OXFORD

Unravelling the biological secrets of microchimerism by single-cell analysis

Anders Ståhlberg, Amin El-Heliebi, Peter Sedlmayr, and Thomas Kroneis

Corresponding author: Thomas Kroneis, Medical University of Graz, Institute of Cell Biology, Histology & Embryology, Neue Stiftingtalstraße 6/II, 8010 Graz, Austria. E-mail: thomas.kroneis@gu.se

Abstract

The presence of microchimeric cells is known for >100 years and well documented since decades. Earlier, microchimeric cells were mainly used for cell-based non-invasive prenatal diagnostics during early pregnancy. Microchimeric cells are also present beyond delivery and are associated to various autoimmune diseases, tissue repair, cancer and immune tolerance. All these findings were based on low complexity studies and occasionally accompanied by artefacts not allowing the biological functions of microchimerism to be determined. However, with the recent developments in single-cell analysis, new means to identify and characterize microchimeric cells are available. Cell labelling techniques in combination with single-cell analysis provide a new toolbox to decipher the biology of microchimeric cells at molecular and cellular level. In this review, we discuss how recent developments in single-cell analysis can be applied to determine the role and function of microchimeric cells.

Key words: microchimerism; single-cell analysis; RNA sequencing; next-generation techniques

Introduction

Analysis at the single cell level has been around for ages with its advent at the end of the 16th century when Hans and Zacharias Jansen invented the first compound microscope. In the second half of the 17th century, Anton van Leeuwenhoek and Robert Hooke made their discoveries of 'single-celled organisms' and cellular structures in thin slices of cork, respectively [1–3]. Cytology on smear preparation was already performed in the mid-19th century, and the cytology's analytical potential was stimulated by Joseph von Gerlach, who elaborated on differential staining [4, 5]. Cell studies were further improved by Camillo Golgi and Santiago Ramón y Cajal, who

developed silver staining as well as by Paul Mayer and Gustav Giemsa, who applied basic and acidic staining, which became a key diagnostic stain [6, 7]. At the same time, Georg Schmorl made the first observations of chimeric cells in humans. Schmorl thoroughly autopsied women who died from pre-eclampsia during pregnancy and found thrombi in their lung capillaries. These thrombi contained multinucleated cells. By similarity, he suggested that these cells were of placental origin [8, 9]. Based on this observation, Schmorl was able to reject several hypotheses of the origin of eclampsia, including being a form of uraemia, a neurological disease, or a consequence of infection. Instead, he considered eclampsia to be a systemic disease with a link to pregnancy and, especially, to the presence of

Anders Ståhlberg is a principal investigator at the Sahlgrenska Cancer Center, University of Gothenburg, Sweden and Wallenberg Centre for Molecular and Translational Medicine, University of Gothenburg, Sweden. He is experienced in tumour biology, liquid biopsies and single-cell analysis.
Amin El-Heliebi is a researcher at the Institute of Cell Biology, Histology and Embryology Medical University Graz, Austria. His research focus lies in liquid biopsies, tumour heterogeneity and single-cell analysis technologies to characterize rare cells at a genomic and transcriptomic level.
Peter Sedlmayr is an associate professor at the Institute of Cell Biology, Histology and Embryology at the University of Graz, Austria. He is experienced in reproductive immunology, maternal immune cells at the endometrium and mechanisms of foeto-maternal tolerance including aspects of microchimerism.
Thomas Kroneis is a university assistant holding a VINNOVA/Marie Curie Fellowship working at Medical University in Graz, Austria and Sahlgrenska Cancer Center in Gothenburg, Sweden, working on rare-cell analysis including circulating tumour cells and microchimeric cells.

the placenta, a transient organ. Generalizing his observations, he speculated that cell trafficking also occurred during normal pregnancies and thereby launched (micro)chimeric research. His prediction was confirmed decades later for foetal chimeric cells in maternal blood [10–13] and maternal cells in foetal blood [14–17].

In the second half of the 20th century, research focused on the bidirectional trafficking of cells such as erythrocytes, leukocytes and trophoblast cells, providing the first hints of their rare presence [18]. Erythrocytes and leukocytes were found in maternal circulation with a frequency of 1 foetal cell in 50 000 maternal red blood cells and 1 foetal cell in 1000 maternal white blood cells, respectively. Despite the analytical difficulties [19], the data were considered to be accurate. Foeto-maternal trafficking was accepted to be a common phenomenon during pregnancy with a detection rate in ~50% of the women at delivery [15]. However, whether microchimeric cells caused immunological diseases or were a consequence of immunological tolerance could not be answered.

