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MicroRNAs in Differentiation of Embryoid Bodies and the Teratoma Subtype of Testicular Cancer

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Abstract. *Background: Testicular germ cell tumours (TGCTs) are the most frequent tumour type among young, adult men. TGCTs can be efficiently treated, but metastases of the teratoma subtype, for which there are no circulating biomarkers, represent a challenge. Materials and Methods: Global microRNA expression in teratoma tissue and embryoid bodies was assessed using next-generation sequencing. Levels of microRNAs identified as potential biomarkers were obtained from serum of patients with teratoma and matched healthy men. Results: We identified miR-222-5p, miR-200a-5p, miR-196b-3p and miR-454-5p as biomarker candidates from the tumour tissue and embryoid body screening but the expression of these microRNAs was very low in serum and not statistically different between patients and controls. miR-375-3p was highly expressed, being highest in patients with teratoma (p=0.012) but the levels of expression in serum from these patients and healthy controls overlapped. miR-371a-3p was not expressed in serum from patients with pure teratoma, only in patients with mixed tumours. Conclusion: The microRNA profiles of the teratoma subtype of TGCT and embryoid bodies were obtained and assessed for candidate circulating biomarkers, but none with high sensitivity and specificity for teratoma were identified in our study. We conclude that neither the proposed teratoma marker miR-375-3p nor miR-371a-3p are suitable as circulating teratoma markers.*

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Testicular germ cell tumours (TGCT) are rare and account for less than 1% of all male cancer but represent the most common malignancy among young men between 15-40 years (1). The incidence of TGCT has been rising and is highest in men of northern European ancestry (2). Known main risk factors for TGCT are cryptorchism, prior diagnosis, increased adult height and a familial history of TGCT (3, 4). Gain of chromosome 12p and aneuploidy are found in nearly all cases (5) and more than 40 susceptibility loci with moderate risk have been identified (6, 7). These include *KIT* and *KRAS*, but with no major high-risk genes (8-10). Although genetic risk factors are observed, the rapid increase in TGCT incidence seen in Western countries since the 1950s cannot be explained by genetic factors alone. A gene–environmental model has been proposed where genetic factors work in concert with environmental and lifestyle factors (11). The associations of TGCT with environmental/lifestyle factors are debated and are generally considered weak, but include, among others, dietary intake, physical exercise, birth weight, exposure to hormones *in utero*, maternal smoking, parity and reduced gestational age (12).

The majority of TGCTs are thought to arise from intratubular, incompletely differentiated embryonal cells where a failure in control of their latent developmental potential and reprogramming gives raise to germ cell neoplasia *in situ* (13, 14). According to the World Health Organization's 2016 classification of testicular germ cell tumours, germ cell neoplasia *in situ*-derived tumours are further classified into seminomas, embryonal carcinoma (EC), yolk sac tumour, trophoblastic tumours (*e.g.*, choriocarcinoma) and post-pubertal teratoma (15). Seminomas and EC are both undifferentiated,

Table I. *Samples included in the tissue screen for microRNA in teratomas.*

Patient	Primary diagnosis	Sample	Tissue extracted at	Tissue extracted*
ID1	100% Teratoma, extragonadal CS4	1-1 Mediastinum	Surgery after CHT	Teratoma
		1-2 Lung	Surgery after CHT	Teratoma
ID2	99% Teratoma + 1% Sem, CS2	2-1 Testis	Orch prior to CHT	Teratoma
		2-2 Testis	Orch prior to CHT	Normal testis
		2-3 Lymph node, RPLND	pcRPLND	Teratoma
		2-4 Lymph node, RPLND	pcRPLND	Teratoma
ID3	50% Teratoma + 50% EC, CS1	3-1 Testis	Orch prior to CHT	Teratoma
		3-2 Testis	Orch prior to CHT	Normal testis
ID4	20% Teratoma + 79% EC, 1% YST, CS2	4-1 Testis	Orch prior to CHT	Normal testis
		4-2 Lymph node, RPLND	pcRPLND	Teratoma
		4-3 Lymph node, RPLND	pcRPLND	Teratoma
		4-4 Lymph node, RPLND	pcRPLND	Normal lymph node
ID5	20% Teratoma + 70% Sem, 10% EC, CS1	5-1 Testis	Orch prior to CHT	Teratoma
		5-2 Testis	Orch prior to CHT	Teratoma
		5-3 Testis	Orch prior to CHT	Normal testis
ID6	50%+ Teratoma 40% EC + 5% YST+ 5% Sem, CS1	6-1 Testis	Orch prior to CHT	Teratoma
ID7	65% Teratoma, 25% EC,	7-1 Lymph node, RPLND	pcRPLND	Normal lymph node
	8% Sem and 2% Chorio, CS2	7-2 Lymph node, RPLND	pcRPLND	Teratoma
		7-3 Lymph node, RPLND	pcRPLND	Teratoma

CHT: Chemotherapy; CS: clinical stage; Chorio: choriocarcinoma; EC: embryonal carcinoma; Orch: orchiectomy; pcRPLND: post-chemotherapy retroperitoneal lymph node dissection; Sem: seminoma; YST: yolk sac tumour. When two or more samples were extracted from the same sample location, they were spatially separated. *All tissue samples were carefully extracted from areas with pure teratoma or normal tissue.

where EC is pluripotent and the origin of the diverse group of non-seminomas. In oncological treatment, a distinction is made between seminoma and non-seminoma histological types, with seminomas being characterised by higher mean age at diagnosis and better prognosis. All mixed tumours are defined as non-seminomas due to their more aggressive nature and poorer prognosis. The teratoma subtype is the most highly differentiated type of the non-seminomas. In contrast to the prepubertal-type, the postpubertal-type teratomas grow invasively and can metastasise (16-19). The histology of teratomas may show elements of all three germ layers, with the originating germ cell layer differentiated into somatic tissue (20). The presence of a mature teratoma component in the primary tumour has been associated with both early and late mortality from TGCT (21, 22). Furthermore, one study found a high frequency of yolk sac tumours in late relapses and hypothesised that the yolk sac tumours may have developed from residual teratoma (23). Recently, it was shown through genetic analyses of teratomas and adjacent somatic transformed tumour cells that it is more likely that progenitor cells in teratomas differentiate into diverse cellular lineages rather than progeny cells dedifferentiating into stem-like cells through genetic mutations (24).

Teratomas with their features of highly differentiated somatic tissue have been linked to cisplatin resistance (25-28). Slightly enlarged lymph nodes that do not shrink as expected following chemotherapy represent a challenge in the management of TGCT and surgical removal is the only available treatment (29). The question is usually whether the mass visible at images represents teratoma, viable tumour or non-viable deposits. The classical markers in TGCT, namely human choriogonadotropin type beta (hCG_{β}) and alpha-fetoprotein (AFP) are not expressed by teratomas. The teratomas are not reactive in fluorodeoxyglucose positron-emission tomography (30). Circulating miR-371a-3p has been shown to be highly sensitive in detecting TGCT cells except for the teratoma sub-type (31). No biomarker for teratoma has been identified so far. miR-375-3p has been proposed as a teratoma marker (32, 33) but the clinical utility of this microRNA as a circulating biomarker is debated (34-36). Thus, research for new circulating markers for teratoma is highly warranted.

