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Intratubular germ cell neoplasia of the human testis

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1 **Intratubular germ cell neoplasia** of the human testis: heterogeneous protein
2 **expression and relation to invasive potential**

3
4 Mitchell RT^{1,3}, Camacho-Moll M¹, Macdonald J¹, Anderson RA¹, Kelnar CJH³, O'Donnell M², Sharpe RM¹,
5 Smith LB¹, Grigor KM², Wallace WHB³, Stoop H⁴, Wolffenbuttel KP⁴, Donat R⁵, *Saunders PTK¹ and
6 *Looijenga LHJ⁴.

7
8 ¹ MRC Centre for Reproductive Health, The University of Edinburgh, Queen's Medical Research Institute, 47 Little
9 France Crescent, Edinburgh, EH16 4TJ, Scotland, UK

10 ² Department of Pathology, Western General Hospital, Crewe Road, Edinburgh, EH4 2XU, Scotland, UK

11 ³ Edinburgh Royal Hospital for Sick Children, 9 Sciennes Road, Edinburgh, EH9 1LF, Scotland, UK

12 ⁴ Department of Pathology (LHJL, HS) and Pediatric Urology (KPW), Erasmus MC-University Medical Center
13 Rotterdam, Josephine Nefkens Institute, Room 430b, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

14 ⁵ Department of Urology, Western General Hospital, Crewe Road, Edinburgh, EH4 2XU, Scotland, UK

15 * These authors contributed equally to this study.

16
17

18 **Running Title:** Proliferation in intratubular germ cell neoplasia

19

20 **Correspondence and reprint requests**

21 Rod Mitchell, MRC Centre for Reproductive Health, The University of Edinburgh, The Queen's Medical

22 Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, Scotland, UK. FAX: 44 131 242 6197.

23 e-mail: rod.mitchell@ed.ac.uk

24

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25 **Abstract:** Testicular germ cell cancer develops from pre-malignant intratubular germ cell neoplasia,
26 unclassified cells that are believed to arise from failure of normal maturation of fetal germ cells from
27 gonocytes (OCT4⁺/MAGEA4⁻) into pre-spermatogonia (OCT4⁻/MAGEA4⁺). Intratubular germ cell neoplasia
28 cell subpopulations based on stage of germ cell differentiation have been described, however the importance
29 of these subpopulations in terms of invasive potential has not been reported. We hypothesised that cells
30 expressing an immature (OCT4⁺/MAGEA4⁻) germ cell profile would exhibit an increased proliferation rate
31 compared to those with a mature profile (OCT4⁻/MAGEA4⁺). Therefore, we performed triple
32 immunofluorescence and stereology to quantify the different intratubular germ cell neoplasia cell
33 subpopulations, based on expression of germ cell (OCT4, PLAP, AP2γ, MAGEA4, VASA) and proliferation
34 (Ki67) markers, in testis sections from patients with pre-invasive disease, seminoma and non-seminoma. We
35 compared these subpopulations with normal human fetal testis and with seminoma cells. Heterogeneity of
36 protein expression was demonstrated in intratubular germ cell neoplasia cells with respect to gonocyte and
37 spermatogonial markers. It included an embryonic/fetal germ cell subpopulation lacking expression of the
38 definitive intratubular germ cell neoplasia marker OCT4, that did not correspond to a physiological (fetal)
39 germ cell subpopulation. OCT4⁺/MAGEA4⁻ cells showed a significantly increased rate of proliferation
40 compared with the OCT4⁺/MAGEA4⁺ population (12.8 v 3.4%, p<0.0001) irrespective of histological tumour
41 type, reflected in the predominance of OCT4⁺/MAGEA4⁻ cells in the invasive tumour component.
42 Surprisingly, OCT4⁺/MAGEA4⁻ cells in patients with pre-invasive disease showed significantly higher
43 proliferation compared to those with seminoma or non-seminoma (18.1 v 10.2 v 7.2%, p<0.05 respectively).
44 In conclusion, this study has demonstrated that OCT4⁺/MAGEA4⁻ cells are the most frequent and most
45 proliferative cell population in tubules containing intratubular germ cell neoplasia, which appears to be an
46 important factor in determining invasive potential of intratubular germ cell neoplasia to seminomas.

47

48 Keywords and Topic Category:49 Testicular germ cell tumours, Cell differentiation, Cell proliferation, Germ cells, Carcinoma *in situ*

50

51

52 **Introduction**

53 Testicular germ cell cancer is the most common malignancy in young men and the incidence of these tumours
54 is increasing worldwide [1,2]. The tumours are classified as seminoma or non-seminoma with a distinct cell of
55 origin and pathogenesis compared with spermatocytic seminoma of late adulthood [1]. These tumours result
56 from transformation, usually in young adulthood, of pre-invasive intratubular germ cell neoplasia (also known
57 as carcinoma *in situ*) cells that arise during fetal life [3,4]. Intratubular germ cell neoplasia cells are believed to
58 be germ cells that have failed to undergo normal maturation during fetal or early postnatal life.

59

60 In humans, during fetal life, primordial germ cells migrate into the developing gonad at around 5 weeks of
61 gestation and become gonocytes [5]. These cells express proteins associated with pluripotency (e.g. OCT4 and
62 NANOG) [6,7] and a number of other embryonic markers (e.g. AP2 γ and PLAP) [8,9]. During the remainder
63 of fetal life and into the early postnatal period these cells begin to express germ cell specific proteins (e.g.
64 VASA and MAGEA4) during their transition from gonocytes into spermatogonia and this is associated with a
65 loss of the gonocyte protein markers [7]. This transition occurs in an asynchronous manner such that cells at
66 different stages of development may be present in an individual seminiferous cord during this period and some
67 of these cells may co-express both gonocyte and a spermatogonial markers [10].

68

69 Intratubular germ cell neoplasia cells express many of the same proteins as gonocytes (e.g. OCT4, PLAP,
70 AP2 γ) and these are often used in conjunction with histological evaluation to diagnose the condition in
71 testicular biopsies [11]. It is also recognised that intratubular germ cell neoplasia cells may express proteins
72 indicative of spermatogonia (e.g. MAGEA4, VASA, TSPY) [3,12,13]. The clinical significance of the
73 differing protein expression profiles amongst intratubular germ cell neoplasia cells is not known.

74

75 Proliferation of pre-invasive cells is important for the development of an invasive tumour and proliferation has
76 been shown to occur in intratubular germ cell neoplasia cells prior to the development of an invasive tumour
77 [12]. However, proliferation in the different sub-populations of intratubular germ cell neoplasia cells, based on
78 germ cell differentiation profile, has not previously been investigated.

79

80 The aim of this study was to characterise the heterogeneous protein expression profiles of intratubular germ
81 cell neoplasia cells using co-localisation of multiple proteins simultaneously and to compare this to the

82 expression profiles of normal germ cells in the human fetal testis. In addition we aimed to quantify the
83 different intratubular germ cell neoplasia sub-populations associated with different testicular germ cell cancer
84 histological types and to investigate whether the protein expression profile of intratubular germ cell neoplasia
85 cells is related to proliferation of these cells and hence to their invasive potential.

