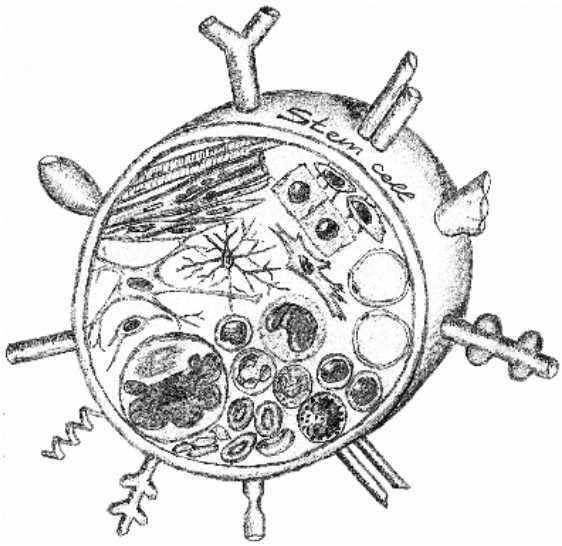
IN VIVO ANALYSIS OF HOMING PATTERN AND DIFFERENTIATION POTENTIAL OF CELLS DERIVING FROM EMBRYONIC AND ADULT HAEMATOPOIETIC REGIONS

Dissertation

For completion of the Doctorate degree in Natural Sciences at the
Bayerische
Julius-Maximilians-Universität Würzburg



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The hereby submitted thesis was completed from June 2000 until April 2004 at the Institut für Medizinische Strahlenkunde und Zellforschung, Bayerische Julius-Maximilians Universität, Würzburg under the supervision of **Professor Dr. Albrecht Müller** (Faculty of Medicine) and **Professor Dr. Georg Krohne** (Faculty of Biology).

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1 Summary

The experimental work of this thesis addresses the questions of whether established cell lines injected into murine blastocysts find their way back home and seed preferentially at the site of their origin. Furthermore, can they change their fate and differentiate to unrelated cell types when exposed to the embryonic environment. This survey was based on the fact that different cell lines have different potentials in developing embryos, dependent on their cellular identity. The cell lines used in this survey were AGM region-deriving DAS 104-4, DAS 104-8 cells, yolk sac-deriving YSE cells and bone marrow-deriving FDCP mix cells. These cells were injected into mouse blastocysts. Donor cells were traced in developing embryos via specific markers.

Analysis of the embryos revealed that DAS cells are promiscuous in their seeding pattern, since they were found in all analysed tissues with similar frequencies. YSE cells showed preferences in seeding yolk sac and liver. YSE donor cells in chimaeric tissues were not able to change their immuno-phenotype, indicating that they did not change their destiny. Analysis of adult mice did not reveal any of YSE-derived cells donor contribution.

In contrast, FDCP mix cells mostly engrafted haematopoietic tissues, although the embryos analysed by *in situ* hybridization had donor signals frequently in cartilage primordia, heads, and livers. Analysis of whether FDCPmix-derived cells found in foetal livers were of haematopoietic or hepatocytes nature showed that progeny of injected FDCP mix cells do not differentiate into cells that express a hepatocyte-specific marker. Further analysis showed that FDCPmix-derived donor cells found in brain express neural or haematopoietic markers. In order to reveal if they transdifferentiate to neurons or fuse with neurons/glial cells, nuclear diameters of donor and recipient cells were determined. Comparison of the nuclear diameters of recipient and donor cells revealed no differences. Therefore this suggests that progeny of FDCP mix in brain are not fusion products. Analysis of adult mice tissues revealed that presence of FDCP mix-derived cells was the highest in brains.

These results confirmed the assumption that the developmental potential of the analysed cells cannot be easily modified, even when exposed to early embryonic environment. Therefore one can conclude that the analysed cell types had different homing patterns depending on their origins.

2 Zusammenfassung

In der vorliegenden Arbeit wurde als zentrale Frage untersucht, wie Zellen verschiedener etablierter Zelllinien nach Injektion in murine Blastozysten an der Entwicklung der resultierenden chimären Tiere beitragen. Insbesondere wurde untersucht, ob injizierte Zellen bevorzugt oder ausschließlich Gewebe ihres Ursprungs besiedeln ("Homing"), oder ob Donorzellen auch heterologe Gewebe infiltrieren und gegebenenfalls gar unter dem Einfluß der frühembryonalen Blastozysten-Umgebung ihr Zellschicksal ändern und in andere Zelltypen transdifferenzieren können. Diese Studie basiert auf früheren Arbeiten, in denen gezeigt wurde, daß unterschiedliche Zelllinien - in Abhängigkeit ihrer Identität - verschiedene Entwicklungspotentiale im frühen Embryonalstadium haben.

Folgende Zelllinien wurden in der vorliegenden Arbeit untersucht: DAS 104-4 und DAS 104-8 (beide aus der sogenannten AGM Region isoliert), YSE (aus dem Dottersack) und FDCP mix (aus Knochenmark abstammend). Zellen dieser Zelllinien wurden in Maus Blastozysten injiziert und der Chimärismus in sich entwickelnden

Embryonen oder adulten Tieren mit Hilfe von Donorzell-spezifischen Markern analysiert.