Grown in suspension culture, human embryonic stem cells (hESCs) and human induced pluripotent stem cell (hiPSCs) both have the ability to spontaneously form three-dimensional aggregates of differentiated embryoid bodies (EBs) comprising all the three embryonic germ layers (37). EBs thus serve as a model of human embryos to study early differentiation events (38). In fact, the first experiment to demonstrate the pluripotency of embryonal carcinoma cells showed that single cells from EBs were able to generate tumours containing embryonal carcinoma cells and somatic tissues in a murine model (39). Individual EC cells were shown to be able to differentiate into well-differentiated, adult-appearing somatic tissue, representing all three germ layers. Today, the formation of spontaneously or (chemically)

Table II. *Characteristics of patients included in the serum microRNA analyses.*

Patient	Primary diagnosis	Stage	Histological diagnosis at time of serum sample	Sample timepoint	FFPE screen	Serum RT-qPCR screen	Serum RT-qPCR panel
ID1	100% Teratoma, mediastinum	CS4	-	Pre-treatment	x	x	x
ID2	99% Teratoma, 1% Sem	CS2	-	Pre-orch	x	x	x
ID3	50% Teratoma, 50% EC, necrosis	CS1	-	Pre-orch	x	x	x
ID4	Teratoma, EC and YST	CS2	100% Teratoma	Pre-RPLND	x	x	x
ID5	60% Teratoma, 40% Sem	CS2	100% Teratoma	Pre-RPLND	x	x	x
ID6	50% Teratoma, 40% EC, 5% YST, 5% Sem	CS1	-	Pre-orch	x	-	-
ID7	65% Teratoma, 25% EC, 8% Sem, 2% Chorio	CS2	100% Teratoma	Pre-RPLND	x	-	-
ID8	50% Teratoma, 50% YST	CS2	-	Pre-orch	-	x	x
ID9	55% Teratoma, 45% YST	CS1	-	Pre-orch	-	x	x
ID10	100% Teratoma	CS2	-	Pre-orch	-	x	x
ID11	100% Teratoma	CS1	-	Pre-orch	-	x	x
ID12	80% Teratoma, 20% EC	CS1	-	Pre-orch	-	x	x
ID13	EC, focal YST	CS3	100% Teratoma	Pre-RPLND	-	x	x
ID14	80% EC, 15% teratoma, 5% chorio	CS2	100% Teratoma	Pre-RPLND	-	x	x
ID15	Sem, EC, YST and teratoma	CS2	100% Teratoma	Pre-RPLND	-	x	x
ID16	EC + GCNIS	CS2	100% Teratoma	Pre-RPLND	-	x	x
ID17	100% Teratoma	CS2	100% Teratoma	Pre-RPLND	-	-	x
ID18	100% Teratoma, extragonadal	CS4	100% Teratoma	Pre-RPLND	-	-	x

Chorio: Choriocarcinoma; CS: clinical stage; EC: embryonal carcinoma; FFPE: formalin-fixed paraffin-embedded tissue; GCNIS: germ cell neoplasia *in situ*; Pre-orch; sample drawn before orchiectomy or start of treatment; Pre-RPLND; sample drawn <48 h prior to RPLND; Pre-treatment; sample drawn prior to start of any treatment; RPLND: retroperitoneal lymph node dissection; RT-qPCR: reverse transcriptase–quantitative polymerase chain reaction; Sem: seminoma; YST: yolk sac tumour. Patients ID6 and ID7 did not have serum samples available for the timepoints in question.

induced EBs in suspension culture is used to demonstrate pluripotency of stem cells [reviewed in (40)], as an alternative to testing teratoma formation capability after injection into the neck of mice.

In the present study, we aimed to identify one or several microRNAs as potential novel, clinical biomarkers for teratoma using EBs as a model, due to the histological similarity between teratomas and EBs and the possibility to study EBs in a time-course manner including early phases during their development from pluripotent stem cells. Specifically, we aimed to correlate microRNA expression in evolving EBs to their expression in teratoma tissue and sera from patients with teratoma in order to identify novel disease-specific microRNA markers.

Materials and Methods

Formation of EBs. In addition to the hESC line H1, pluripotent stem cells were obtained from fibroblasts from two healthy control donors (named N26 and N904), and reprogramming of fibroblasts into induced pluripotent stem cells was performed, as previously described (41), by episomal reprogramming. EBs were aggregated from stem cells by following the instruction of the AggreWell™ 800 starter kit (Stem Cell Technologies, Vancouver, Canada). Cells were harvested with gentle dissociation reagent (Stem Cell Technologies) and resuspended in EB formation medium (Stem Cell Technologies) with 10 μM Y-27632. In each well, 1.2 million cells were plated and incubated for 24 h in a cell incubator. The EBs were harvested and spontaneous differentiation was induced by culturing the EBs in primate ES cell media (Tebu bio, Le Perray-en-Yvelines, France) first 10 days in suspension plates (Sarstedt, Nümbrecht, Germany) followed by 30 days on plates (Sarstedt) coated with Matrigel (Fisher Scientific, Corning, NY, USA). Sampling was performed at days 0, 8, 20 and 30 of the spontaneous differentiation.

Upon harvesting, the EBs were disrupted and homogenized in 600 μl Buffer RLT Plus (Qiagen, Venlo, the Netherlands) and homogenized using a TissueLyzer LT (Qiagen) at 30 Hz for 2 min before exaction of total RNA using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen) according to the manufacturer's instructions. An on-column DNase-treatment step was included in the protocol. RNA was eluted in 30 μl nuclease-free water.

Tissue and serum samples. Patients with at least 50% teratoma in the primary tumour or metastases were identified among patients included in the SWENOTECA MIR study (ClinicalTrials.gov Identifier: NCT04914026). Archival paraffin tissue was retrieved from the Department of Pathology, Haukeland University Hospital (Table I), while blood samples were drawn in the SWENOTECA MIR study (Table II), before orchiectomy or before post chemotherapy retroperitoneal lymph node dissection (pcRPLND).

As a control group for serum analyses, we used serum from 30 healthy, age-matched male blood donors (42). We also downloaded datasets with global circulating microRNA expression data from healthy individuals in order to filter out microRNAs that are expected to be highly expressed in serum from healthy individuals (43, 44).

Table III. *List of miRCURY LNA miRNA polymerase chain reaction assays used for screening of serum samples.*

Target RNA	GeneGlobe Cat. No.
hsa-miR-196b-3p	YP00206018
hsa-miR-455-3p	YP00204035
hsa-miR-181a-2-3p	YP00204142
hsa-miR-301b-3p	YP00204390
hsa-miR-1226-3p	YP02102736
hsa-miR-205-5p	YP00204487
hsa-miR-205-3p	YP00205602
hsa-miR-490-5p	YP00206077
hsa-miR-490-3p	YP00205999
hsa-miR-133b	YP00206058
hsa-miR-221-5p	YP00204032
hsa-miR-452-5p	YP00204301
hsa-miR-224-5p	YP00204641
hsa-miR-320d	YP00205667
hsa-miR-200c-5p	YP02119294
hsa-miR-429	YP00205901
hsa-miR-141-5p	YP00206088
hsa-miR-934	YP02119292
hsa-miR-223-5p	YP00204529
hsa-miR-27a-5p	YP00206021
hsa-miR-148a-3p	YP00205867
hsa-miR-326	YP00204512
hsa-miR-301a-3p	YP00205601
hsa-miR-492	YP00204053
hsa-miR-421	YP00204603
hsa-miR-520a-3p	YP00204074
hsa-miR-26b-3p	YP00204117
hsa-miR-193a-5p	YP00204665
hsa-miR-6510-3p	YP02105880
hsa-miR-196a-5p	YP00204386
hsa-miR-365b-5p	YP00204654
hsa-miR-222-5p	YP00204314
hsa-miR-200a-5p	YP00206063
hsa-miR-200b-5p	YP00204144
hsa-miR-130b-3p	YP00204317
hsa-miR-133b	YP00206058
hsa-miR-181d-5p	YP00204789
hsa-miR-203a-3p	YP00205914
hsa-miR-21-3p	YP00204302
hsa-miR-425-5p	YP00204337
hsa-miR-23a-3p	YP00204772
hsa-miR-451a	YP02119305
hsa-miR-191-5p	YP00204306
hsa-miR-30b-5p	YP00204765

RNA extraction from formalin-fixed paraffin-embedded (FFPE) tissue. Tumour areas containing pure teratoma were identified by an experienced uropathologist (OJH). Two to four 1.0 mm diameter tissue cores were extracted from the paraffin blocks guided by encircled teratoma areas from corresponding hematoxylin and eosin-stained slides. The FFPE tissue cores were disrupted in 600 μl Deparaffinization Solution using Tissue Ruptor with disposable probes (all from Qiagen). RNA was further extracted according to the manufacturer's protocol for purification of total RNA including small RNAs using an AllPrep DNA/RNA FFPE kit (Qiagen), on-column DNase treatment was performed, and RNA was eluted in 30 μl nuclease-free water.