Non-invasive prenatal diagnostic approaches

The relative low abundance of microchimeric cells in combination with unspecific detection methods posed an obvious drawback as foetal cells were considered in cell-based non-invasive prenatal diagnostics. To improve the target-to-background cell ratio, foetal cells were enriched using a combination of methods including density gradient centrifugation, fluorescence- and magnetic-activated cell sorting (FACS and MACS), filtration, laser capture microdissection or dielectrophoresis [20–23]. Processed samples represented an enriched but not pure foetal cell population. However, reducing the initial number of background cells by several orders of magnitude allowed foetal cells to be identified and studied. Foetal erythroblasts were chosen as target cells for diagnostics, as their lifespan is short. Consequently, the likelihood that these cells originate from an earlier pregnancy is minimal [24]. However, detection of foetal cells was biased against male pregnancies, as most techniques targeted the Y-chromosome including fluorescence *in situ* hybridization (FISH), primed *in situ* labelling [25] and polymerase chain reaction (PCR) not allowing for prenatal diagnosis of female pregnancies. Secondly, X- and Y-FISH probes yielded false-positive signals overestimating the presence of foetal cells [26]. Although false-positive events could be overcome by using two different Y-chromosome probes or reverse-colour XY-FISH [27–30], sample enrichment methods are at risk of target cell loss. Performing erythrocyte lysis of 3 ml of maternal blood without any further enrichment results and subsequent reverse XY-FISH results in >30 slides, each containing 10 00 000 nuclei, to be processed and analysed. However, these cumbersome analyses resulted in concordant numbers of circulating male cells ranging between one and four cells per ml of maternal blood [26, 31]. In contrast, when using foetal enrichment methods, such as MACS, the number of successfully isolated cells dropped to 3 in 573 ml of maternal blood [26, 32]. Hence, target cell recovery based on the aforementioned methods was insufficiently specific and sensitive for cell-based non-invasive prenatal diagnostics [26, 32]. Sample enrichment based on filtration by size seems to be less prone to target cell loss, as its diagnostic sensitivity and specificity were reported to be 100% in 63 pregnancies at risk of having a child affected by either cystic fibrosis or spinal muscular atrophy [33]. Parallel to cell-based non-invasive prenatal diagnostics, the analysis of circulating cell-free foetal DNA was developed and optimized for

its use in clinical applications, in a way outselling cell-based analysis for its use in prenatal diagnostics [34–36].

Established microchimerism

When extensive research was done to move cell-based non-invasive prenatal diagnostics towards clinical implementation, another striking consequence of pregnancy came into awareness. While it was discovered that most circulating foetal cells are cleared from maternal circulation within hours after delivery [37], several groups noticed that microchimeric cells persisted after delivery [38, 39]. Following these reports, foetal and maternal microchimerism was detected across all human and murine organs [40, 41]. How could these cells survive in an immune-challenging environment and what did their presence mean to human life? Early findings linked the presence of microchimeric cells to immunological tolerance [42, 43]. As the transplacental passage of cells is bidirectional, the immune system of both the mother and the foetus may be challenged. It was noticed that only every fifth woman pregnant for their first time produced antibodies directed against foetal-specific human leukocyte antigens (HLAs), although 95% of them differ in HLA loci compared with their foetuses [18]. It is known that the foetal immune system tolerates maternal microchimeric cells: Rhesus-negative mothers of Rhesus-positive babies are less likely to form anti-Rh-antibodies if their own mothers have been Rh-positive [44]. Multiply transfused, highly sensitized patients awaiting renal transplantation frequently fail to make antibodies against the non-inherited HLAs of their mothers (non-inherited maternal antigens, NIMAs) [45]. Graft survival is higher in recipients of kidneys from siblings expressing NIMA than in recipients of kidneys from siblings expressing non-inherited paternal antigens [46]. Breastfeeding contributes to the tolerance of NIMA, exemplified by improved outcome of allogeneic bone marrow transplantation in mice because of a breastfeeding-induced tolerogenic effect depending on regulatory T cells [47]. However, the consequence of the presence of microchimeric cells appears to be janiform. While on the one hand microchimeric cells are able to induce tolerance to antigens shared with the microchimeric cells, on the other hand, they also may cause sensitization leading to graft rejection [48].