Die Analyse chimärer Embryonen ergab, daß DAS Zellen diese promiskuitiv besiedeln: DAS Zellen wurden in allen untersuchten Geweben mit ähnlichen Häufigkeiten gefunden.

YSE Zellen hingegen wurden bevorzugt in der fötalen Leber und im Dottersack nachgewiesen. YSE Donorzellen in chimären Geweben zeigten keine Änderungen in ihrem Immunphänotyp und damit keine Hinweise auf eine mögliche Transdifferenzierung. In adulten Mäusen konnten keine YSE-abstammende Zellen mehr identifiziert werden.

FDCP mix Zellen besiedelten vor allem hämatopoetische Gewebe. In Embryonen wurden allerdings auch häufig Donorzell-spezifische *in situ* Hybridisierungssignale in Knorpelvorläufergewebe, der Leber und in der Kopfregion erhalten. Die FDCP mix Marker positiven Zellen der fötalen Leber wurden negativ auf die Expression von Hepatozyten-Markern getestet. Dies spricht auch in diesem Fall gegen einen Wechsel der Donorzellidentität und Transdifferenzierung. Im Gegensatz dazu wurden im Gehirn FDCP mix abstammende Spenderzellen identifiziert, die entweder neurale oder hämatopoetische Marker tragen. Die Zellkerndurchmesser wurden für die Donor-abstammenden Zellen und für die endogenen Gehirnzellen bestimmt und wiesen keinen signifikanten Unterschied auf. Dieser Befund läßt vermuten, daß FDCP mix abstammende Zellen mit Expression von neuralen Markern nicht das Produkt von Zellfusion von Donorzellen mit Neuronen oder

Gliazellen sind. In der Analyse von adulten Mäusen wurden FDCP mix abstammende Zellen am häufigsten im Gehirn identifiziert.
Die Ergebnisse dieser Arbeit zeigen für die analysierten Zelllinien, daß sich deren Entwicklungspotential auch bei Exposition in frühembryonalem Milieu nicht leicht modifizieren läßt. Es wurde weiterhin gezeigt, daß die analysierten Zelltypen in Bezug auf ihren Ursprung ein sehr unterschiedliches “Homing“-Verhalten aufweisen.

3 Introduction

3.1 Early development of mammals

Mammalian eggs are among the smallest in the animal kingdom, produced in the least number, and the development is within the mother organism, which makes them hard to manipulate. Only recently it has been possible to maintain internal conditions and observe development in vitro.

The mammalian ovary releases the oocytes, which are taken by fimbriae into the ampula of the oviduct were fertilization occurs. During murine preimplantation development the first cleavage starts the next day, and it is the slowest in the animal kingdom (takes 12-24 hours). The cleavage is meridional, while two following are meridional and equatorial, respectively. This type of cleavage is called **rotational cleavage** (Gulyas et al 1975). Typical for mammalian cleavage is asynchrony of early cell division and that the genome is activated, producing the proteins necessary to continue the development. The phenomenon of **compaction** happens after the third cleavage (8-cell embryo) when the cells get tightly packed together. This maximize the contact in the compact ball, and outer cells form tight junctions and inner gap junctions, thereby enabling small molecules and ions to pass in between (Peyrieras et al 1983; Fleming et al 2001). The 16-cell embryo is called a **morula** (Barlow et al 1972), and it consists of smaller inner and larger external cells where most of the descendants become the **trophoblast (trophoectoderm)**. The trophoblast cells will later form the extraembryonic structure **chorion.** During the process called **cavitation** a trophoblast cells pump sodium ions via sodium pumps (Na^+/K^+ ATP-ase) into the morula which draws water osmotically in, and creates a blastocoel (Borland, 1977; Wiley, 1984). This type of blastula is called the **blastocyst**. Inner cells of the morula, together with some trophoblast cells generate the **inner cell mass (ICM),** which will later give rise to the embryo and extraembryonic structures **yolk sac**, **allantois, and amnion.** Forming of the blastocyst is the first differentiation event during mammalian development, and during that process ICM and trophoectoderm become separate cell layers (Dyce et al 1987, Fleming, 1987). **The zona pellucida** is the extracellular matrix that surrounds the egg, it is essential for sperm binding during fertilization, and it prevents the blastocyst from binding to the oviduct walls. The hatching of the mouse blastocyst from the zona pellucida is provided by a trypsin like protease on the trophoblast cell membranes, that lyses a hole in the zona pellucida through which blastocyst cells squeeze out (O'Sullivan et al 2001). Direct contact between the endometrium (uterine epithelium) and the blastocyst is provided by integrins on the

trophoblast cells, which bind to the uterine extracellular matrix that contains collagen, fibronectin, laminin, hyaluronic acid and heparan sulphate receptors (Carson et al 1993). The digesting enzymes secreted by trophoectoderm lyse the extracellular matrix of the endometrium and allow the blastocyst to implant within the uterus (Strickland et al 1976; Brenner et al 1989).