86

87 **Materials and Methods**

88 **Tissue collection**

89 *Human intratubular germ cell neoplasia/testicular germ cell cancer tissue:* Ethical approval was obtained for
90 the use of archived human testicular tissue from the Pathology Departments at the Western General Hospital in
91 Edinburgh (REC Reference number - 10/S1402/33) and Erasmus MC-University Medical Center, Rotterdam
92 (Institutional review board - MEC 02.981 and CCR2041). Samples were randomly selected from the testicular
93 germ cell tumour database and analysed by light microscopy for the presence of intratubular germ cell
94 neoplasia cells. The diagnosis included pre-invasive disease (childhood, n=4; adulthood, n=7), seminoma
95 (n=9) and non-seminoma (n=8). Patient details are described in Table 1. The specimens had been fixed in
96 formalin for 24 hours.

97

98 *Human Fetal Testes:* Human fetal testes were obtained following termination of pregnancy during 2nd
99 trimester (14-19 weeks, n=5). Women gave consent in accordance with national guidelines, and ethical
100 approval was obtained from the Local Research Ethics Committee (Reference number – LREC08/S1101/1).
101 No terminations were due to fetal abnormalities. Gestational age was determined initially by ultrasound,
102 followed by measurement of foot length. Testes were fixed for 2h in Bouins, transferred into 70% ethanol and
103 then embedded in paraffin. Sections of 5µm thickness were prepared.

104

105 **Immunohistochemistry**

106 Details of antibodies, dilutions and requirement for antigen retrieval are shown in Table 2. Sections were
107 dewaxed in xylene, rehydrated in graded alcohols and washed in tap water. Antigen retrieval involved pressure
108 cooking in 0.01M citrate (pH 6.0) buffer as described previously [14]. Sections were treated with 3% (v/v)
109 H₂O₂ in methanol for 30 min and washed in water, followed by Tris-buffered saline (TBS, 0.05M Tris and
110 0.85% NaCl, pH 7.6) for a further 5 min. Endogenous biotin was blocked using an avidin/biotin blocking kit
111 (Vector Laboratories, Peterborough, UK), according to the manufacturers instructions. Sections were

112 incubated in appropriate normal serum (diluted 1:5 with TBS containing 5% (w/v) bovine serum albumin
113 (BSA) (Sigma, Poole, Dorset, UK) for 30 min. Sections were incubated overnight with primary antibody
114 diluted in serum at 4°C in a humidified chamber. Sections were washed in TBS (2x5min) and incubated for 30
115 min with the appropriate biotinylated secondary antibody (swine anti-rabbit, rabbit anti-mouse; both Dako,
116 Ely, UK or rabbit anti-goat; Vector Laboratories), diluted in normal serum. This was followed by two further 5
117 min washes in TBS and incubation for 30 min with Streptavidin-HRP at 1:1000 (Dako), diluted in TBS.

118

119 Visualisation was performed using 3,3-diaminobenzidine tetrahydrochloride (DAB) (Dako) and sections were
120 counterstained with haematoxylin, dehydrated in graded alcohols, immersed in xylene and mounted in Pertex
121 medium (CellPath, Hemel Hempstead, UK). For each experiment a negative control (primary antibody
122 replaced with the appropriate normal serum) was included. Images were captured using an Olympus Provis
123 microscope (Olympus, London, UK) and Canon DS126131 camera with Canon EOS image capture software
124 (Canon, Woodhatch, Surrey, UK).

125

126 **Immunofluorescence**

127 Sections were initially treated as described for single staining as far as the primary antibody stage, with
128 Phosphate Buffered Saline (PBS; Sigma) washes between each step. Antigen retrieval was required for all
129 experiments. Details of antibodies, serum and visualisation method are listed in Table 2.

130

131 Following overnight incubation with primary antibody in serum, sections were incubated with secondary
132 antibody for 30 min, followed by fluorescently labelled Tyramide (1:50; Perkin Elmer, Cambridge, UK) in
133 dilution buffer. For this and subsequent steps, the sections were kept in darkness. Sections were then
134 microwaved in 0.01M citrate (pH 6.0) for 2.5 min and left to cool for 30 min, before being washed in water
135 and PBS for 5 min. Sections were incubated for 30 min in serum. They were incubated with secondary
136 antibody for 30 min, followed by the labelled Tyramide (1:50) using a different fluorescent label. After the
137 second visualisation sections were microwaved again as described above and incubated with the third
138 secondary antibody for 30 min, followed by the third labelled Tyramide (1:50). DAPI (Sigma) was applied to
139 the sections at 1:1000 in PBS for 10 min and the slides were mounted using Permafluor (Immunotech,
140 Marseille, France). Images were captured using an LSM 510 Confocal microscope (Carl Zeiss, Hertfordshire,
141 UK).

142

143 Quantification of germ cell differentiation and proliferation

144 Quantification of germ cell subpopulations and proliferation indices were performed for the triple-stained
145 sections as previously described [10]. For each sample, a minimum of 10 randomly selected fields with tubules
146 containing intratubular germ cell neoplasia were counted and included an average of 1000 cells per section.
147 Images were obtained using an Axiovert 200M microscope with attached AxioCam HRC camera and
148 Axiovision 4.6 software (all Carl Zeiss). All germ cells within each section were manually counted and
149 quantified according to their protein expression profile and proliferation status by marking cells in layered
150 images using Adobe Photoshop 7.0 (Adobe, San Jose, CA, USA).

151

152 Statistics

153 Statistical analysis was performed using Graphpad Prism 5 software (La Jolla, CA, USA). Groups were
154 compared using Student's t-test. Multiple groups were analysed using one-way analysis of variance (ANOVA).
155 Statistical significance was set at $P < 0.05$.

156

157 Results

158 In order to characterise the heterogeneity of expression of germ cell proteins in putative intratubular germ cell
159 neoplasia cells we first compared the expression of a range of germ cell-specific proteins in testicular tissue
160 from patients with testicular germ cell cancer (including tubules containing intratubular germ cell neoplasia
161 cells and those with apparently normal spermatogenesis) with that of the normal human fetal testis.

162

**163 Expression of gonocyte markers in human fetal testis, intratubular germ cell neoplasia and
164 spermatogonia**

165 OCT4, AP2 γ and PLAP were expressed in germ cells (gonocytes) in the human fetal testis. In sections from
166 patients with testicular germ cell cancer these proteins were also expressed in intratubular germ cell neoplasia
167 cells; however none of the proteins were expressed in spermatogonia in tubules that contained active
168 spermatogenesis (Fig. 1A-I).

169

**170 Expression of spermatogonial markers in human fetal testis, intratubular germ cell neoplasia and
171 spermatogonia**

172 MAGEA4 and VASA were expressed in germ cells (pre-spermatogonia) in the human fetal testis. These
173 proteins were also expressed in intratubular germ cell neoplasia cells. There was also expression of these
174 proteins in germ cells (MAGEA4 in spermatogonia and early spermatocytes, VASA in all germ cells) of
175 tubules that contained active spermatogenesis in patients with testicular germ cell cancer (Fig 1J-O).

176

177 **Co-expression of gonocyte and spermatogonial markers in intratubular germ cell neoplasia cells and** 178 **normal testis**

179 For the identification of intratubular germ cell neoplasia cells and to attempt to distinguish these cells from
180 normal germ cells, AP2 γ and VASA co-expression was investigated (Fig 2). In tubules with normal-appearing
181 spermatogenesis there was expression of VASA in the cytoplasm of germ cells, but no expression of AP2 γ
182 (Fig 2A). In tubules containing a mixture of germ cells characteristic of either intratubular germ cell neoplasia
183 or normal spermatogonia the putative intratubular germ cell neoplasia cells (located on the basement
184 membrane) were identified as AP2 γ^+ /VASA $^-$, whilst a small proportion of these cells were AP2 γ^+ /VASA $^+$ (Fig
185 2B). AP2 γ /VASA $^+$ cells, located nearer the lumen were also identified in intratubular germ cell neoplasia
186 tubules. These putative spermatocytes were also identified in tubules in which the majority of the cells were
187 intratubular germ cell neoplasia cells (AP2 γ^+ /VASA $^-$; Fig 2C). Similar populations of germ cells were also
188 identified within the human fetal testis, based on co-staining for OCT4 and VASA (Supp. Fig. 1; [7]).