Maternal and foetal microchimerism is associated with autoimmune diseases [49], such as systemic sclerosis [50], rheumatoid arthritis [51], Hashimoto's disease [52], Graves' disease [53] and type 1 diabetes mellitus [54]. Beyond that, microchimeric cells have been reported to contribute to tissue repair and regeneration [55] as well as to cancer [56]. Autoimmune diseases were initially thought to be caused by chimeric maternal T lymphocytes that trigger chronic inflammation in a manner similar to graft versus host disease. This hypothesis was recently modified [57]. Recent data suggest that initial host tolerance takes place *in utero* when regulatory T cells, which respond to maternal antigens, are induced and start producing anti-inflammatory response. In contrast, chronic inflammation may occur through host T-cell activation in response to maternal antigens within tissues: injury or infection may lead to proliferation of microchimeric cells present in the affected tissue. Consequently, the number of maternal HLA increases, too. Loss of tolerance would then result from maternal HLA exceeding T-cell activation threshold.

In cancer, microchimeric cells are considered a cell type with progenitor-like properties that may differentiate into target tissue-specific cell types. Reported data indicate that

microchimeric cells differentiating along the haematopoietic lineage act in a tumour destructive manner, whereas epithelial derivatives probably are engaged in tissue repair [56, 58]. Furthermore, pregnancy is associated with a long-term protective effect in breast cancer. However, after giving birth, there is also a transient increased risk of breast cancer [59]. This double-edged effect is reported for breast, cervical and colon cancer [60, 61], and to date, it is not clear what changes drive microchimeric cells to act protectively or destructively [56, 58].

Characterizing microchimeric cells

Non-invasive prenatal diagnostics were restricted to enriched foetal erythroblasts and trophoblasts with downstream genetic analysis of the nuclei. Thus, most studies and methods focusing on microchimerism were based on a diagnostic point of view. None of the methods used for diagnostics represented a holistic approach trying to analyse all aspects of microchimerism as a phenomenon. In principle, detection of microchimeric cells can be accessed in two ways. In samples obtained from individuals with established microchimerism, detection methods must be based on genomic differences, allowing discrimination between host and microchimeric source. Currently, the portfolio of these markers include genetic differences such as the Y-chromosome (FISH and PCR) in sex-mismatched samples [62–65], short tandem repeats (STRs) [66, 67], mismatched HLA loci, single-nucleotide alterations (SNAs), copy number alterations (CNAs) and (in/del) mutations (reviewed in [68] and [69–72]). In patients receiving stem cells Schumm *et al.* [73] used a flow cytometry to monitor chimerism and minimal residual disease levels based on HLA mismatches. Similarly, Drabbels and colleagues improved an HLA-based approach to separate microchimeric from background cells [74, 75]. They manufactured a series of antibodies directed against HLA antigens. Testing artificial spikings of maternal and foetal cells, they separated microchimeric cells present a 0.01%. Using two HLA-antibodies, one specific for the foetal cells and the other specific for the maternal background cells proved to be more sensitive than using only antibody against foetal-specific HLA. With their panel of eight different HLA-specific antibodies, they calculated to cover >90% of the foeto-maternal HLA-mismatches present in Caucasians [74]. This approach may become useful to address basic questions about microchimerism, targeting most types of microchimeric cells. Trophoblast cells do not express the classical major histocompatibility complex molecules, i.e. HLA-A and HLA-B. Thus, the panel need to include HLA-C antibodies [76].

In animal experiments, fluorescently labelled offspring can be generated by mating mouse reporter strains giving rise to immunofluorescence (IF)-positive, foetal microchimeric cells [77–83]. In these mice, reporter genes such as green fluorescent protein (GFP) will be constitutively expressed allowing microchimeric foetal cells to be detected without further staining throughout and after pregnancy [80, 84]. The GFP signal can be used for enrichment [66] and combined with additional microchimerism- and cell-type-specific labelling, allowing specific contextual analysis within the microenvironment [85]. In addition, GFP-positive cells allow analysis by means of flow cytometry from blood or single-cell suspensions obtained from organs [80]. In mice, the detection of transgenic GFP can be accompanied by targeting additional mismatched markers such as CD45.1 and H-2 D reducing the false-positive rate of microchimeric cell detection [86, 87]. Cell-type independent isolation of microchimeric cells from the background cells seems to be feasible but could be challenging in embryonic

stem cells and multipotent adult progenitor cells, which show only weak expression of HLAs [88–90]. Nonetheless, isolation of microchimeric cells with stem cell-like properties will be essential as current hypothesis suggests that establishment of microchimerism is based on the exchange of cells with stem cell properties [85, 91–95].