The process of embedding the embryo into the lining of the uterus is called **implantation** and it occurs at embryonic day (E) 4.5 of development of the mouse. The embryo survives in the uterine environment by forming a vital organ, the **placenta,** for the exchange of gases, nutrients and waste products with the mother. The placenta acts as a source of hormones and growth factors and it is involved in immune protection of the foetus. The trophoectodermal cells overlaying the ICM- **polar trophoectoderm** give rise to the **extraembryonic ectoderm** (which expands to **chorionic epithelium**) and **ectoplacental cone** (which develops into **spongiotrophoblast** of the placenta). The trophoectoderm cells further away from ICM form primary **trophoectoderm giant cells** which develop into **syncytiotrophoblast** of the placenta. The **chorion** is formed from chorionic epithelium, lined with a thin layer of mesothelim. The **allantois** derives from the mesoderm of the posterior end of the embryo. It later gives rise to the placental blood vessels and the umbilical cord. It makes contact with the **chorion** forming **chorioallantoic fusion** (no cell fusion) at E8.5, which undergoes villous branching and creates a structure of the placenta called the **labyrinth,** the area of direct exchange between the foetus and maternal blood supply. The maternal portion of the placenta is called **decidua** (Rossant et al 2001).

The first segregation within the ICM forms the **hypoblast** (**primitive endoderm)** which gives rise to the **extraembryonic endoderm** that creates the **yolk sac**. The rest of the ICM forms the **epiblast,** which separates into **embryonic epiblast,** that will generate all three germ layers of the embryo (**ectoderm, mesoderm and endoderm**) and **amniotic ectoderm** which gives rise to the **amnion**. The amnion is an extraembryonic membrane that lines the embryo and secretes amniotic fluid to the amniotic cavity that serves as a shock absorber (Gilbert, 2003).

3.2 Differentiation

Differentiation is the process by which an unspecialized cell becomes specialized, develops specific structures and performs specific function in the body (Figure 1). During differentiation certain genes become activated and the other genes inactivated in a regulated fashion. Adult mammals consist of more then 200 different kinds of cells. The fertilized egg is totipotent (Latin *totus* means entire) because it has the potential to generate all cells and tissues of an embryo and the entire cell types that support its development in the uterus. The fertilized egg further divides and differentiates into the blastocyst (previously described). The ICM of the blastocyst contains embryonic stem cells that can self-renew and are pluripotent.

Embryonic stem cells that are deriving from the inner cell mass of E3.5 embryos are pluripotent stem cell types (Latin *plures* means several or many), as they can differentiate into cells of all three germ layers (ectoderm, mesoderm and endoderm) (see table 1). Adult type stem cells are generated at precise developmental stages in the individual tissues.

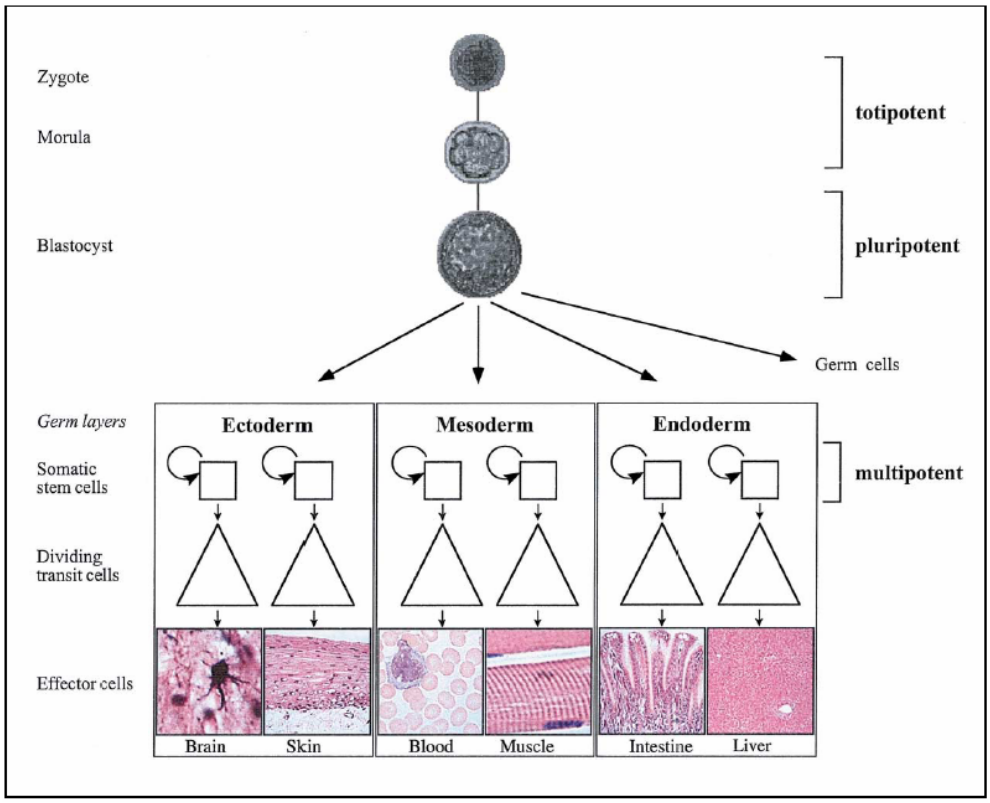


Figure 1. Differentiation of the tissues from zygote. During the mammalian embryonic development, the zygote gives rise to the morula- and blastocyst-stage embryo, which in a process called gastrulation generates the three germ layers (ecto-, meso-, and endoderm). These layers further differentiate to the terminal effector cells. But not all cell divisions generate terminally differentiated cells; some special cells are retained as somatic stem cells maintaining a high proliferation and differentiation potential. Throughout ontogeny, stem cells with characteristic developmental potentials are generated (totipotent, pluripotent and multipotent stem cells). Source Kirchhof et al 2002.