189

190 **Heterogeneity of expression of ‘classical’ intratubular germ cell neoplasia markers in patients with** 191 **testicular germ cell cancer**

192 In order to demonstrate the heterogeneity of expression of gonocyte proteins in intratubular germ cell
193 neoplasia, co-localisation of OCT4, AP2 γ and PLAP was undertaken. OCT4 and AP2 γ were always co-
194 expressed and were localised to the nuclei of intratubular germ cell neoplasia cells (Supp. Fig. 2). A similar
195 pattern of co-expression was demonstrated for OCT4 and PLAP, with co-expression of OCT4 (nuclear) and
196 PLAP (cytoplasm +/- nuclear) in the majority of cells (OCT4 $^+$ /PLAP $^+$) within these tubules, however there
197 were also germ cells that were OCT4 $^+$ /PLAP $^-$ (Fig. 3A). These two populations were also identified in the
198 human fetal testis (Fig. 3B). However within the intratubular germ cell neoplasia containing tubules we also
199 identified a rare population of cells that were OCT4 $^+$ /PLAP $^+$ (Fig. 3C), whilst this population of germ cells was
200 not identified in any of the human fetal testis sections (Fig. 3B).

201

202 The expression of spermatogonial markers in putative intratubular germ cell neoplasia cells

203 To further characterise the germ cells within intratubular germ cell neoplasia containing tubules that either did
204 or did not express OCT4, triple immunofluorescence staining was undertaken for MAGEA4/VASA, OCT4
205 and PLAP (Fig. 4). In tubules containing intratubular germ cell neoplasia cells, the majority of germ cells
206 expressed nuclear OCT4 and most of these were co-stained with PLAP (cytoplasmic +/- nuclear). A small
207 proportion of presumptive spermatocytes expressed VASA only (Fig. 4A). However there were infrequent
208 germ cells which expressed VASA and PLAP, but which did not express OCT4 (Fig. 4A), suggesting that a
209 proportion of the VASA expressing cells (VASA⁺/OCT4⁻) are not 'normal' spermatogonia/spermatocytes and
210 may represent gonocytes that have downregulated OCT4 and begun to express VASA but retain PLAP
211 expression (VASA⁺/OCT4⁻/PLAP⁺). Similar sub-populations were found when the co-expression of MAGEA4
212 with OCT4 and PLAP was undertaken (Figure 4B).

213

**214 Quantification of intratubular germ cell neoplasia phenotypes depending on histological testicular germ
215 cell cancer type**

216 The proportion of intratubular germ cell neoplasia cells (identified by expression of OCT4 and/or PLAP) with
217 different expression profiles based on OCT4/PLAP/MAGEA4 co-staining was determined (Fig. 5). By far the
218 most common phenotype was OCT4⁺/PLAP⁺/MAGEA4⁻, which was found in a significantly higher proportion
219 (68%) of cells compared with the other phenotypes (Fig. 5; b versus a,c). A smaller proportion (7.7%) of
220 intratubular germ cell neoplasia cells were OCT4⁺/PLAP⁻/MAGEA4⁻. Overall 82% of intratubular germ cell
221 neoplasia cells expressed OCT4 with the remaining 18% of putative intratubular germ cell neoplasia cells
222 expressing PLAP (but no detectable OCT4). In terms of spermatogonial markers, MAGEA4 expression was
223 found in 6% of putative intratubular germ cell neoplasia cells (defined by expression of OCT4 and/or PLAP;
224 Fig. 5) and this represented a significantly lower proportion of cells compared to those not expressing
225 MAGEA4 (Fig 5; c versus a,b). There was a shift towards an increasing proportion of these MAGEA4⁺ cells
226 from pre-invasive (child) to pre-invasive (adult) and seminoma, whilst very few putative intratubular germ cell
227 neoplasia cells in non-seminoma expressed this protein profile, however the differences in expression were not
228 significant (Supp. Fig. 3).

229

230 **Proliferation of intratubular germ cell neoplasia cells according to germ cell expression profile and**
231 **histological testicular germ cell cancer type**

232 In order to investigate whether the germ cell expression profile of the putative intratubular germ cell neoplasia
233 cells might determine their proliferation rate, which might in turn affect their invasive potential, triple
234 immunofluorescence for OCT4/MAGEA4/Ki67 was performed (Supp. Fig. 4). Overall, the proportion of
235 proliferating (Ki67⁺/OCT4⁺) intratubular germ cell neoplasia cells was 8.1% (Fig. 6A). There was a shift
236 towards increased proliferation in intratubular germ cell neoplasia cells from patients with pre-invasive disease
237 compared with those with a seminoma or non-seminoma, but this was not statistically significant (Supp. Fig.
238 5). However, when intratubular germ cell neoplasia (identified by expression of OCT4⁺) cell proliferation was
239 analysed according to whether or not the cells also expressed MAGEA4, a significantly higher proliferation
240 rate was found for the OCT4⁺/MAGEA4⁻ population compared to OCT4⁺/MAGEA4⁺ intratubular germ cell
241 neoplasia cells (12.8 v 3.4%, p<0.0001; Fig. 6B). Moreover, the significant difference in proliferation rate
242 between the two intratubular germ cell neoplasia phenotypes was consistent when the same analysis was
243 performed according to whether the intratubular germ cell neoplasia cells were from patients with pre-invasive
244 disease (child or adult), seminoma or non-seminoma (Fig. 6C-F). Furthermore, when the proliferation rates in
245 the two sub-populations of intratubular germ cell neoplasia cells (OCT4⁺/MAGEA4⁻ or OCT4⁺/MAGEA4⁺)
246 were compared according to the histology of the adjacent testicular germ cell cancer, there was a significantly
247 higher proliferation rate in the OCT4⁺/MAGEA4⁻ cells in pre-invasive disease compared with these cells in
248 seminoma and non-seminoma (Fig. 7A); in contrast, there was no difference in the proportion of
249 OCT4⁺/MAGEA4⁺ that were proliferating for the different tumour types (Fig. 7B).

250

251 **Proliferation of seminoma cells according to germ cell expression profile**

252 Given that the OCT4⁺/MAGEA4⁻ population of intratubular germ cell neoplasia cells were more proliferative
253 than the OCT4⁺/MAGEA4⁺ population, we investigated MAGEA4, OCT4 and PLAP expression in seminoma
254 cells in order to determine whether this results in a predominance of OCT4⁺/MAGEA4⁻ cells in the resulting
255 tumours. Indeed we found that in the majority of intra-tubular seminoma cells MAGEA4 was not expressed
256 and that the majority of cells were OCT4⁺/PLAP⁺/MAGEA4⁻ (Fig. 8A). MAGEA4 positive cells were seen in
257 tubules with normal appearance adjacent to areas of invasive seminoma (Fig. 8B), however MAGEA4
258 expression was not seen in the invasive seminoma cells (Fig. 8C). The OCT4⁺/MAGEA4⁻ seminoma cells

259 were highly proliferative, whilst a smaller proportion of OCT4⁺/MAGEA4⁻ intratubular germ cell neoplasia
260 cells were proliferative. In contrast MAGEA4 expressing cells were rarely proliferative (Supp. Fig. 6).