The origin of microchimeric cells

Today, there is an ongoing search for the source of cells responsible for lifelong microchimerism. Microchimeric cells represent derivatives of multi-lineage origin; therefore, it was hypothesized that seeding microchimeric cells are stem cells or progenitor cells that exhibit stem cell properties [94, 96, 97]. It is believed that these cells manage to cross the foeto-maternal interface if it is dysfunctional or injured [98]. Thus, termination of pregnancy or miscarriage enables direct transfer of the cells [38, 99–101]. Differentiated microchimeric cells were found to be derived from the mesodermal [94], ectodermal [41, 79, 102] and endodermal [91, 103, 104] lineage. Although all these cells originate from the epiblast, it cannot be ruled out that microchimerism establishes from placental cells. Of all cell types containing the foetal genome, trophoblast cells are the only ones being in direct contact with maternal blood and tissues. Extravillous trophoblast cells may even escape the foetal tissues remodelling vascular endothelium [105]. In addition, cells of the extravillous trophoblast express markers specific for trophoblast stem cells [106]. Apart from their structural integration into maternal arteries, trophoblast cells were also isolated from maternal blood [20, 33]. However, to establish cells from mesodermal, ectodermal or endodermal lineage, trophoblast-derived cells need to switch lineage. Recently, Schorle's group established a trophoblast stem cell line and reprogrammed it to a pluripotent cell state *in vitro* [107, 108]. Studies in mice presented first evidence that microchimeric cells might be derived from a trophoblastic origin: Kara and colleagues [83] reported that 40% of foetal cells contributing to tissue repair in maternal myocardium expressed caudal type homeobox 2 (*CDX2*). In blastocysts, *CDX2* is required for trophectoderm fate commitment [109–111]. The trophectoderm, in turn, gives rise to trophoblast stem cells, which differentiate down the placental lineage only [112, 113], suggesting a link between *CDX2* and trophoblast stem cells. Sunami *et al.* used a transgenic mouse model generating mice pregnant with GFP-positive foetuses and terminated the pregnancies by hysterectomy before delivery. It is way, they excluded cell trafficking occurring during delivery. Still, they detected GFP-positive cells in dam tissues [81]. These findings support the idea that extra-embryonic cells contribute to the establishment of microchimerism. Pritchard and colleagues [114] used a similar approach sorting GFP-positive foetal cells from maternal lungs in late pregnancy. They detected epiblast- and trophoblast-derived cells to be present. Thus, at least in theory, both could also give rise to long-term microchimerism. Despite the widely accepted use of animal models, we need to consider differences in murine and human placental anatomy and carefully check our findings in human studies (e.g. with human preeclamptic samples). However, when taking a closer look, the possibility of *in vivo* lineage conversion needs to be tested alongside the possibility that cells with different origins are fused. As fused cells will result in a GFP-positive cell and thereby simulate microchimerism. Harbouring two genetically different nuclei, they can be identified as false positive by means of DNA profiling [66, 67, 115].