Table 1. Embryonic germ layers from which differentiated tissues develop. Modified after Stem Cells Scientific Progress and Future Research Directions (www.nih.gov/news/stemcell/scireport.htm)

Embryonic Germ Layer	Differentiated Tissue
Ectoderm	Skin Neural tissue (neuroectoderm) Adrenal medulla Pituitary gland Connective tissue of the head and face Eyes, ears
Mesoderm	Bone marrow (blood) Adrenal cortex Lymphatic tissue Skeletal, smooth, and cardiac muscle Connective tissues (bone and cartilage) Urogenital system Hearth and blood vessels (vascular system)
Endoderm	Thymus Thyroid, parathyroid glands Larynx, trachea, lung Urinary Bladder, vagina, urethra Gastrointestinal organs (liver, pancreas) Lining of the gastrointestinal tract Lining of the respiratory tract

They represent an undifferentiated cell types found in specialized tissues capable of self-renewal for the life time of the organism and generating the cells from the tissue from which they originate. They are found in bone marrow, brain, the dental pulp of the tooth, cornea and retina of the eye, skin, liver, gastrointestinal tract, pancreas and heart.

It has been recently described that adult stem cells can “transdifferentiate”, i.e. generate cells of unrelated tissues. Several reports described that haematopoietic stem cells (HSCs) from bone marrow can generate skeletal muscle (Gussoni et al 1999; Ferari et al 1998; Bittner et al 1999), neural cells (Brazelton et al 2000; Eglitis and Mezey, 1997; Mezey et al 2000), cardiac muscle (Orlic et al 2001a; Orlic et al 2001b; Jackson et al 2001), hepatic cells (Lagase et al 2000; Petersen et al 1999; Alison et al 2000), epithelia of the gut, skin, lung and kidney (Perez et al 2001). But these reports are still controversially discussed, because in most of the studies unpurified or only partially purified cell populations have been analysed, and almost none have performed analysis on single isolated stem cells. Additionally, the most of the early studies did not exclude the possibility of cell fusion. Recent evidence suggests that is possible to isolate a multipotent adult progenitor cell (MAPC) from adult bone marrow, which can contribute to nearly every tissue in the body (Jiang et al 2002). However it is still in discussion whether MAPC cells exist in the adult organism or whether they are generated during extended time in cell culture.

3.3 Haematopoiesis

A highly orchestrated process of blood cell development and homeostasis is termed **haematopoiesis** (Smith, 2003). In the embryo the haemangioblast cells of the lateral mesoderm give rise to the angioblast, the precursor of blood vessels and to haematopoietic stem cells (HSCs), precursors of all blood cell types. Interestingly, the earliest blood cells and the earliest blood vessel cells share many of the same rare proteins on their cell surfaces (Choi et al 1998). All mature blood cells originate from relatively small numbers of HSCs and their immediate progenitors.

HSCs are defined as cells that are capable of both self-renewal and multilineage reconstitution of the entire haematopoietic system. They were discovered when irradiated bone marrow was injected into mice that had a hereditary deficiency of haematopoiesis. Abramson *et al* showed in 1977 that the same chromosomal abnormalities after irradiation are present in the cells of myeloid and lymphoid lineage. Phenotypically, HSCs are relatively small cells with a minimal cytoplasm. They express high levels of multidrug resistant (MDR) proteins, and aldehyde dehydrogenase (ALDH) (Smith 2003). Most methods for isolating of HSCs are based on cell-surface staining with monoclonal antibodies, followed by fluorescence-activated cell sorting (FACS). Some of these combinations are markers Lin^{-}, c-kit^{+}, Sca-1^{+} or $CD34^{low/neg}$, or $Lin^{neg/low}$, Thy1.1^{low}, Sca-1^{+} and Rhodamine 123^{low} or c-kit^{high}, Thy1.1^{low}, $Lin^{neg/low}$, Sca-1^{+} or $Lin^{neg/low}$, c-kit^{high}, Sca-1^{hi}, Thy1.1^{low}, $Flk2^{-}$. By using c-kit , Thy1.1, Lin, Sca-1 markers ~1:10000 mouse bone marrow cells has long term, multilineage, repopulating capability (**LT-HSC**), and ~1:2000 cells has short term repopulating capability (**ST-HSC**). One engrafting HSC is principally sufficient to repopulate the entire haematopoietic system; however the delayed reconstitution originating from a single HSC is not compatible with life. Therefore, 100 injected HSCs are considered a protective dose for 95-100% of lethally irradiated transplant recipients (Domen and Weissman, 1999). The LT-HSC is capable of indefinite self-renewal, while the ST–HSC is capable of self-renewal for a shorter time frame (~ 8 weeks). Upon further differentiation ST-HSCs give rise to briefly self-renewing multipotent progenitors (MPPs). MPPs then generate oligolineage-restricted progenitors. These are common lymphoid progenitors (CLPs) which generate B, T and natural- killer (NK) cells and common myeloid progenitors (CMPs), which produce granulocytes, monocytes, macrophages, erythrocytes. A functional definition of a committed progenitor cell that is able to generate the cells of myeloid lineage was first demonstrated by Till and McCulloch in 1961. They injected in their seminal experiments bone marrow cells into lethally irradiated mice of the same genetic strain as the marrow donors. Irradiation kills the haematopoietic