261

262 **Discussion**

263 The present study has characterised the heterogeneity of germ cell protein expression in the human testis based
264 on co-expression of germ cell proteins involved in differentiation from gonocyte to pre-spermatogonia. We
265 have demonstrated an infrequent population of cells within intratubular germ cell neoplasia containing tubules
266 with an expression profile distinct from germ cells in the normal human fetal testis. We have also
267 demonstrated that the most common sub-population in intratubular germ cell neoplasia containing tubules,
268 which displays a 'gonocyte' expression profile (OCT4⁺/MAGEA4⁻), is associated with an increased
269 proliferation rate compared to the subpopulation expressing a 'pre-spermatogonial' profile
270 (OCT4⁺/MAGEA4⁺), and that this (OCT4⁺/MAGEA4⁻) population represents the true intratubular germ cell
271 neoplasia cell and precursor for invasive seminoma and non-seminoma. The findings of the present study are
272 summarised in Fig 9.

273

274 Intratubular germ cell neoplasia cells are thought to originate in fetal life from abnormally differentiated
275 gonocytes [3,15]. This is supported by similarities in morphological, immunohistochemical and genetic
276 profiles [3,16,17]. Intratubular germ cell neoplasia cells share expression of a variety of proteins that are
277 involved in pluripotency and early germ cell fate, such as OCT4 [18-20], PLAP [9,21] and AP2γ [8]. As
278 gonocytes differentiate into spermatogonia during fetal life these markers have been shown to be
279 downregulated [3,6,7,20,22,23]. OCT4, AP2γ and PLAP are described as 'classical' markers of intratubular
280 germ cell neoplasia cells in adulthood and persistence of expression of these proteins is routinely used for
281 diagnostic purposes for patients at risk of, or with suspected, testicular germ cell cancer [11]. These markers
282 are considered highly sensitive and specific for intratubular germ cell neoplasia cells. Whilst we found co-
283 expression of AP2γ and OCT4 in intratubular germ cell neoplasia cells with no cells expressing a single
284 marker alone, there was heterogeneity in the co-expression of PLAP and OCT4 in intratubular germ cell
285 neoplasia cells. Expression of OCT4 has been reported to be expressed by all intratubular germ cell neoplasia
286 cells [19], whilst PLAP has been reported to be expressed in 83-99% of intratubular germ cell neoplasia cells
287 [9]. The present co-localisation studies demonstrate that the majority of the cells expressing OCT4 also

288 express PLAP. Overall co-localisation was seen in 68% of cells in tubules with intratubular germ cell
289 neoplasia. Both of these sub-populations are also present in the normal human fetal testis during the transition
290 from gonocyte to spermatogonia. However, our co-localisation studies have demonstrated the presence of a
291 sub-population of intratubular germ cell neoplasia cells with a protein expression profile distinct from the
292 germ cells in the normal human fetal testis. This OCT4⁺/PLAP⁺ sub-population represented 18% of the total
293 cells in tubules with intratubular germ cell neoplasia. PLAP is expressed in most of the germ cells in a first
294 trimester fetal testis, but is rare by the start of the second trimester [23]. OCT4 is also present in most of the
295 germ cells of the first trimester, but is downregulated later in gestation in comparison to PLAP [24]. This study
296 has shown that an OCT4⁺/PLAP⁺ population can be identified in tubules with intratubular germ cell neoplasia,
297 whilst our results confirm that PLAP is not expressed without co-expression of OCT4 in the normal human
298 fetal testis [23]. As these cells do not occur as part of normal germ cell development they may represent
299 impaired maturation of gonocytes with loss of OCT4 and retention of PLAP expression as a result of an
300 altered germ cell niche.

301

302 In addition to the proteins that are found in undifferentiated germ cells, intratubular germ cell neoplasia cells
303 have also been reported to express proteins characteristic of differentiated germ cells such as VASA [25] and
304 MAGEA4 [26]. These markers begin to be expressed in germ cells during fetal life in increasing proportions
305 as the cells differentiate [24,26-28]. We have shown that the majority of intratubular germ cell neoplasia cells
306 (based on the expression of OCT4 and/or PLAP) do not express the spermatogonial proteins MAGEA4 and
307 VASA. We have quantified the expression of these differentiated germ cell markers in putative intratubular
308 germ cell neoplasia for the first time and shown that MAGEA4 is only expressed in 6% of OCT4 and/or
309 PLAP-expressing cells and therefore is not a common phenotype for putative intratubular germ cell neoplasia
310 cells. Heterogeneous expression of MAGEA4 in intratubular germ cell neoplasia has been described
311 previously, however VASA expression was reported to be expressed in all intratubular germ cell neoplasia
312 cells [3]. We found that VASA was expressed heterogeneously in a similar proportion of putative intratubular
313 germ cell neoplasia cells as those expressing MAGEA4.

314

315 Previous studies have indicated that differentiated germ cells (e.g. spermatogonia) may be present within
316 intratubular germ cell neoplasia containing tubules [12,22]. Co-staining for OCT4 and VASA/MAGEA4
317 identified cells that had an OCT4⁺/VASA⁺ phenotype [22]. These cells would be considered differentiated

318 germ cells rather than intratubular germ cell neoplasia cells. We have described similar populations in our
319 samples, however triple co-localisation has demonstrated that some VASA or MAGEA4 expressing cells that
320 do not express OCT4, express PLAP and therefore may not represent 'normal' spermatogonia. As a result it is
321 likely that only cells expressing neither OCT4 nor PLAP may represent normally matured germ cells that have
322 not undergone pre-invasive change. The OCT4⁻/VASA⁺/PLAP⁺ or OCT4⁻/MAGEA4⁺/PLAP⁺ populations may
323 represent pre-invasive germ cells that have undergone a degree of maturation towards pre-spermatogonia (due
324 to downregulation of OCT4 and expression of VASA/MAGEA4), alternatively they may represent pre-
325 spermatogonia that have aberrantly retained PLAP expression following the downregulation of OCT4. In order
326 to determine whether these populations could represent intratubular germ cell neoplasia cells we investigated
327 expression during the development of invasive disease. OCT4 (without MAGEA4) was expressed in all intra-
328 tubular and invasive seminomas, indicating that intratubular germ cell neoplasia cells with invasive potential
329 express OCT4 and do not express MAGEA4. Therefore we conclude that the OCT4⁻/MAGEA4⁺/PLAP⁺ cells
330 do not represent intratubular germ cell neoplasia cells with malignant potential and are more likely to be a
331 separate population of abnormally differentiated germ cells that are present in intratubular germ cell neoplasia
332 containing tubules.