Blood sampling and RNA extraction from serum. Blood was collected by standard venipuncture technique into 10-ml Vacutainer tubes with clot activator (BD, Franklin Lakes, NJ, USA). The samples were allowed to clot for 1 h prior to centrifugation at 2,000 × *g* for 10 min. Serum was carefully removed, aliquoted and frozen immediately at −80°C.

Prior to RNA extraction, serum was thawed, and haemolysis was assessed spectrophotometrically at 414 nm for all serum samples (45). Total RNA including small RNAs was extracted from 200 μl serum using miRNEasy (Qiagen) and the supplementary protocol RY43 (Version Feb-11). Briefly, five volumes of Qiazol Lysis Reagent were added and vortexed for homogenization. Prior to addition of 1 volume of chloroform and centrifugation, 3 μg glycogen (Thermo Fisher Scientific, Waltham, MA, USA) were added as carrier. The upper phase was carefully transferred to a new tube where 1.5 volumes of 96% ethanol was added, and column purification performed. RNA was eluted in 30 μl nuclease-free water.

miRNA profiling by next-generation sequencing, from FFPE tissue and EBs. The RNA samples were submitted to Qiagen's next-generation sequencing service for microRNA profiling. Briefly, libraries were prepared using a QIAseq miRNA Library Prep kit (Qiagen) from 100 ng total RNA. Adaptors containing unique molecular markers were ligated to the RNA before conversion into cDNA, PCR amplification and sequencing using a NextSeq500 instrument with an average number of reads of 75 million reads/sample. Raw data were de-multiplexed and FASTQ files generated using bcl2fastq software (Illumina Inc., San Diego, CA, USA). Reads were mapped and annotated using Bowtie2 v 2.2.2 (46) with GRCh37 as the reference genome and the miRbase 20 as annotation reference. Normalized counts were obtained using edgeR (47). The microRNA profiling results were analysed and visualised using the software Qlucore Omics Explorer version 3.6 (Qlucore, Lund, Sweden). Dimensionality reduction by principal component analysis (PCA) was used to identify expression patterns. Thresholds for the identification of differentially expressed microRNAs were absolute fold change >2.0 and false-discovery rate <0.05.

Target identification and Gene Ontology (GO) analysis. The list of microRNAs found to be up-regulated and down-regulated in EBs were loaded into the open source software platform Cytoscape (version 3.8.2, cytoscape.org) (48) with the ClueGO app (v2.5.7) (49) installed to identify biological processes associated with the targets of the microRNAs found to be up- and down-regulated at day 30 vs. day 0.

Panel screening of serum microRNAs by reverse transcriptase–quantitative polymerase chain reaction (RT-qPCR). RNA from the serum samples of seven patients with teratoma in the primary tumour and nine patients in whom teratoma was found in lymph nodes at pcRPLND in addition to 10 healthy controls (Table II) were submitted to RT-qPCR screening using a custom-made panel consisting of 39 microRNAs (See Table III) with hsa-miR-30b-5p and hsa-miR-191-5p as endogenous controls. RNA, 2 μl, was transcribed to cDNA in 10-μl reactions using a miRCURY LNA RT Kit (Qiagen). qPCR was performed with 45 cycles using a

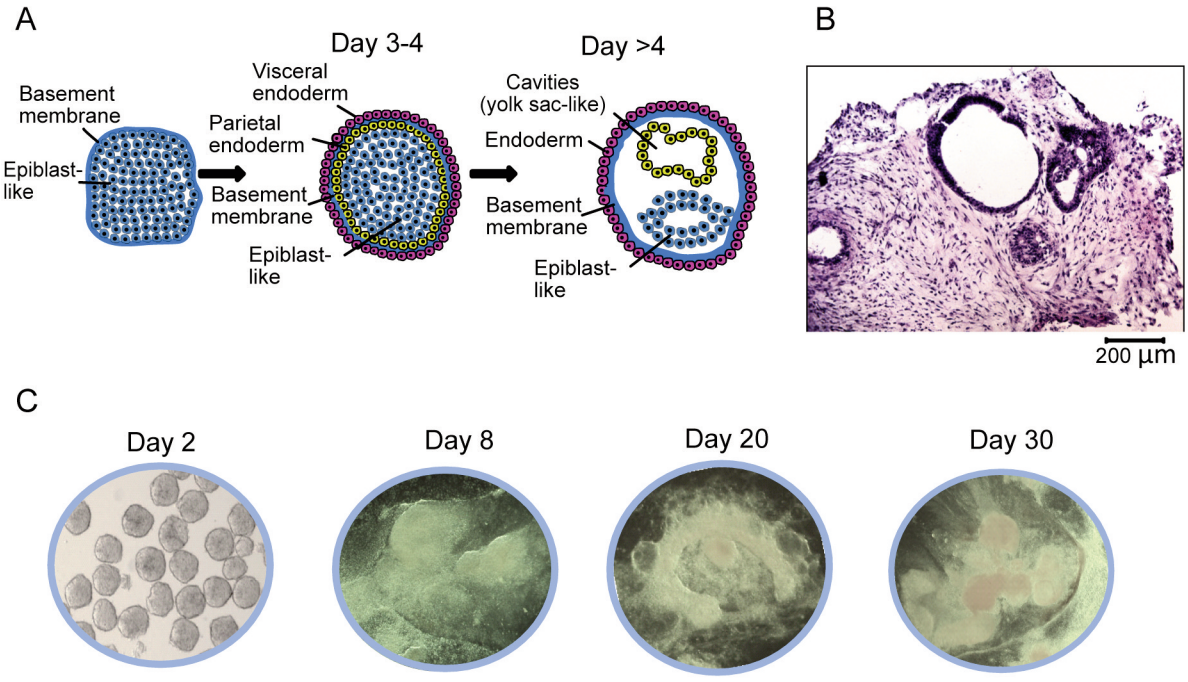


Figure 1. *Structural development of embryoid bodies (EBs) from human induced pluripotent stem cells. A: Schematic showing the growth of cells from simple spherical aggregates to cystic EBs with one or more cavities. B: Cryosectioned and hematoxylin and eosin-stained EBs with cavities. C: Images of EB morphology at the timepoints of sampling for microRNA analysis, showing simple spheres at day 2, aggregated spheres at day 8 and tissue-like structures at days 20 and 30.*

LightCycler 480 Real-Time PCR System (Roche, Basel, Switzerland) in 384-well plates. Haemolysis was assessed using the hsa-miR-23a-3p/hsa-miR-451a ratio (50).