cells of the host, so that any new blood cell must come from the transplant. The donor cells produced discrete nodules or colonies in the spleens and microscopic studies showed that the colonies consist of erythrocyte, granulocyte and platelet precursors originating from the transplanted cells. The assay is called **colony-forming-units-spleen assay** and the cell colony-forming-unit of the spleen (**CFU-S**). CMPs give rise to myelomonocytic progenitors (GMPs) which produce monocytes/macrophages and granulocytes. They also give rise to megakaryotic/erythroid progenitors (MEPs) which differentiate into megakaryocytes/platelets and erythrocytes, but maintain at low frequency potential for B cell lineage differentiation. Both CMPs and CLPs can give rise to dendritic cells, suggesting the existence of alternative differentiation pathways (Figure 2) (Passegue, 2003).

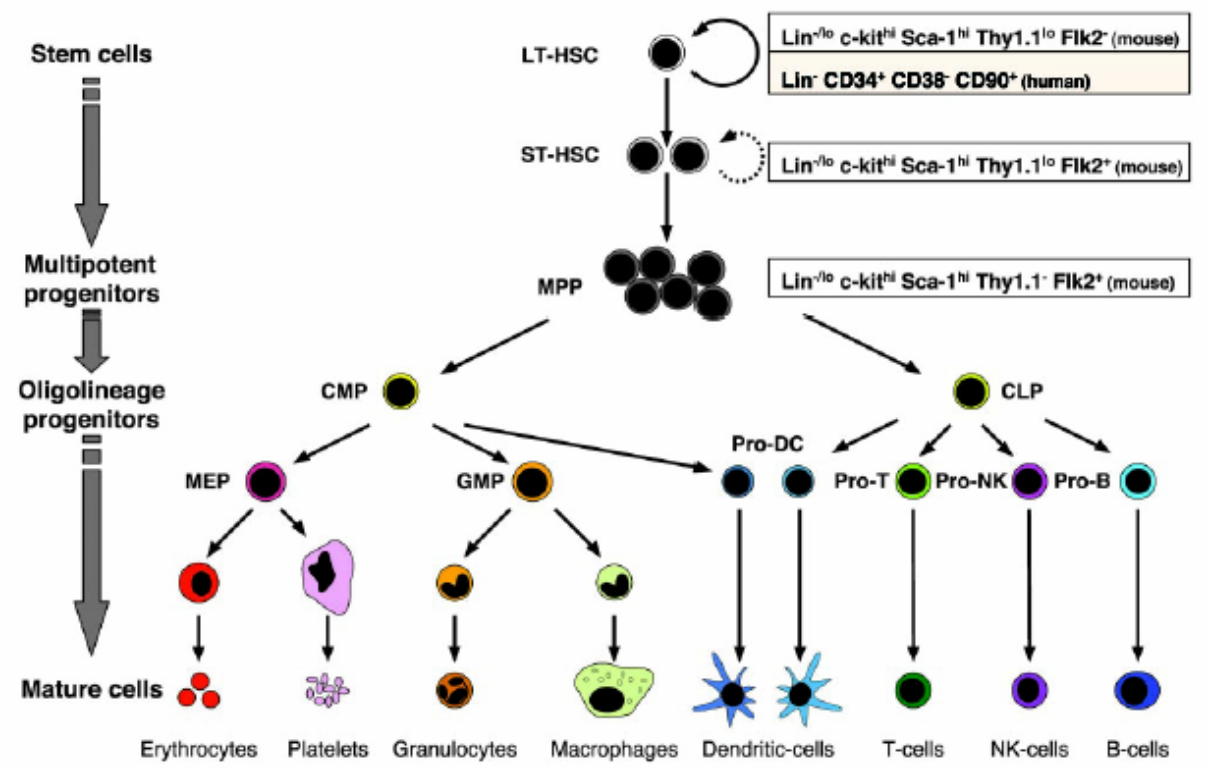


Figure 2. Haematopoietic stem system. HSCs can be divided into LT-HSCs, and ST-HSCs. ST-HSCs differentiate into MPPs, which have the ability to differentiate into oligolineage-restricted progenitors - CLPs and CMPs that ultimately give rise to differentiated progeny through functionally irreversible maturation steps. The CLPs give rise to T lymphocytes, B lymphocytes, and natural killer (NK) cells. The CMPs generate GMPs, which then differentiate into monocytes/ macrophages and granulocytes, and to megakaryotic/erythroid progenitors (MEP), which produce megakaryocytes/platelets and erythrocytes. Both CMPs and CLPs can give rise to dendritic cells. All of these stem and progenitor populations are separable as pure populations by using cell surface markers and FACS sorting (Modified after Passegue et al 2003)

3.4 Sites of embryonic and adult haematopoiesis

The different cell lines used in this study are deriving from different haematopoietic regions of embryos and adult mice. Therefore, the origin of the haematopoietic system is briefly discussed.