333

334 Uncontrolled proliferation of cells is a hallmark of invasive tumours [29]. Previous studies have demonstrated
335 proliferation in intratubular germ cell neoplasia and overt testicular germ cell cancer [12,30-32], and a
336 previous study has shown that Ki67 expression is found in intratubular germ cell neoplasia cells in 14/16 non-
337 seminomas and 14/17 seminomas, although the proportion of intratubular germ cell neoplasia cells that were
338 proliferating was not quantified [31]. A detailed analysis of proliferation in the various intratubular germ cell
339 neoplasia sub-populations in relation to the underlying tumour type has not previously been performed.
340 Intratubular germ cell neoplasia cells have previously been reported to proliferate at a relatively high rate. In a
341 study of sections taken from patients with testicular germ cell cancer, using Ki67 as a marker of proliferation,
342 17.42% of intratubular germ cell neoplasia cells were found to be Ki67 positive [30]. Overall we found that
343 8.1% of intratubular germ cell neoplasia cells were positive for Ki67, however we have shown that the
344 proliferation rate is dependent on which intratubular germ cell neoplasia sub-population is investigated. We
345 have shown that PLAP is not expressed in ~20% of intratubular germ cell neoplasia (OCT4⁺) cells and this
346 may partially explain the differences seen between proliferation of intratubular germ cell neoplasia cells in the
347 present study compared to previous studies which relied on PLAP expression to identify intratubular germ cell

348 neoplasia cells [12,30]. The OCT4⁺/MAGEA4⁺ (and also OCT4⁻/MAGEA4⁺, not shown) populations are less
349 proliferative than those expressing OCT4⁺/MAGEA4⁻ which provides further evidence supporting the view
350 that the OCT4⁺/MAGEA4⁻ cells have more invasive potential than those expressing a more mature phenotype.
351 This hypothesis is supported by our finding of little or no expression of MAGEA4 in the OCT4⁺ seminoma
352 cells of an invasive tumour. We therefore propose that the OCT4⁺/MAGEA4⁻ population of intratubular germ
353 cell neoplasia cells give rise to the invasive tumour, whilst the OCT4⁺/MAGEA4⁺ population has a lower
354 capacity to progress to invasiveness and may represent germ cells that are arrested in the transition from
355 gonocyte to spermatogonia and do not contribute to the invasive tumour. OCT4⁻/MAGEA4⁺ cells are
356 occasionally seen within the seminomatous component but are likely to represent spermatogonia that have
357 become enclosed in the invasive tumour.

358

359 Differences in the proliferation of intratubular germ cell neoplasia cells have recently been investigated with
360 respect to one of the key regulators of the mitosis-meiosis switch, DMRT1 [12]. This study demonstrated that
361 intratubular germ cell neoplasia cells expressing DMRT1 were significantly less proliferative than those
362 intratubular germ cell neoplasia cells that did not express DMRT1 and that progression from ‘early-stage’
363 (Ki67⁻) intratubular germ cell neoplasia cell to invasive disease (Ki67⁺) is associated with a down-regulation
364 of DMRT1. In order to test the hypothesis that certain subpopulations of intratubular germ cell neoplasia cells
365 display differences in invasive potential, future studies involving isolation of the different intratubular germ
366 cell neoplasia sub-populations followed by germ cell transplantation or xenografting may be performed.

367

368 The present study has demonstrated the presence of proliferating intratubular germ cell neoplasia cells in testis
369 tissue from patients with pre-invasive disease, seminoma and non-seminoma with a higher rate of proliferation
370 in the OCT4⁺/MAGEA4⁻ population in pre-invasive samples compared to those with an invasive tumour
371 (either seminoma or non-seminoma). The finding of higher rates of proliferation of intratubular germ cell
372 neoplasia cells in pre-invasive disease compared to tumours samples might be considered surprising given
373 previous reports of low rates of proliferation in pre-invasive intratubular germ cell neoplasia cells [12]. In
374 adults with pre-invasive disease this may be explained by an increase in proliferation around the time of
375 progression to invasive disease, however this would not explain the proliferation rate for intratubular germ cell
376 neoplasia cells in the pre-invasive childhood patients in which it might be expected that these cells are
377 relatively quiescent. However, we have demonstrated previously that a higher proportion of OCT4⁺ germ cells

378 in the second trimester human fetal testis are proliferating compared with the MAGEA4⁺ population and that
379 the rates of proliferation in the present study are similar to those in the normal human fetal testis for each sub-
380 population [10], indicating that the proliferation in the germ cell sub-populations in children with pre-invasive
381 disease may simply reflect the proliferation rates in the normal human fetal testis.

382 In conclusion, we have described in detail the heterogeneity of germ cell protein expression in cells within
383 intratubular germ cell neoplasia tubules. We have demonstrated sub-populations of OCT4⁻ cells that do not
384 correspond to an equivalent stage of normal human fetal germ cell differentiation, suggesting that these cells
385 may have lost expression of proteins that may determine their malignant potential. We have also demonstrated
386 that a more undifferentiated/pluripotent expression profile is associated with an increased proliferation rate
387 compared with a differentiated phenotype. These results indicate that germ cells expressing an
388 OCT4⁺/MAGEA4⁻ phenotype are those that will ultimately lead to tumour formation.

389

390 **Disclosure/Conflict of Interest:** The authors have no conflicts of interest to disclose.

391

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397

398 Supplementary information is available at *Modern Pathology*'s website

399

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468

469 **Figure Legends**

470 **Figure 1** - Expression of gonocyte markers (OCT4, AP2 γ and PLAP; A-I) and spermatogonial markers
471 (MAGEA4 and VASA; J-O) in human fetal testis, intratubular germ cell neoplasia containing tubules (Adult –
472 intratubular germ cell neoplasia) and tubules from adult testis with active spermatogenesis (Adult – ‘Normal’).
473 Gonocyte proteins are detected in human fetal germ cells and intratubular germ cell neoplasia cells, but are
474 absent from the germ cells in tubules with apparently normal spermatogenesis; whilst spermatogonial proteins
475 are expressed in germ cells in all tissue types. Human fetal samples are 14 (A,D), 16 (J) and 18 weeks (G,M)
476 gestation. Scale bar = 50 μ m.

477

478 **Figure 2** - Representative image for expression of VASA (green) and AP2 γ (red) in tubules from adult
479 patients with testicular germ cell cancer. A) Tubule with apparently normal spermatogenesis: VASA is
480 expressed in the germ cells with no expression of the intratubular germ cell neoplasia/gonocyte protein AP2 γ .
481 B) Tubule with abnormal spermatogenesis: VASA expression is seen in the presumptive spermatocytes
482 towards the lumen (white arrowhead), however germ cells along the basement membrane express VASA
483 (yellow arrowhead) or AP2 γ (yellow arrow). A small proportion of cells co-express VASA and AP2 γ (white
484 arrow). C) Intratubular germ cell neoplasia tubule: The majority of cells express the intratubular germ cell
485 neoplasia protein AP2 γ (yellow arrow) with a small number of cells adjacent to the basement membrane
486 expressing VASA (presumptive spermatogonia; pink arrow). A small number of germ cells expressing VASA
487 are located towards the lumen (presumptive spermatocytes; white arrowhead).

488

489 **Figure 3** - Expression of OCT4 (red) and PLAP (green) in intratubular germ cell neoplasia containing tubules
490 from men with testicular germ cell cancer (A,C) and in normal human fetal testis tissue (B). The majority of
491 intratubular germ cell neoplasia cells co-express OCT4 and PLAP (A,C; yellow arrowheads). Occasional
492 OCT4 positive intratubular germ cell neoplasia cells are negative for PLAP expression (A; white arrow).
493 Similar sub-populations of OCT4⁺/PLAP⁻ (B, white arrow) and OCT4⁺/PLAP⁺ (B, yellow arrowheads) cells
494 are also identified in the human fetal testis. In tubules with intratubular germ cell neoplasia, occasional OCT4⁻
495 /PLAP⁺ cells are identified (C; yellow arrows), however no similar population is seen in the human fetal testis.
496 Counterstain (DAPI; blue).