Table 1. Overview on single-cell analysis

Tissue	Isolation	Analysis	Purpose	Reference
Peripheral blood	Filtration, LMD	HC, ICC, STR, PCR, FISH	Genetic disease	[20]
Peripheral blood	Micromanipulation	ICC, IF,STR, FISH	Chromosomal aneuploidy	[29]
Peripheral blood	LMD	HC, STR, PCR	Cystic fibrosis, spinal muscular atrophy	[33]
Artificial spiking	LMD	IF, STR	Non-identical cells[a], haploidentical cells[b]	[66]
Artificial spiking	LMD	IF, STR, mCGH, Sanger-seq	Non-identical cells[a]	[67]
Cardiac and skeletal muscular tissue	FACS	IF, RT-qPCR, qPCR, IF, FISH	Organ injury model	[83]
Blastomeres	Micromanipulation	mRNA-seq	Oocytes (knockout)	[120]
Blastomeres	Micromanipulation	aCGH; FISH	CNAs	[121]
Embryonic stem cells, embryonic fibroblasts	Micromanipulation	mRNA-seq	Cell-type-specific transcriptome analysis	[122]
Cancer cell line	Micromanipulation	Sanger-seq, NGS	Evolution of SNAs	[123]
Cancer cell lines	Micromanipulation	mRNA-seq	Transcriptome analysis	[124]
Artificial spiking	LMD, Micromanipulation	aCGH, NGS	Non-identical cells[a]	[125]
Astrocytes	FACS	RT-qPCR	Effect of direct lysis protocol on expression analysis	[126]
Cancer cell line	FACS	HT-qPCR	Effect of preamplification on expression analysis	[127]
Spleen	FACS	MARS-seq	*Ab initio* cell-type characterization	[128]
Peripheral blood	FACS	Smart-seq2	Characterization of multiple specialized human blood dendritic cell and monocyte subtypes	[129]
Cancer cell line, mouse retinal cells	Microfluidics	Drop-seq	Characterizing transcriptionally distinct cell populations from complex tissue (retina)	[130]
Pancreas	Microfluidics	inDrop	Characterizing transcriptionally distinct cell populations from complex tissue (pancreas)	[131]
Mouse brain	Microfluidics	Drop-seq	Characterizing transcriptionally distinct cell populations from complex tissue (brain)	[132]
Embryonic stem cells	Microfluidics, FACS	CEL-seq, Smart-seq, Drop-seq, MARS-seg, SCRB-seg, Smart-seq2	Effect of single-cell RNA-seq methods on sensitivity, accuracy and precision of transcriptome analysis	[133]
Embryonic stem cells	FACS	M&T-seq	Parallel sequencing linking transcriptional and epigenetic heterogeneity in single cells	[134]
Hepatocellular carcinoma	Micromanipulation	Trio-seq	Simultaneous genetic, epigenetic and transcriptomic heterogeneity in single-cell analysis	[135]
Cancer cell line	FACS	PLA, qPCR, RT-qPCR	Quantitative analysis of DNA, RNA and protein in the same single cells	[136]
Mouse lung	FACS	Microarray	Characterization of microchimeric cells present in lungs during late pregnancy	[114]

Note: HC, histochemistry; ICC, immunocytochemistry; mCGH, metaphase comparative genomic hybridization; RT-qPCR, reverse transcription quantitative PCR; mRNA-seq, mRNA sequencing; aCGH, array comparative genomic hybridization; Sanger-seq, Sanger sequencing; NGS, next-generation sequencing; HT-qPCR, high-throughput quantitative PCR; Drop-seq, droplet sequencing; inDrop, droplet sequencing; M&T-seq, methylome and transcriptome sequencing; Trio-seq, triple omics sequencing; PLA, proximity ligation assay.
[a]Tumour cells in peripheral blood.
[b]Haploidentical cells, foetal cells in maternal background.

Conclusion and future aspects

The combined efforts to (a) isolate cells based on individual-specific markers (e.g. HLA-mismatches), (b) analyse whole single-cell suspensions obtained from dissected tissues or blood and (c) high-throughput techniques addressing genetics, transcriptomics and epigenetics at the single-cell level will help to further investigate controversially discussed effects of microchimeric cells in cancer [56, 61] and to re-evaluate underpowered aspects in earlier studies [50, 54]. Techniques, such as ultrasensitive sequencing and digital PCR, will allow us to detect the presence of microchimerism, i.e. individual microchimeric cells in bulk tissue. Tissue sectioning, tissue digestion and enrichment techniques in combination with labelling and FACS, LMD and micromanipulation enable isolation of candidate microchimeric cells at the single-cell level. Analysis of STRs, single polymorphism and CNAs, methylated DNA and other allele-specific differences will unravel

the microchimeric status allowing us to exclude false positives and forward true microchimeric cells towards comprehensive molecular analysis. Single-cell methodologies have dramatically improved over the recent years allowing even multiple analytes to be analysed in the same cells, enabling microchimeric cells to be characterized and defined in depth. Ultimately, we will be able to isolate living microchimeric cells to test their properties by functional *in vivo* and *in vitro* assays, deciphering their biology.

Key Points

- Microchimerism occurs naturally as a physiological consequence of pregnancy either temporarily or life-long. Foetal microchimerism originates from cells of embryonic, foetal and/or extra-embryonic (e.g. tropho-blast) origin that cross the placental barrier to reside in maternal blood and tissues, whereas maternal microchimerism relates to maternal cells being present in their offspring.
- Microchimeric cells comprise cell types derived from ectodermal, endodermal and mesodermal lineages, suggesting that the founder cells giving rise to microchimerism exhibit stem cell-like properties.
- Microchimerism is associated with autoimmune diseases, cancer, immune tolerance and surveillance as well as tissue repair. Microchimeric cells can affect microenvironment both positively and negatively.
- Single-cell analysis enables detailed cell characterization and to decipher the true biological properties of microchimeric cells. Today, techniques for cell enrichment, isolating and characterizing of rare cells are available.

Funding

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