RT-qPCR with pre-amp using TaqMan hydrolysis probes. MicroRNAs with positive amplification signal for serum from at least one patient with teratoma and no signal in the samples from the healthy donors in the panel screen were verified in a larger cohort consisting of seven patients with >50% teratoma in the primary tumour, nine with viable teratoma in one or more lymph nodes at pcRPLND and 30 healthy males. cDNA was synthesised using a TaqMan MicroRNA Transcription Kit (Thermo Fisher Scientific) as recommended by the manufacturer. qPCR with pre-amplification was performed with a pool of the 20X TaqMan microRNA Assays TaqMan assays (See Supplementary Table I for assay list and protocol). Relative quantification was performed using miR-30b-5p as endogenous control, $\Delta Cq = Cq_{\text{target microRNA}} - Cq_{miR\text{-}30b\text{-}5p}$. The relative quantities are given as $2^{-\Delta Cq}$.

Ethics statement. The patient samples analysed were included through the SWENOETCA-MIR study which was approved by the Regional Ethics Committee (REC Central Norway 2015/1475) and Regional Ethics Committee (REC Stockholm 2018/1730-31). Written, informed consent was obtained from all patients at inclusion, in accordance with the Declaration of Helsinki. We reprogrammed fibroblast cells from two donors from the Norwegian MODY Registry after written informed consent and with experiments approved by the Regional Committee of Medical Ethics (REK 2010/2295).

Statistical analysis. Expression levels in two groups were compared using the Mann–Whitney non-parametric *t*-test. All *p*-values were two-tailed and considered statistically significant when $p<0.05$. Statistical analyses and plots were generated using Graph Pad Prism 9 (Graph Pad Software LLC, La Jolla, CA, USA) and figures were prepared using Affinity Designer (Serif Software Ltd, Nottingham, UK).

Results

MicroRNA expression in EBs. To identify potential novel teratoma biomarkers, we studied the microRNA profile at several timepoints during differentiation of hESCs and hiPSCs into EBs. Firstly, we developed EBs during a 30-day protocol and assessed a time-course microRNA expression profile to both validate the model and to allow subsequent correlative studies with microRNA gene expression in teratoma tissue and sera from patients with teratoma. We used two hiPSC lines from two healthy donors (N26 and N904), and the H1 hESC line to generate EBs. The EBs were grown as described from single cells into three-dimensional tissue-resembling cultures (Figure 1) containing ectodermal, mesodermal and endodermal germ components. We harvested cells for microRNA expression analysis on days 0, 8, 20 and 30. After microRNA sequencing, very low-abundant microRNAs were filtered out leaving 872 microRNAs in the dataset. We used differential expression

analysis comparing days 8, 20 and 30 to baseline day 0 to characterise the microRNA expression in the EBs at these cell harvest time points.

PCA identified a strong clustering by time points (Figure 2A) across different cell sources (H1 hESCs, hiPSCs) supporting our assumption of a predictable pace of differentiation regardless of the cell source and hence justifying our grouping of time point data at the selected time points. The PCA plot showed differences in the expression levels of microRNAs between H1 hESCs and hiPSCs at the early timepoints, but the global expression profiles converged towards the late timepoints (Figure 2A). We identified the most down-regulated (Figure 2B) and the most up-regulated (Figure 2C) microRNAs in the samples from day 30 compared to day 0 and day 8. As expected, expression of miRNA pluripotency markers, the miR-302/367 and miR-520 clusters, faded with differentiation from day 0 to day 30 in all three EB cultures (Supplementary Figure 1). The miR-371-373 cluster was stably expressed in all three EB cultures at all timepoints. miR-375-3p was weakly expressed at day 0 but the expression increased and remained relatively high from day 8 for the hiPSC EB cultures (N26 and N904), and from day 20 for the H1 hESC EB culture.

The microRNAs with increased expression at days 20 and 30 included miR-10b, miR-10a, miR-143, miR-145, miR-23a, miR-23b, miR-125b and miR-100, which have previously been shown to be highly expressed in differentiated hiPSCs (51, 52), validating our model. Consistent with the development of more differentiated tissue during EB development, we identified the GO terms "epithelial cell proliferation and development", "tube formation" and "mesenchymal cell differentiation" for the top down-regulated microRNAs (Supplementary Figure 2 and Supplementary Table II). The GO terms "cell differentiation", "circulatory system development", "tube morphogenesis", "odontogenesis", "embryonic morphogenesis", and "urogenital system development" and "brain development" were identified for the top up-regulated microRNAs (Supplementary Figure 3 and Supplementary Table III). Furthermore, GO terms and pathways detected include regulation of the apoptotic signalling pathway, regulation of the extracellular signal-regulated protein kinase 1 (ERK1) and ERK2 cascade, regulation of the mitogen-activated protein kinase 3 (MAPK3), erythroblastic leukemia viral oncogene (ERBB) signalling pathway, among others in the top down-regulated microRNAs and regulation of WNT-signaling pathway, immune system development, cellular response to oxidative stress, among others in the top up-regulated microRNA.

microRNA expression in teratoma tissue. RNA was extracted from FFPE tissue from 19 tissue specimens from seven patients diagnosed with teratoma. Of these, five specimens were from primary teratomas in the testes of five of the patients (Figure 3), six specimens were from teratomas in lymph node metastases of three of the patients, one specimen was from a primary teratoma in the mediastinum and one specimen was from teratoma in the lung in the same patient (See Table I for more information). The study also included RNA from normal testicular tissue from four of the patients in addition to two specimens from normal lymph nodes.

Differential expression analysis of normal testicular tissue compared to teratoma tissue showed that the normal tissue was clearly separated from teratoma tissue based on the PCA analysis of microRNA expression (Figure 4A). The microRNAs most up-regulated in teratoma versus normal testicular tissue are shown in Figure 4B (fold change >3, false-discovery rate <0.05). Figure 4C shows the microRNAs down-regulated in teratoma versus normal tissue. We next compared these disease-specific miRNAs with the miRNA expression in the EB model by overlap analysis (Figure 5), in particular the miRNAs appearing in the latest phase (day 30) of EB differentiation. Of the microRNAs up-regulated in the teratomas, 19 were also up-regulated in the EBs at day 30, among them miR-193a-3p, miR-196a-5p, miR-196b-3p, miR-223-5p and miR-490-3p and miR-490-5p. Eleven microRNAs were down-regulated in both the teratomas and the EBs (Figure 5A, Supplementary Table IV). Among these were miR-520c-3p and miR-520-3p, which are members of the primate-specific microRNA gene cluster (C19MC). Members of the miR-17-92 cluster, miR-19a-3p, miR-19-1-5p, miR-20a-5p and miR-92a-1-5p, were also down-regulated in both the differentiated EBs and teratoma FFPE. miR-454-5p and miR-363-3p were up-regulated in the teratomas, but down-regulated in the EBs at day 30. The miR-371-373 cluster, showing stable expression in the EBs (Supplementary Figure 1) and the miR-302/367 cluster, showing decreasing expression with time in the EBs, were expressed in testicular teratoma tissue and to some degree in normal testicular tissue, but not in the teratomas from the lymph node metastases (Figure 5B and Figure 6). The previously proposed teratoma marker, miR-375-3p, was expressed at higher levels in primary teratoma from the testes and teratoma metastases in lymph nodes, mediastinum and lungs than in normal tissue from testis and in normal lymph nodes (Figure 6A). The expression was, however, not statistically significant different, with overlapping confidence intervals for testicular teratoma tissue, normal testicular tissue and teratoma from lymph node metastases (Figure 6B).

microRNA screening in serum. MicroRNAs found to be up-regulated in teratoma tissue and in the differentiated EBs (Figure 5C and D) were filtered against serum microRNA profiles from healthy individuals to remove microRNAs known to be highly expressed in the serum compartment of healthy individuals. MicroRNAs reported to be up-regulated in teratoma datasets published by others were also included in the screening (53, 54) (See Supplementary Table V for full list of