497

498 **Figure 4** - Representative image for expression of OCT4 (red), VASA (A; blue), MAGEA4 (B; blue) and
 499 PLAP (green) in intratubular germ cell neoplasia containing tubules from patients with testicular germ cell
 500 cancer. A) VASA expression is demonstrated in putative 'spermatogenic' germ cells that are negative for
 501 intratubular germ cell neoplasia cell proteins PLAP and OCT4 (white arrow). A small proportion of the
 502 VASA⁺ cells that are negative for OCT4 express PLAP (white arrowhead). B) The majority of cells co-express
 503 OCT4 and PLAP without MAGEA4, other sub-populations are identified including PLAP⁺/OCT4⁺/MAGEA4⁺
 504 (pink arrow) and PLAP⁺/OCT4⁻/MAGEA4⁻ (pink arrowhead). Counterstain (DAPI; pale blue) in merged
 505 panels.

506

507 **Figure 5** - Quantification of putative intratubular germ cell neoplasia phenotypes. Expression (+) of OCT4,
 508 PLAP and MAGEA4 for intratubular germ cell neoplasia containing tubules (n=9; pre-invasive, seminoma and
 509 non-seminoma; n=3 each). Bars with different letters are significantly different from each other (p<0.05).
 510 Mean +/- SEM.

511

512 **Figure 6** - Proliferation in putative intratubular germ cell neoplasia cells. A) Overall proliferation in all
 513 intratubular germ cell neoplasia cells. B) Proliferation (Ki67⁺) of intratubular germ cell neoplasia (OCT4⁺)
 514 cells based on the co-expression with MAGEA4 in tubules from all patients (B), children with pre-invasive
 515 disease (C; Pre-Inv. Child; n=4), adults with pre-invasive disease (D; Pre-Inv. Adult; n=6), seminoma (n=7)
 516 and non-seminoma (n=8). Mean +/- SEM. * p<0.05, ** p<0.01, **** p<0.0001.

517

518 **Figure 7** - Proliferation of intratubular germ cell neoplasia cells based on diagnosis of pre-invasive disease
 519 (PRE INV; n=7), seminoma (SEM; n=7) or non-seminoma (NON-SEM; n=8). A) Proliferation of OCT4⁺
 520 intratubular germ cell neoplasia cells. B) Proliferation of OCT4⁺/MAGEA4⁺ intratubular germ cell neoplasia
 521 cells. Mean +/- SEM. * p<0.05, ** p<0.01, in comparison with pre-invasive intratubular germ cell neoplasia.

522

523 **Figure 8** - Representative images for expression of OCT4 (red), PLAP (green) and MAGEA4 (blue) in testis
 524 sections from patients with A) Intra-tubular seminoma, B) Seminoma with surrounding 'normal' (*) tubules
 525 and C) Seminoma. Note the lack of expression of MAGEA4 in both intra-tubular seminoma and invasive
 526 seminoma. Counterstain (DAPI; pale blue) in merged panels (bottom right).

527

528 **Figure 9** - Schematic for germ cell maturation and proliferation in germ cells during transition from
529 gonocyte to intratubular germ cell neoplasia and testicular germ cell cancer (bottom). Germ cell maturation
530 from gonocyte to initiation of spermatogenesis is represented in the testis during the different stages of life
531 (middle). For comparison, germ cell differentiation in the normal testis is also shown (top). Germ cells in the
532 fetal testis may exhibit delayed maturation with persistence of gonocyte markers through childhood. A
533 variety of germ cell protein profiles are present in the intratubular germ cell neoplasia tubule, however it is
534 the cells expressing exclusively gonocyte proteins (with no spermatogonial proteins) that are more
535 proliferative and contribute to the majority of the cells in intratubular seminoma and subsequently invasive
536 seminoma. Cells expressing spermatogonial proteins are occasionally seen in the tubule or resultant tumour
537 but exhibit low proliferation rates. Expression of OCT4 (red), PLAP (green) and MAGEA4 (blue) is shown
538 for individual cells and cells with high rates of proliferation are indicated (yellow asterisk).

539 Supplementary Figures**540 Figure S1**

541 Expression of OCT4 (red) and VASA (green) in a seminiferous cord from a 14 week gestation human fetal
542 testis. Sub-populations of gonocytes (OCT4⁺/VASA⁻; white arrowhead), pre-spermatogonia (OCT4⁺/VASA⁺;
543 white arrow) and spermatogonia (OCT4⁻/VASA⁺; yellow arrowhead) are present within the tubule.

544

545 Figure S2

546 Representative image for expression of AP2γ (B; green) and OCT4 (C; red) in an intratubular germ cell
547 neoplasia containing tubule from patients with testicular germ cell cancer. Intratubular germ cell neoplasia
548 cells co-express both proteins in all cells (D; yellow). Nuclear counterstain with DAPI (A; blue).

549

550 Figure S3

551 Proportion of putative intratubular germ cell neoplasia cells expressing MAGEA4 in pre-invasive disease in
552 childhood (PRE-INV. Child; n=4) and adulthood (PRE-INV. Adult; n=6), seminoma (SEM; n=9) and non-
553 seminoma (NON-SEM; n=8). Mean +/- SEM.

554

555 Figure S4

556 Example of triple immunofluorescence used for quantification of proliferation in sub-populations of
557 intratubular germ cell neoplasia cells. Expression of Ki67 (green), OCT4 (red) and MAGEA4 (blue) in
558 tubules containing intratubular germ cell neoplasia from a patient with testicular germ cell cancer.
559 Arrowheads indicate proliferating OCT4⁺/MAGEA4⁻ intratubular germ cell neoplasia cells, whilst the arrow
560 indicates a proliferating OCT4⁺/MAGEA4⁺ intratubular germ cell neoplasia cell.