Blood development in vertebrates occurs in two phases: The **embryonic** ("primitive") **phase** and the **definitive** ("adult") **phase.** These phases differ in the sites of blood cell production, also the morphology of the produced cells and in the types of expressed genes. During the embryonic phase of haematopoiesis the embryo is transiently provided from the extraembryonic yolk sac with primitive type blood cells. During the definitive phase of haematopoiesis the embryo is supplied with blood cells from HSC that originate from the intraembryonic aorta-gonad-mesonephros (AGM) region. These definitive-type HSCs last for the entire lifetime. At each stage of development different subsets of mature haematopoietic cells are generated. Definitive erythroid cells that are small and enucleated in adult organisms, while primitive erythroid cells produced during embryonic yolk sac haematopoiesis, are large and nucleated. Specific types of B cells and T cells are produced by progenitors in the foetal liver (Bonifer et al 1998; Dzierzak et al 1998). There are differences between embryonic/foetal and adult-type macrophages (Faust et al 1997).

The first extraembryonic sites of haematopoiesis, where primitive erythrocytes are generated in E7.5 embryos, are blood islands of the yolk sac. Blood islands of the yolk sac are the close associations of endothelial and haematopoietic cells, which led to the proposal of a common progenitor cell for both cell types, the haemangioblast. At the beginning of E7 granulocyte-macrophage progenitors and at E8.5 T- and B-lymphoid progenitors can be detected in the yolk sac (Cumano et al 1993; Liu and Auerbach 1991). The cells from intraembryonic para-aortic splanchnopleura seed the blood system of the embryo and the yolk sac at E8.5. Only short-term repopulating haematopoietic progenitors are generated in the yolk sac. At mid-E9 colony forming unit spleen (CFU-S) are first detected in the AGM region, and shortly after at E10.5 HSCs that can repopulate the entire haematopoietic system are produced in the AGM region (Müller et al 1994).

The first intraembryonic region of haematopoiesis is the para-aortic splanchnopleural (PAS) mesoderm and associated tissue at E7. At E7.5 myeloid-erythroid, B- and T- lymphoid cells are found there (Cumano, 1996). The first CFU-S progenitors appear in the dorsal aorta gonads-mesonephros (AGM) region at E9 (Medvinsky, 1993) and the first long-term repopulating haematopoietic cells are detected at late E10 (Müller 1994 and Medvinsky 1996). Thus, the first definitive HSCs are produced in the AGM.

The rudiment of the liver is formed at E9 from the evagination of the gut into septum transfersum. The liver does not generate its own haematopoietic cells but it is seeded from the yolk sac and AGM region at this stage of development. At E9 the liver contains erythroblasts and at E10 it starts to produce differentiated erythroid cells (Dzierzak and Medvinsky, 1995). At mid-E9 myeloid progenitors appear, at E10 T-cell progenitors and at E10-11 granulocyte-macrophage and B-lineage cells (Velardi et al 1984). At late E10, early E11 HSCs and CFU-S progenitors are detected in the developed foetal liver (Medvinsky et al 1993).
Haematopoietic progenitors and stem cells in the adult mouse are found in bone marrow and spleen. The differentiated cells move quickly through the circulation to the other tissues and organs. Growth factors induce the efficient mobilization of haematopoietic precursor and stem cells into the blood (Bodine, 1995). Colonization of bone marrow with HSCs is believed to take place around E16 of gestation (Ogawa, 1988). According to Cumano 1996 at E15 prior to bone formation haematopoietic progenitors have already accumulated in bone marrow, but active B lymphopoiesis does not appear before E17.

3.5 Cell lines derived from specific haematopoietic regions

In this survey different, well described cell lines were used. The specific characteristics of these cell lines play an important role in their capacity to contribute to the organs of developing embryos. These characteristics are described in detail below.

3.5.1 Dorsal aorta stromal (DAS) 104-4 and DAS 104-8 cell lines

These two cell lines are of endothelial origin. They were isolated from the murine AGM region as supportive cell lines for haematopoietic progenitors and HSCs. To obtain endothelial cell lines, the AGM region of E11 murine embryo was isolated by dissection and cells were transformed using polyoma virus middle T- expressing retroviruses. This oncogene transforms endothelial cells and maintains them in an endothelial-like state (Bautch et al 1987; Williams et al 1989). Two morphologically similar CD34+ adherent cell lines DAS 104-4 and DAS 104-8 were selected.
Analysis revealed that DAS 104-8 efficiently induced foetal liver HSCs to differentiate down erythroid, myeloid and B-lymphoid pathways, but did not mediate self renewal of the pluripotent cells. The DAS104-4 cell line was inefficient at the induction of haematopoietic

differentiation, but provoked expansion of early haematopoietic progenitor cells and was proficient at maintaining foetal liver derived HSCs. The morphology of these cell lines shows typical flattened, endothelial-like structure. They expressed a number of endothelial markers: CD34, FLT1, FLK1 (both are VEGF receptors), vWF and CD31 (platelet endothelial cell adhesion marker- PECAM). These cells produce the endothelial growth factor VEGF which may allow autocrine-mediated proliferation of these cell lines upon binding to the FLT1 and FLK1. The formation of capillary-like structures when these cells are grown in Matrigel also supports the fact that they are endothelial cells (Ohneda et al 1998).