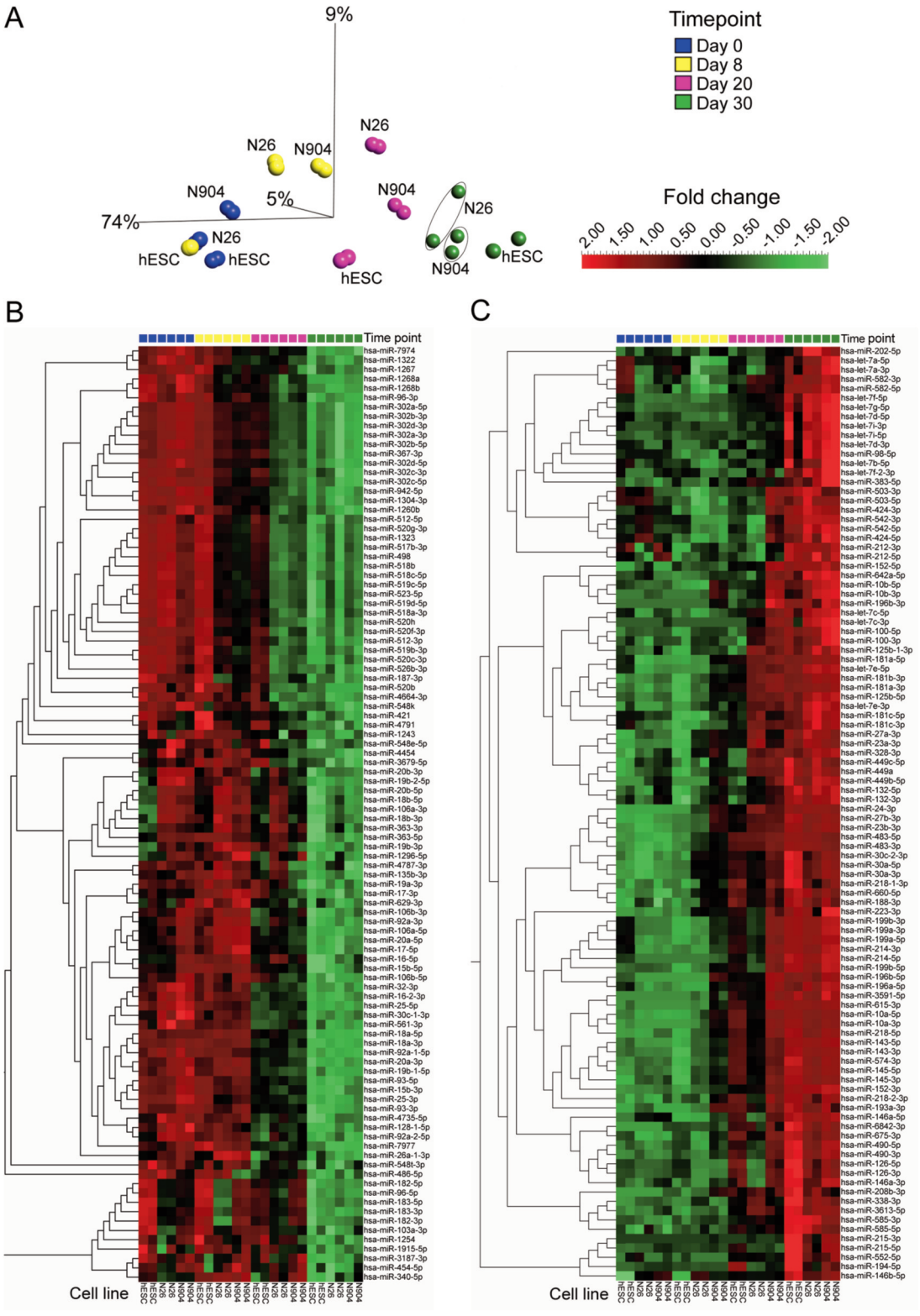


Figure 2. *MicroRNA expression in the embryoid bodies (EBs) at different timepoints. A: Principal component analysis (PCA) plot based on similarities in microRNA expression showing the clustering of samples from the two human induced pluripotent stem cell cultures (N26 and N904) and the human embryonic stem cell culture (hESC) at days 0, 8 and 20 versus day 30 (fold change >3, false-discovery rate <0.05). B and C: Heatmaps indicating the relative expression at days 0, 8, 20 and 30 for the most down-regulated and up-regulated microRNAs, respectively.*

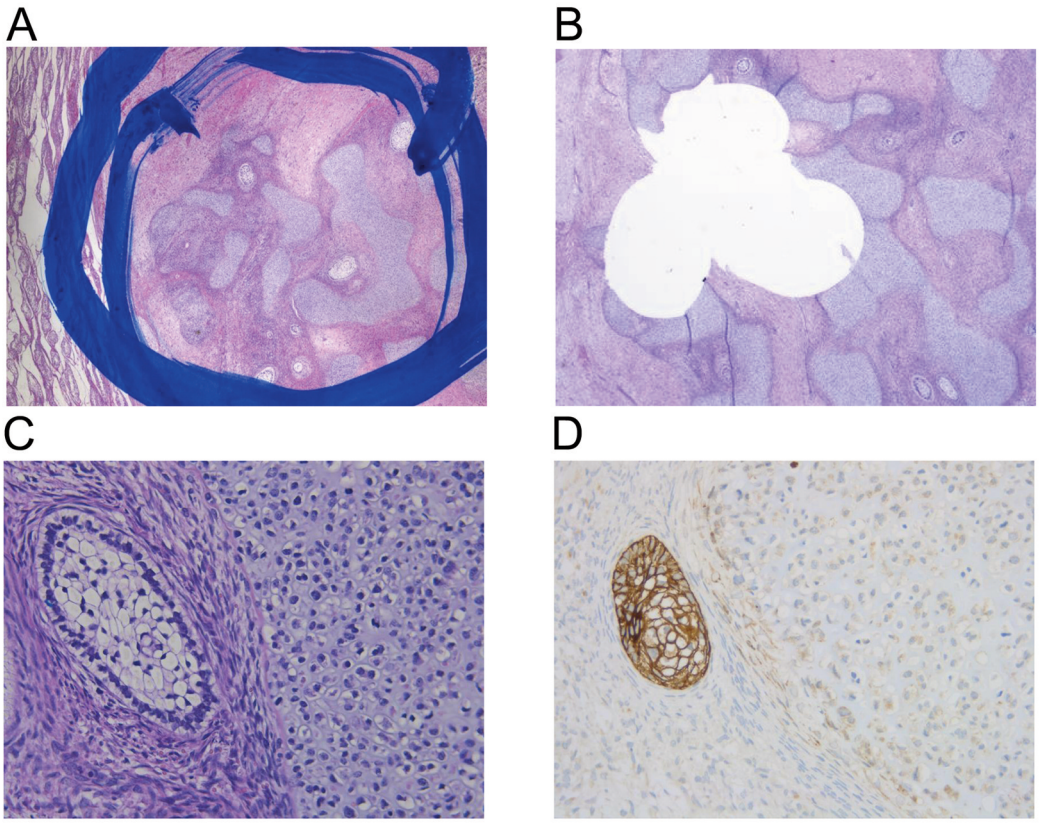


Figure 3. *Extraction of tissue cores from formalin-fixed paraffin-embedded teratoma tissue. A: Encircled area of teratoma before extraction [hematoxylin and eosin (HE)]. B: Area of teratoma after extraction of three tissue cores (HE). C: Epithelial (left) and chondroidal (right) components of teratoma. D: Immunohistochemical staining showed positivity for cytokeratin (CKAE1/AE3) in the epithelial component.*