561

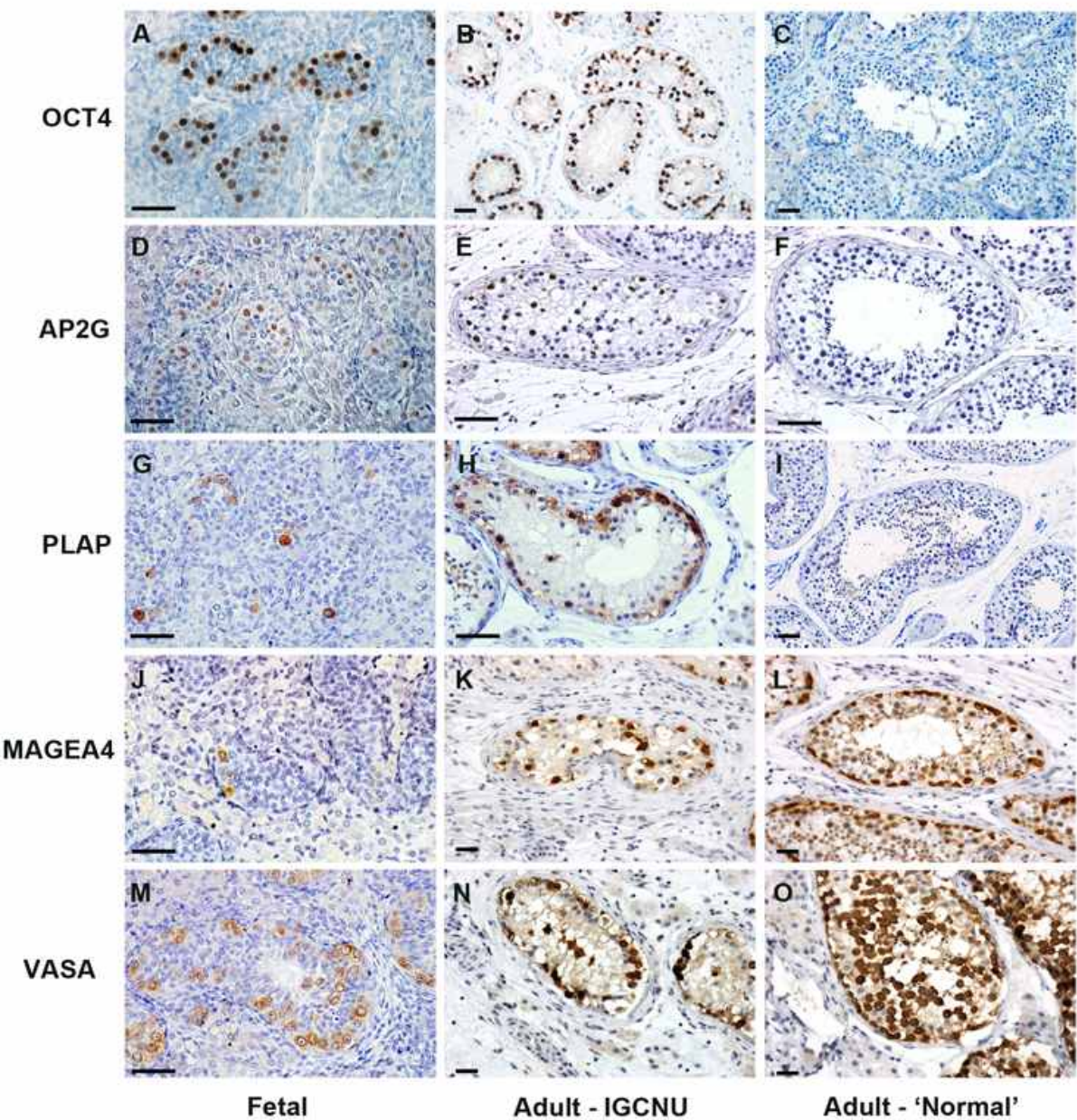
562 Figure S5

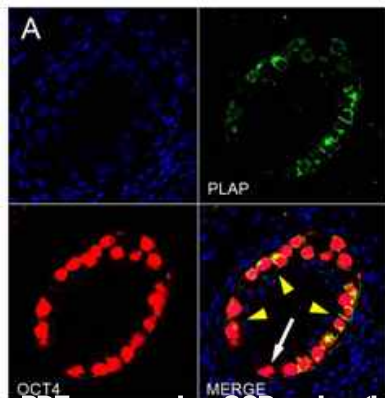
563 Proliferation (Ki67⁺) in MAGEA4 expressing intratubular germ cell neoplasia (OCT4⁺) cells in children with
564 pre-invasive disease (C; Pre-Inv. Child; n=4), adults with pre-invasive disease (D; Pre-Inv. Adult; n=6),
565 seminoma (n=7) and non-seminoma (n=8). Mean +/- SEM.

566

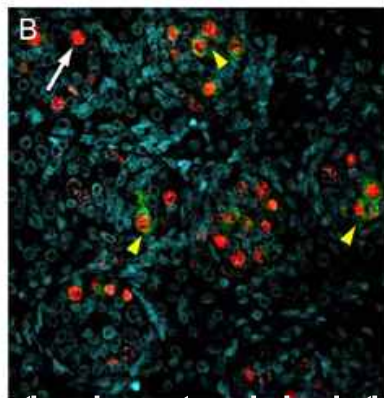
567 Figure S6

568 Representative image of Ki67 (green), OCT4 (red) and MAGEA4 (blue) expression in seminoma (n=9).
569 Seminoma cells express OCT4 (but not MAGEA4) and a large proportion also express Ki67, whilst the
570 OCT4⁺ intratubular germ cell neoplasia cells (*) are less proliferative. MAGEA4⁺ cells are present in
571 adjacent tubules (#) and are Ki67⁻. MAGEA4⁺/OCT4⁻ cells are occasionally seen interspersed between the
572 seminoma cells (arrow; inset). Counterstain (DAPI; pale blue) in merged panel (bottom right).
573
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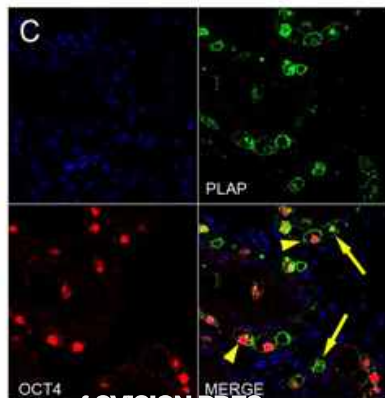




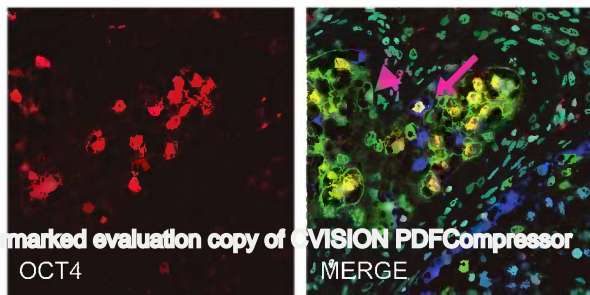
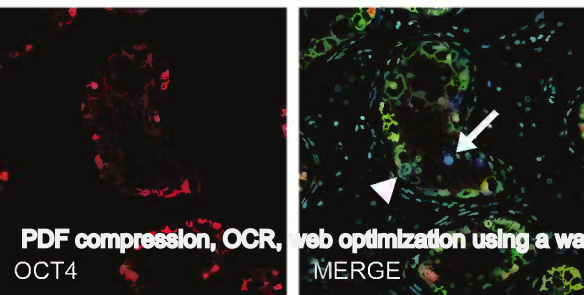
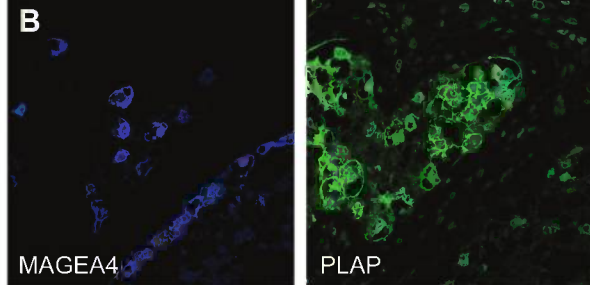
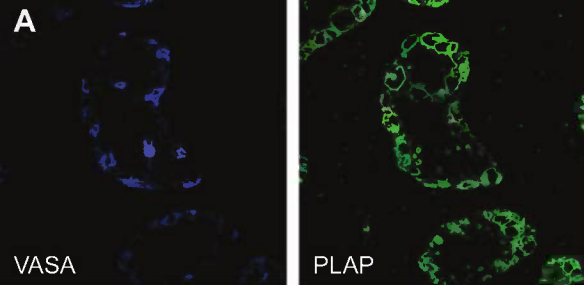
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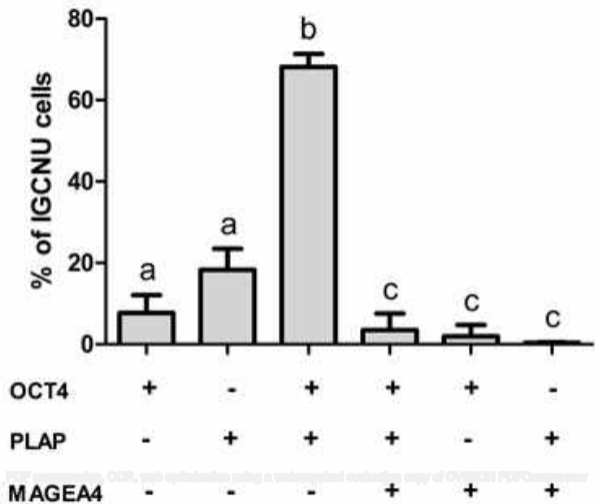
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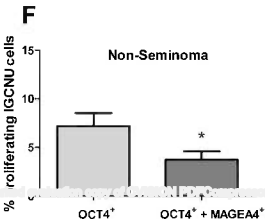
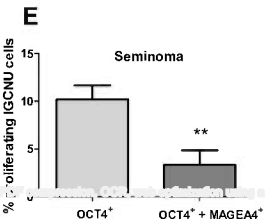
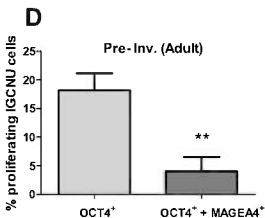
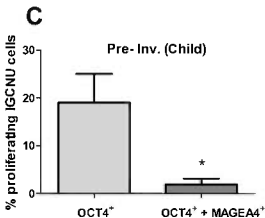
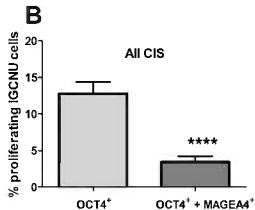
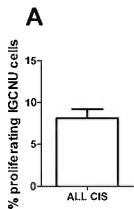