3.5.2 Yolk sac endoderm (YSE) cell line

This cell line was established from the visceral endoderm of the yolk sac to better define the role of the murine yolk sac in differentiation and proliferation of haematopoietic cells (Yoder et al 1994).

Yolk sacs from E9.5 murine embryos were isolated and separated endoderm and mesoderm layers were cultured. Adherent cells were infected with a recombinant retrovirus encoding SV40 large T antigen. Permanent lines from yolk sac endoderm were established. The morphology of the cell lines was similar to freshly isolated visceral endoderm cells. They showed polygonal morphology with formation of tight cell clusters. None of these cell lines were contact inhibited, and none showed anchorage-independent growth in soft agar. A polarized morphology, tight junctions, cell surface apical microvilli, coated pits and cytoplasmic coated vesicles were identified in these cells, which is characteristic of endocytically active yolk sac endoderm cells. Markers expressed on these cells are the extracellular matrix proteins fibronectin, collagen IV, laminin, Endo-B cytokeratin, zonulla occludens-1 and GATA4. They do not express macrophage cell surface molecules Mac-1, F4/80, vimentin intermediate filaments, H513E3 marker for stromal endothelial cells and one of the major secreted proteins in visceral yolk sac - α fetoprotein. Expression of laminin and collagen IV by these cells are not usual characteristics of visceral endodermal cells. Non-expression of α-fetoprotein indicates that the cells do not show fully differentiated visceral endoderm phenotype, although other investigators noted the rapid loss of α-fetoprotein expression with in monolayer cultures of yolk sac visceral endoderm in vitro (Hogan and Taylor, 1981; Hogan et al 1983). It seems that this cell line has an intermediate phenotype between visceral and parietal endoderm.

YSE cell line supported less neutrophil and greater macrophage differentiation from BM cells depleted of committed progenitors cells by 5-FU treatment. This suggests that these cell lines recapitulate microenvironmental influences that exist in normal yolk sac (Yoder at al 1994).
This cell line significantly stimulated the proliferation of adult murine bone marrow high proliferative potential colony forming cells (HPP-CFC) in coculture (Yoder et al 1995).

3.5.3 Factor dependant cell Paterson (FDCP) mix A4 cell line

The FDCP mix cell line was generated from long-term bone marrow cultures (of B6D2F1 mice) which had been infected with a recombinant of the Molony murine leukaemia virus and the *src* oncogene of the Rous sarcoma virus (*src*-MoMuLV). Such cultures show 20-50-fold increase in the concentration of spleen-colony forming cells (CFU-S), and they are IL-3-dependant progenitor cells. These cultures can be serially transferred *in vivo* and *in vitro* with no loss of clonogenic potential (Boettiger, 1984).
All FDCP cell lines initially isolated had primitive blast morphology. The cells are absolutely dependant upon IL-3 for proliferation and other haematopoietic cell growth factors are not able to replace it. The cells have a diploid karyotype and are non-leukaemogenic. They produce spleen colonies in irradiated mice. However after 3 months of culture the spleen colony-forming ability was lost. The late isolates of FDCP mix cells can establish haematopoiesis when injected directly into the spleen, which suggests that they lost spleen-homing capacity. The FDCP mix cells are multipotent *in vitro* (Spooncer at al 1986). Under specific conditions they can differentiate to macrophages, granulocytes, megacaryocytes, erythrocytes, osteoclasts and mast cells, but it was not possible to induce lymphocyte differentiation *in vitro* (Ford et al 1992). They die without IL-3, but bone marrow stromal cells can replace IL-3. Cell attachment and cell to cell association is probably the mechanism, by which FDCP mix cells respond to marrow stromal cells in the absence of IL-3. FDCP mix cell line does not contain an integrated *src* oncogene (Spooncer et at 1986).

3.6 The process of migration and homing

Migration and homing are necessary events for normal development and homeostasis in the embryonic and adult organism, and deregulation of both processes has many dramatic consequences. During development primordial germ cell (PGS) precursors migrate from the extraembryonic mesoderm through the allantois (E 7.5) to the yolk sac. Then the PGCs move caudally from the yolk sac through the newly formed hindgut and up the dorsal mesentery

into the genital ridge. They seed developing mouse gonads (homing organ) up to E11 (Gilbert, 2003).

Neural crest cells (NCCs), a second migrating cell type , emerge from the dorsal part of neural tube at vagal (somites 1-7) and truncal (somites 8-28) levels and migrate through the embryo following two main pathways: dorso-ventral and dorso-lateral. Cells that migrate along the dorso-ventral route give rise to neurons and Schwann cells of the peripheral nervous system (PNS) and some endocrine cells (chromaffin cells of adrenal medulla). Cells that migrate along the dorso-lateral pathway give rise to melanocytes. In the hindbrain and cranium NCCs proliferate and migrate throughout the head mesenchyme and give rise to glial cells and cranial PNS neurons. When NCCs migrate through the branchial arches or the face they give rise to dermis and facial muscles, cartilage or bones (Pla et al 2001).

During ontogeny, as already mentioned, HSCs arise in the AGM region and migrate to the foetal liver, and later from the liver to the bone marrow.