microRNAs). The microRNAs were measured in serum samples from healthy controls and patients using a custom RT-qPCR panel for microRNA screening. The serum screen included samples from ten healthy males and 16 teratoma patients of whom seven cases had primary tumour with > 50% teratoma cells and nine cases had teratoma in lymph nodes from pcRPLND (see Table II for patient characteristics). The results from the screening showed that most of the microRNAs in our candidate panel were either highly expressed in serum from both healthy individuals and patients or not detected at all in any of them (Supplementary Table VI), hence these miRNAs were omitted from further analysis. However, miR-222-5p, miR-200a-5p, miR-454-5p and miR-196b-3p were not expressed in any of the sera from healthy donors but were weakly expressed in at least one of the teratoma patient samples and kept for further analysis. These four microRNAs were further analysed by RT-qPCR with pre-amplification in an extended set of 16 patients (Table II) and 30 healthy blood donors as controls. miR-222-5p (Figure 7A) was expressed at equal levels in sera from the patients with teratoma and the healthy controls (p=0.39), and hence is not a potential circulating biomarker. miR-200a-5p (Figure 7B) and miR-196b-3p (Figure 7C) were both expressed at low levels in serum after the pre-amplification procedure but were not statistically differentially expressed when comparing patients with teratoma and healthy controls in the extended cohort (p=0.76 and p=0.11, respectively). miR-454-5p (Figure 7D), which was up-regulated in teratoma tissue but down-regulated in the EBs at day 30, was also found to be expressed at the same low magnitude in serum from both controls and patients (p=0.53). miR-371a-3p (Figure 7E) was weakly expressed in the serum from one patient with primary tumour described as pure teratoma, in two patients where teratoma was mixed with EC, and in three patients with teratoma mixed with yolk sac tumour. None of the patients with teratoma in lymph nodes at the time of pcRPLND expressed miR-371a-3p. For miR-375-3p (Figure 7F) the mean serum expression level was statistically significant higher in patients compared to controls (p=0.012). The expression was high in serum from both patients and controls, with overlap of the 95% confidence intervals between controls (95% confidence interval=0.05-0.13) and teratoma cases (95% confidence interval=0.11-0.36).

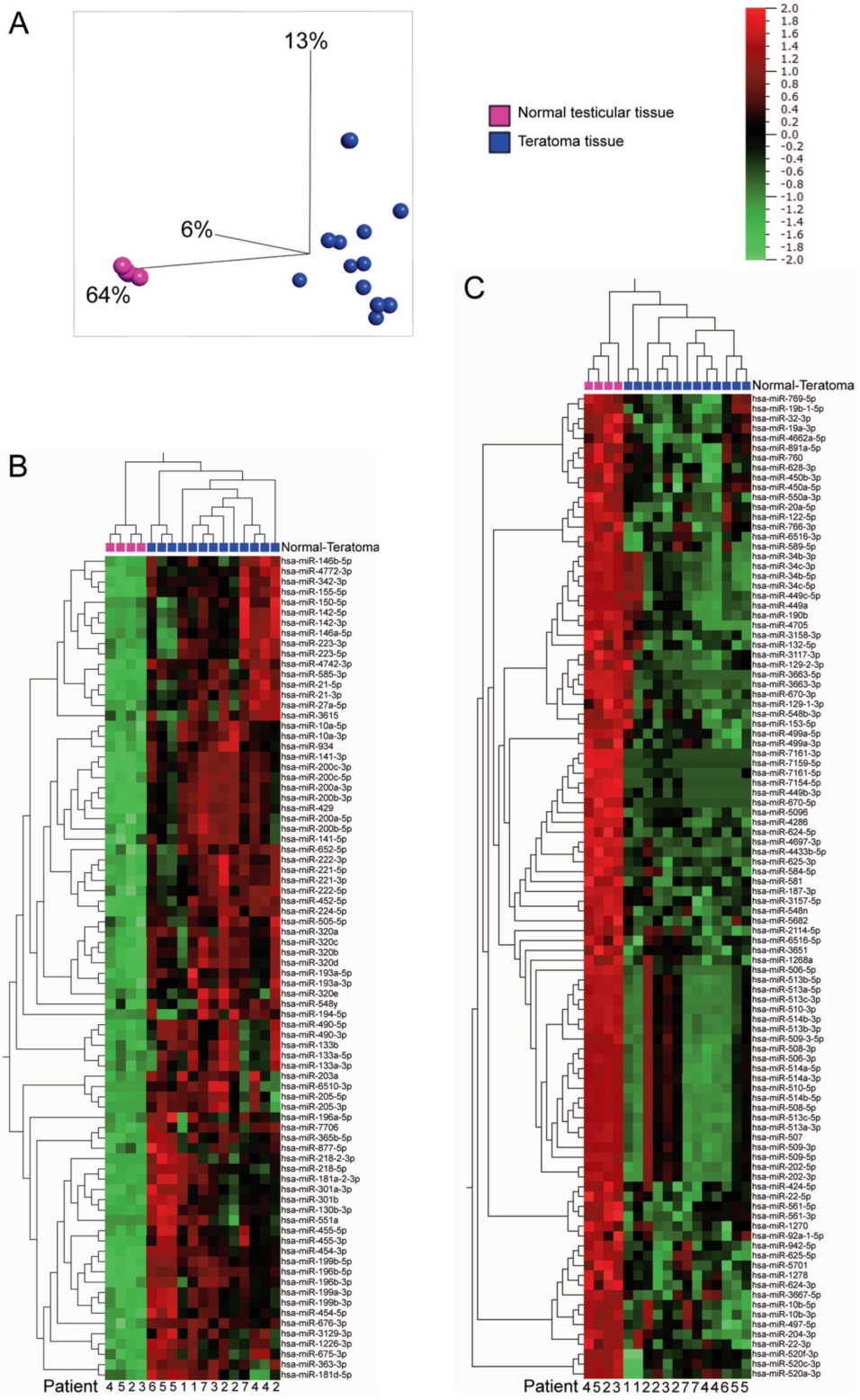


Figure 4. *MicroRNA expression in formalin-fixed paraffin-embedded samples of testicular teratoma and normal testicular tissue. A: Principal component analysis plot showing the clustering of normal testicular tissue versus teratoma tissue based on the microRNA expression (fold change >3, false-discovery rate <0.05). Heatmaps showing the microRNAs most up-regulated (B) and down-regulated (C) in teratoma tissue versus normal testicular tissue. Several patients are represented with multiple samples, as indicated at the bottom of the heatmaps.*