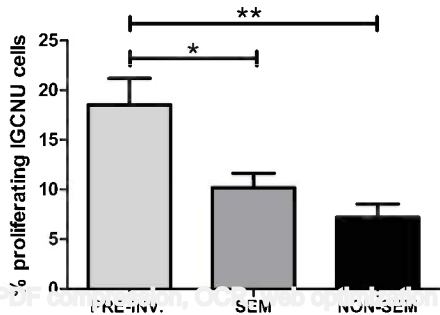
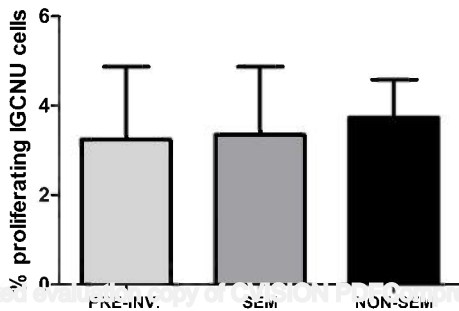
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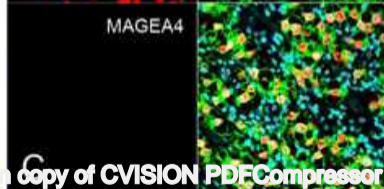
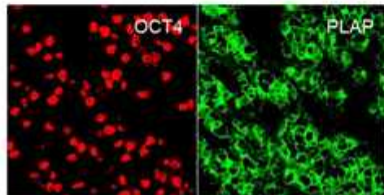
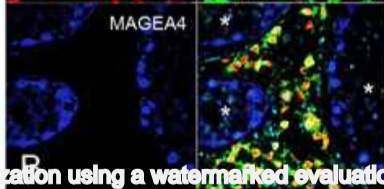
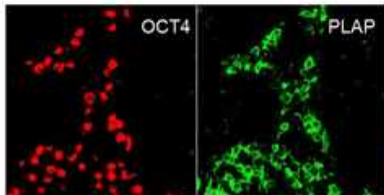
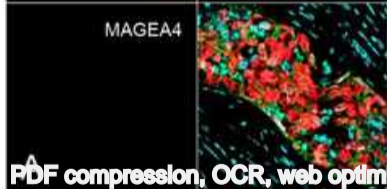
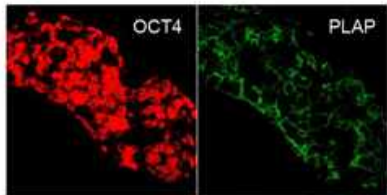


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A**OCT4⁺****B****OCT4⁺ + MAGEA4⁺**



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Intra-tubular Seminoma

'Normal' + Seminoma

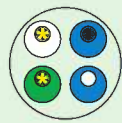
Seminoma

Fetal/early postnatal

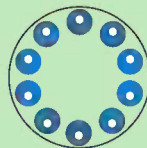
Childhood

Adulthood

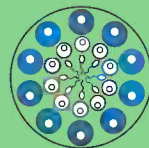
Normal



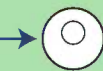
Normal maturation



Normal maturation



Germ cells present in the normal testis



Gonocyte

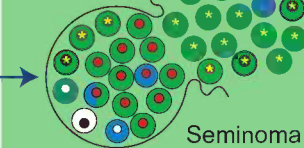
Spermatogonium

Spermatogonium

Spermatocyte



Delayed maturation



Seminoma



Table 1. Clinical characteristics of patients from whom tissue was obtained

Sample	Age (y)	Diagnosis
1	0.8	Maturation delay, intra-abdominal testis
2	1	Maturation delay, intra-abdominal testis
3	7	Intratubular germ cell neoplasia, intra-abdominal testis
4	12	Intratubular germ cell neoplasia
5	21	Intratubular germ cell neoplasia only
6	31	Intratubular germ cell neoplasia, atrophy
7	34	Intratubular germ cell neoplasia only
8	36	Intratubular germ cell neoplasia only
9	36	Intratubular germ cell neoplasia only
10	32	Intratubular germ cell neoplasia, intra-tubular seminoma
11	Adult	Seminoma
12	Adult	Seminoma
13	Adult	Seminoma
14	Adult	Seminoma
15	Adult	Seminoma
16	Adult	Seminoma
17	Adult	Seminoma
18	Adult	Seminoma
19	Adult	Seminoma
20	Adult	Embryonal carcinoma with teratoma
21	Adult	Mixed- embryonal carcinoma, teratoma and seminoma
22	Adult	Embryonal carcinoma with teratoma
23	Adult	Embryonal carcinoma with teratoma
24	Adult	Mixed- embryonal carcinoma, yolk sac tumour, teratoma and seminoma
25	Adult	Embryonal carcinoma with teratoma
26	Adult	Teratoma
27	Adult	Teratoma

Table 2. Antibodies and conditions for immunohistochemistry. All antibodies were raised against human peptide sequences

Antigen	Retrieval	Source	Species	Dilution	
				DAB	Fluorescence
AP-2 γ	Y	Santa Cruz ^a	Mouse	1:20	1:60
OCT 4	Y	Santa Cruz ^a	Goat	1:50	1:150
PLAP	Y	Abcam ^b	Rabbit	1:100	1:200
VASA	Y	Abcam ^b	Rabbit	1:200	1:500
MAGEA4	N	Gift ^c	Mouse	1:20	1:100
Ki67	Y	Dako ^d	Rabbit	1:40	1:100

^a Santa Cruz Biotechnology, CA, USA

^b Abcam, Cambridge, UK.

^c Dr. Giulio Spagoli, University Hospital, Basel, Switzerland

^d Dako, Ely, UK