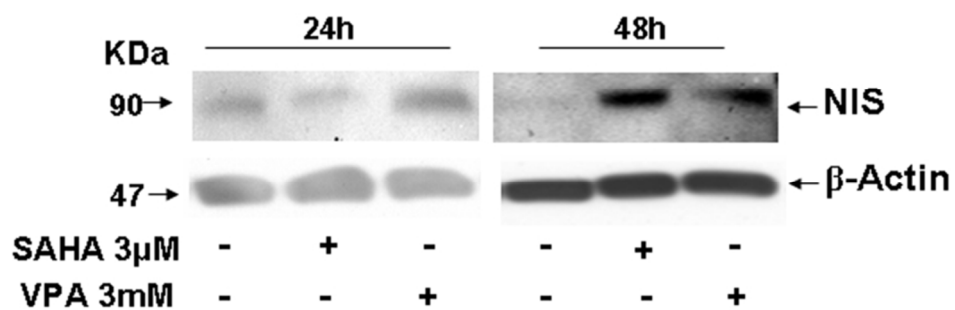


Figure 5

Fig.5 Expression of NIS protein in NTERA cells
 Western blot analysis was performed under reducing conditions using a monoclonal anti-NIS antibody and a monoclonal anti-human β -actin antibody. A representative of three separated experiments is shown. A specific band of approximately 90 kDa, corresponding to human NIS protein, was detected in the total protein extracts of carcinoma testicular cells and increased after treatment with HDAC inhibitors.

78x36mm (300 x 300 DPI)

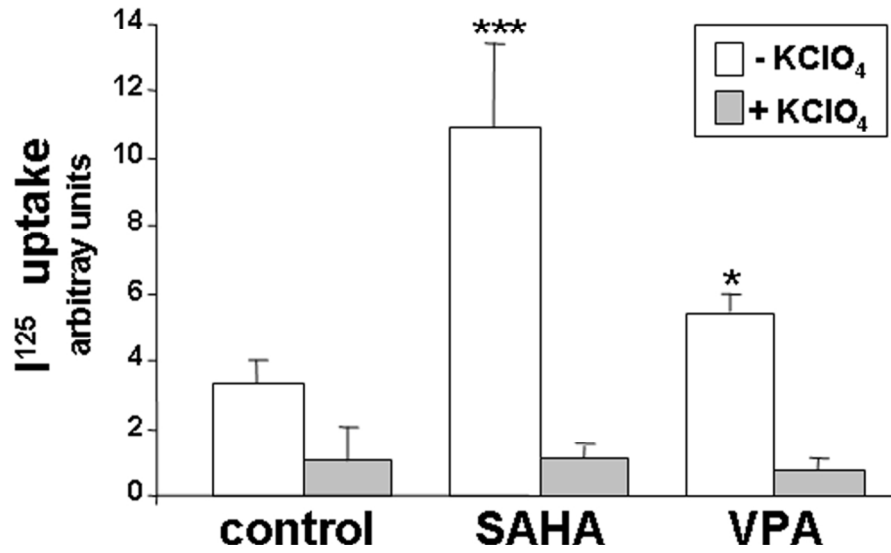


Figure 6

Fig.6 SAHA and valproic acid increase radio-iodine uptake in NTERA cells. NTERA cells were treated with SAHA 3 μM and valproic acid 3 mM for 48 h. Radio-iodine uptake was evaluated as described in Materials and Methods section. Each bar represents the mean value ($\pm\text{SD}$) of three different experiments. ***, statistical significance compared to untreated cells ($p < 0.001$).