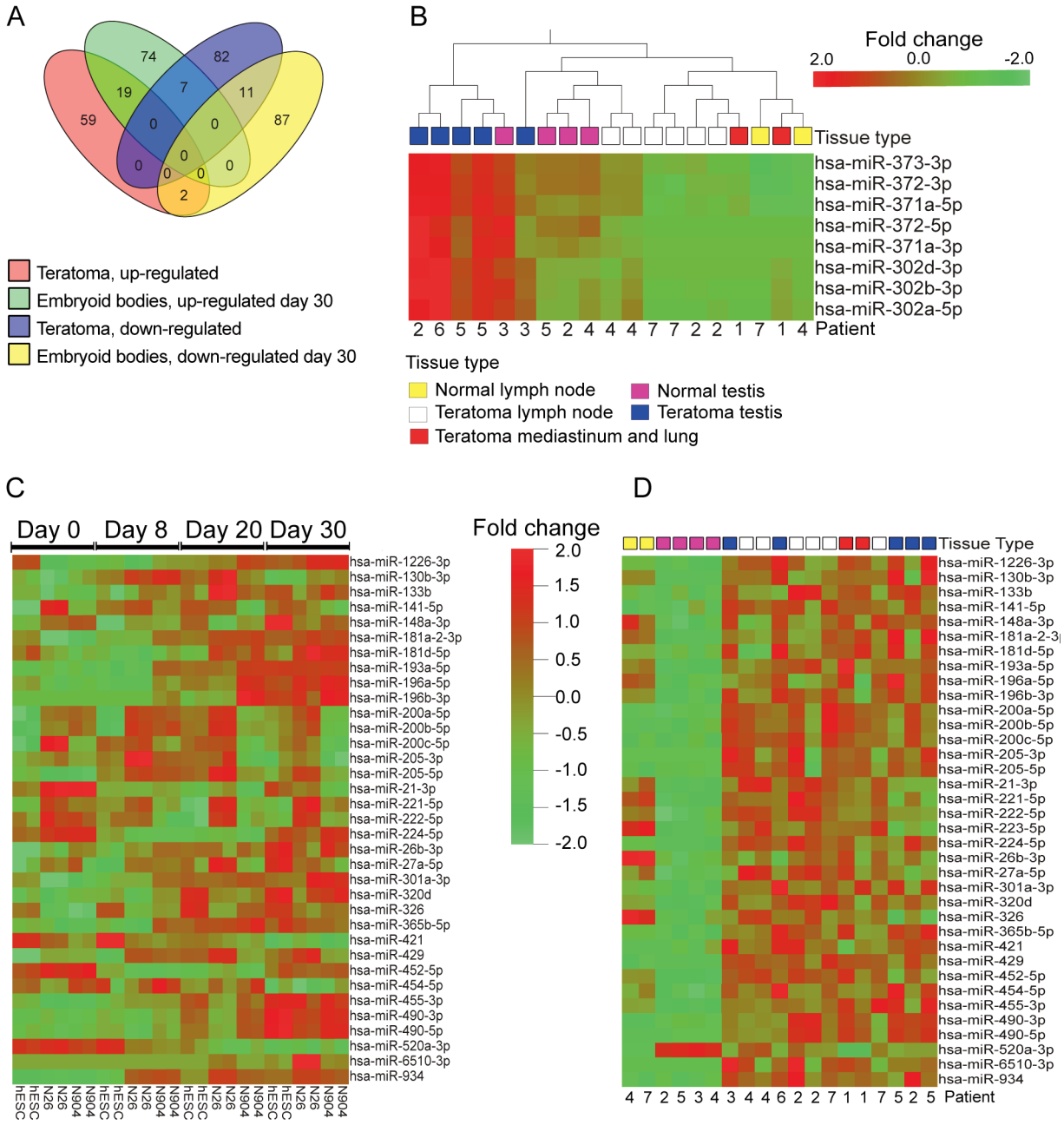
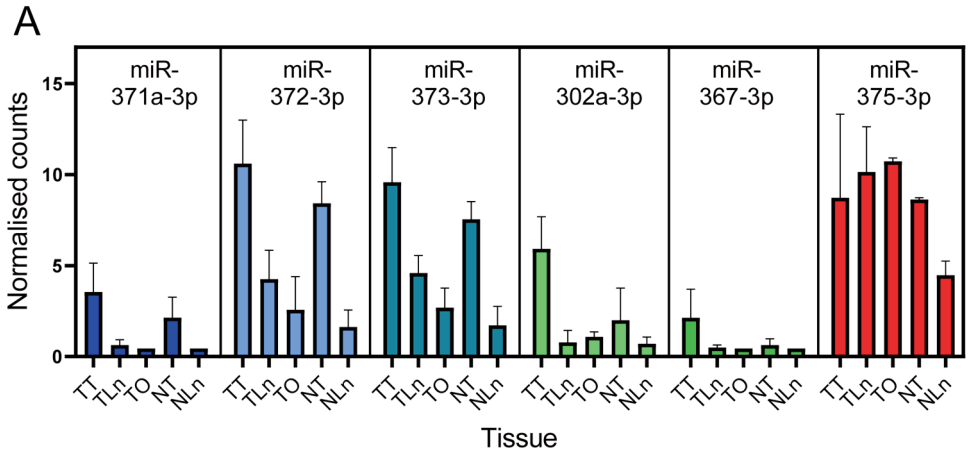


Figure 5. *MicroRNA expression in teratoma tissue compared to embryoid bodies. A: Venn diagram showing the overlap between the microRNAs up-regulated and down-regulated in teratoma tissue and embryoid bodies. B: Expression of microRNAs in the miR-302/367 and miR-371-373 clusters in tissue from teratoma and normal corresponding normal tissue. C: Expression of the microRNAs identified as the best biomarker candidates for teratoma in the embryoid body cultures at different timepoints. D: Expression of the microRNA biomarker candidates in teratoma tissue and corresponding normal tissue.*

Discussion

In the present study, we searched for microRNAs as potential novel biomarkers for the teratoma subtype of TGCT. We employed hES- and hiPSC-derived EBs to model the cellular differentiation that occurs from precursor cells to mature teratomas in TGCT. Investigating overlap with tissue and serum microRNA expression identified miR-222-5p, miR-200a-5p, miR196b-3p and miR-454-5p as potential novel candidate circulating biomarkers of the teratoma subtype of

A



B

Tissue	miR-375-3p
Teratoma, testes N=5	8.7 (3.0-14.4)
Teratoma, lymph node (N=6)	10.2 (7.6-12.8)
Teratoma, other sites (N=2)	10.7 (9.1-12.4)
Normal testicular tissue (N=4)	8.6 (8.5-8.8)
Normal lymph node (N=2)	4.5 (-2.5-11.5)

Figure 6. *A: Expression of miR-375-3p and selected microRNAs of the miR-302/367 and miR-371-373 clusters in formalin-fixed paraffin-embedded teratoma tissue from testicular tumours (TT, N=5), lymph node metastases (TLn, N=6), other metastases (TO, lung N=1, mediastinum N=1), normal testicular tissue (NT, N=4) and normal tissue from lymph nodes (NLn, N=2). Data are the mean expression with standard deviation. B: The mean normalised counts with 95% confidence intervals for miR-375-3p are stated in the table.*

TGCT but validation studies did not confirm a clinically useful role. However, the hiPSC-derived EB model system seemed robust and aligned well with previous miRNA findings, warranting further exploration of this model.

Microscopically, a testicular tissue specimen containing teratoma may appear histologically benign and contain highly differentiated mature, or embryonic immature, tissue from one or more of the three germ layers (mesoderm, ectoderm, endoderm), *e.g.*, cartilage, teeth, and hair. However, these teratomas have the ability to grow invasively and metastasise. The scientific challenge is, in a circulating biomarker test, to identify the changes that occur in teratomas that account for their malignant features. To model this process, we used the ability of human pluripotent stem cells to form differentiated EBs (37-39). We grew EBs from H1 hESCs and hiPSCs and harvested samples from the cultures at baseline, and then after 8, 20 and 30 days in order to study patterns in microRNA expression as these cells differentiated and developed into tissue bodies containing ectoderm, mesoderm and endoderm, resembling post-pubertal teratomas. Our analysis showed a differential expression of microRNAs between EBs from H1 hESCs and hiPSCs at the early timepoints but, in concordance with previously published reports, the microRNA expression pattern of H1 hESCs and hiPSCs became more similar with increasing time in culture (52). Most of the changes in microRNA expression between day 0 and day 30 EBs can be ascribed to cellular and tissue differentiation processes. The microRNAs down-regulated at day 30 were associated with positive regulation of the cell cycle, DNA damage checkpoint, and regulation of programmed cell death, while those up-regulated were associated with tissue development, cell differentiation, regulation of metabolic processes and senescence. These findings suggest that the most differentially expressed microRNAs at day 30 compared to day 0 were typical of the normal differentiation process of cells, but some of the changes may also represent invasive processes. Suh *et al*. discovered in 2004 that the miR-302/367 and miR-371-373 clusters were specific for undifferentiated hESCs and the early embryonic stage (55). A later study designed to test whether microRNAs predicted risk of post transplantation tumour in therapeutic use of hiPSCs showed that miR-371-373 and miR-302/367 clusters were not expressed in xenografts of mice injected with hiPSCs that developed into pure teratomas (teratoma assay) (56). On the other hand, xenografts that contained elements with a minor yolk sac component in addition to the teratoma did express miR-371a-3p. Our findings are in line with these