111x80mm (300 x 300 DPI)

Table 1. Clinical and pathological features of the 107 patients with testicular tumours analyzed

Age	Mean±SD	37.24±11.4	TNM*	T	T1	82 (76.6%)
	Median	35		T2	20 (18.7%)	
	Range	18÷73		T3	5 (4.7%)	
Size	Middle±SD	4.68±2.69	N	N0	97 (90.7%)	
	Median	4		N1-N2	10 (9.3%)	
	Range	1.5÷13		M	M0	99 (92.5%)
Histology	Typical seminoma	98 (91.6%)	Lymphovascular invasion (+/-)	M+	8 (7.5%)	
	Embryonal carcinoma	6 (5.5%)		Follow up	Mean	98.4 months
	Mixed[^]	1 (1%)			Range	12÷144 months
	Leydigoma	2 (1.9%)			Alive	95 (89%)
Stage	I	88 (82.3%)	Died**	4 (4%)		
	II	10 (9.3%)	Recurrences	8 (7%)		
	III	9 (8.4%)				

[^]Embryonal and choriocarcinoma

*TNM: Tumour Nodes Metastases

**Deceased for cause unrelated to the tumour

Table 2. Clinico-pathological characteristics of NIS+ and NIS- seminomas and embryonal testicular carcinomas

		SEMINOMAS		EMBRYONAL CARCINOMAS		LEYDIGIOMAS		TOTAL OF TUMOURS		p value*
		NIS +	NIS -	NIS +	NIS -	NIS +	NIS -	NIS +	NIS -	
TNM °	T1	74 (94.9%)	4 (5.1%)	3 (100%)	0 (0%)	0 (0%)	1 (50%)	77 (94%)	5 (6%)	n.s.
	T2-T3	16 (80%)	4 (20%)	2 (50%)	2 (50%)	0 (0%)	1 (50%)	18 (72%)	7 (28%)	
	N0	84 (92.3%)	7 (7.7%)	3 (75%)	1 (25%)	0 (0%)	2 (100%)	87 (90%)	10 (10%)	n.s.
	N1-N2	6 (85.7%)	1 (14.3%)	2 (66.7%)	1 (33.3%)	0 (0%)	0 (0%)	8 (80%)	2 (20%)	
	M0	86 (91.5%)	8 (8.5%)	2 (66.7%)	1 (33.3%)	0 (0%)	2 (100%)	88 (89%)	11 (11%)	n.s.
	M+	4 (100%)	0 (0%)	3 (75%)	1 (25%)	0 (0%)	0 (0%)	7 (87.5%)	1 (12.5%)	
Lymphovascular invasion	Present	10 (83.3%)	2 (16.7%)	3 (60%)	2 (40%)	0 (0%)	1 (50%)	13 (72%)	5 (28%)	<0.05
	Absent	80 (93%)	6 (7%)	2 (100%)	0 (0%)	0 (0%)	1 (50%)	82 (92%)	7 (8%)	
Recurrence of disease	Present	5 (100%)	0 (0%)	1 (50%)	1 (50%)	0 (0%)	1 (50%)	6 (75%)	2 (25%)	n.s.
	Absent	85 (91%)	8 (9%)	4 (80%)	1 (20%)	0 (0%)	1 (50%)	89 (90%)	10 (10%)	

° TNM= Tumour Node Metastases

* chi-square test

n.s. = not significant

Table 3. Compounds used to stimulate NIS expression

Compounds	Maximum concentration tested	Fold of increment of NIS mRNA levels
Histone deacetylase inhibitors		
SAHA	3 μ M	62.8 \pm 3.5
Valproic Acid	3 mM	36 \pm 2.24
Apha compound 8	5 μ M	5.3 \pm 1.03
Proteasome inhibitor		
Bortezomib	52 nM	2.7 \pm 0.35
Demethylating agent		
5-Azacytidine	5 μ M	3 \pm 1.1
Decitabine	5 μ M	1.5 \pm 0.52
Adenylate cyclase stimulator		
Forskolin	10 μ M	0.8 \pm 0.89
Inhibitor of hydroxymetilglutaril-coenzyme A reductase		
Mevinolin	50 μ M	4.7 \pm 1.7
mTOR inhibitor		
Rapamycin	20 nM	0.6 \pm 0.9