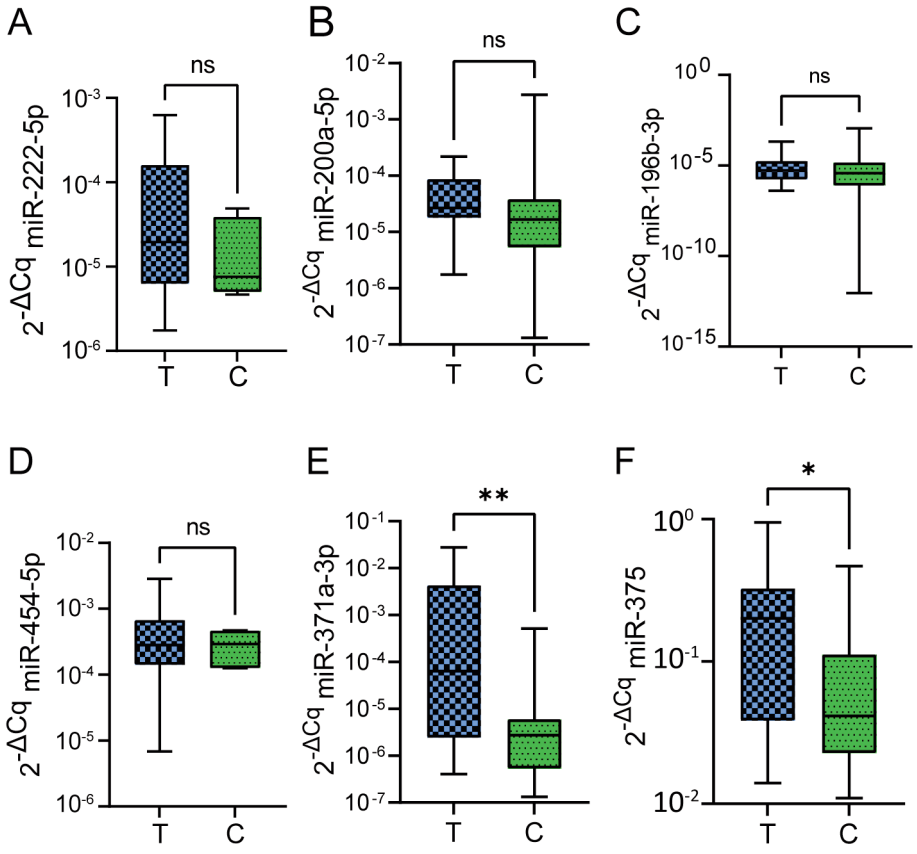


Figure 7. *Expression of miR-222-5p (A), miR-200a-5p (B), miR-196b-3p (C), miR-454-5p (D), miR-371a-3p (E) and miR-375-3p (F) in serum from patients with teratoma (T) (N=16) and healthy , male controls (C) (N=30). Mean relative quantities with 95 % confidence intervals are shown. *Significantly different at p<0,05; ns: not significantly different.*

findings. Our results show that expression of the miR-302/367 cluster is high in undifferentiated cells and diminishes with differentiation, as expected from previous published reports (55, 57, 58). The miR371-373 cluster was expressed at low levels, with no clear decrease or increase of expression during the time course of the EB culture. Members of the primate-specific C19MC, which is located in the vicinity of miR371-373 on chromosome 19, was found to be down-regulated during differentiation of hiPSC into EBs in another study (59). The C19MC has been studied in TGCT and found to represent promising circulating biomarkers in non-seminomas (60, 61). Our results from teratoma tissue indicates that this cluster was lowly expressed in teratomas, which is consistent with the results reported by Flor *et al*. (61).

From our microRNA expression studies in EBs and FFPE tissue from teratomas, we identified a list of miRNAs expressed at high levels in differentiated EBs and teratomas which we further investigated as putative circulating candidate biomarkers. Most of these microRNAs were either undetectable or not different between patients and controls in the RT-qPCR screen of serum samples. We further studied miR-222-5p, miR-454-5p, miR-196b-3p and miR-200a-5p that were identified to be expressed in serum from patients with teratoma but not in healthy controls. To this end, we used an extended cohort analysed by RT-qPCR including a pre-amplification step, which increases the sensitivity of the detection method. The results showed no statically significant difference in expression between patients with teratoma and healthy controls. These five microRNAs were generally weakly expressed and detected in few patients even with the pre-amplification step included in the RT-qPCR method. miR-371a-3p, which has been previously reported as a circulating biomarker (31, 42, 62-68) was found to be weakly expressed in serum from one patient with primary TGCT described as pure teratoma, but was not expressed in any of the serum samples collected prior to pcRPLND from patients with pure teratoma in the post-chemotherapy RPLND specimen. In tissue, our finding of a gradient in expression of miR-371a-3p from tumour, through normal testicular tissue to non-testicular

tissue is in concordance with the report from Belge *et al*. (69). miR-375-3p has been shown in a molecular characterisation of TGCT to be expressed in teratomas (10) and was later proposed as a marker for teratomas in conjunction with miR-371a-3p (32, 67). We found that the expression of miR-375-3p increased after day 8 of the EB cultures. In tissue, we did not find statistically significantly differences in miR-375-3p expression levels in teratoma tissue from tumours localized to the testes, compared to lymph nodes or lungs/mediastinum and normal testicular tissue. Normal lymphoid tissue had lower levels of miR-375-3p than normal testicular tissue. Our sample size was too small to draw any conclusions regarding miR-375-3p expression in tissue but the results from tissue together with our finding from the EBs showing that miR-375-3p expression increased during differentiation, indicates that miR-375-3p is expressed not only in teratoma and testicular tissue, but also in differentiated, normal somatic tissues. In serum, we found the mean expression of miR-375-3p to be higher in patients with teratoma compared to healthy controls, but the 95% confidence intervals overlapped, reducing the discriminatory potential. We conclude that miR-375-3p is not a good serum marker for teratoma in our studies, consistent with other recently published studies (10, 34-36).

To conclude, we identified miR-222-5p, miR-200a-5p, miR-196b-3p and miR-454-5p as candidate biomarkers for teratoma, but further analysis in serum contradicted the discriminatory value of these microRNAs. The fact that the teratomas are often highly differentiated and have traits of normal, somatic tissues makes the search for a circulating biomarker challenging because the plasma compartment of the blood harbours a cross section of molecules shed from cells and tissues throughout the body. A microRNA not normally present in testicular tissue but highly expressed in teratomas may also be found at high levels in other normal and highly differentiated tissue and will thus not be detected above the background signal in a serum or plasma sample, as shown here for miR-375-3p.

Supplementary Material

Supplementary Figures and Tables are available at: https://1drv.ms/u/s!AnUCtk5rLUTliqF7KEg9aSDMn30H9w?e=eVXevS or upon request to the corresponding author.

Conflicts of Interest

The Authors declare no potential conflicts of interest exist.

Authors' Contributions

MPM: Conceptualization, methodology, data curation, funding acquisition, software, formal analysis and writing of the article. AMS: Methodology, laboratory analyses and preparation of the article. OJH: Histological and pathological assessment and preparation of the article. AT: Resources and preparation of the article. OD: Conceptualization, investigation, funding acquisition and preparation of the article. HR: Conceptualization, methodology, funding acquisition and writing of the article.

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