The processes of migration and homing are retained in adult organisms in the haematopoietic system. Leucocytes migrate to the sites of inflammation or trauma. Homing of lymphocytes to the white pulp cords of the spleen is required for their proliferation, and for effective immune responses. For homeostasis of the organism, migration of HSCs to the circulation and their homing back to bone marrow are imperative. A complex interplay between adhesion molecules, chemokines, cytokines, proteolytic enzymes, non-peptide mediators, stromal cells and haematopoietic cells regulates stem cell release and homing to the bone marrow (Lapidot and Petit 2002)

Mesenchymal and haematopoietic cells in BM microenvironment secrete more then 40 different growth factors that are present in cell-bound forms, are bound to the extracellular matrix (ECM) or are in solution. More then 20 different adhesion receptors have so far been identified on stem and progenitor cells. Based on structure and function analysis they are divided into integrins, cadherins, selectins, members of mucin-like family, and members of immunoglobulin family of receptors (Prosper and Verfaillie, 2001). Adhesion receptors on HSCs and progenitor cells provide specific cell-cell and cell-ECM interactions, and retain them in the BM, but also serve as growth or survival signals and modulate growth factor dependant signals. The ECM components such as collagen, fibronectin, proteoglycans, laminin, vitronectin, tenascin provide also support for stem cell survival and proliferation (Whetton and Graham, 1999). Cytokines and growth factors affect adhesive interactions between stem and progenitor cells and their adhesive ligands in the BM.

A number of diseases of the haematopoietic system are based on abnormal adhesive interactions that results in abnormal regulation of growth, differentiation and survival (Prosper and Verfaillie, 2001).

The process of migration and homing is determined in part by the presence of the specific cell type receptors. Their role is shown in Figure 3.

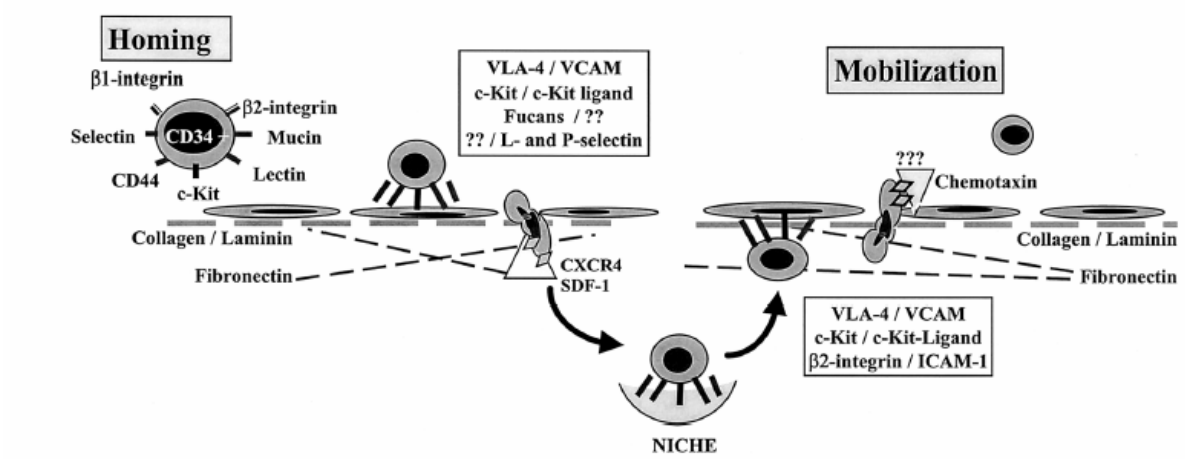


Figure 3. Role of adhesion receptors implicated in homing and mobilization of haematopoietic progenitors. HSCs/HPCs express a number of adhesion receptors on their surfaces that allow them to interact with a large number of adhesive ligands. In the setting of transplantation, it is thought that haematopoietic cells tether to ligands on endothelial cells via selectins and fucans. This then results in the activation of integrins, which allow firm adhesion and transmigration through the endothelium. The chemokine SDF-1a that binds to the CXCR4 receptor has been implicated in providing specificity of this activation process. Adhesion to fibronectin and possibly vascular cell adhesion molecule-1(VCAM-1) through β1-integrins participate in more permanent anchoring of haematopoietic cells to the BM niche. The mobilization process requires de-adhesion of progenitors from the BM. β1, β2, and c-kit are implicated in mobilization of haematopoietic cells through an unknown mechanism. Source Prosper and Verfaillie, 2001.

In the article by Gendron *et al* in 1996, the endothelial cell line IEM derived from embryonic stem cells (ES cells) was analysed. The properties of IEM cell line are expression of a range of endothelial markers, including von Willibrand Factor VII, vascular cell adhesion molecule, platelet-endothelial cell adhesion molecule and receptors for acetylated low-density lipoprotein. Upon exposure to a combination of basic fibroblast growth factor and leukaemia inhibitory factor *in vitro*, these cells chimaerize the microvascular endothelium *in vivo* following injection into blastocysts.

Further analysis revealed that heterochronically transplanted stem/progenitor cells from brain and BM engraft embryos and adults. In particularly, after injection of HSCs into murine

blastocysts preferentially haematopoietic tissues were seeded, while neural tissues were engrafted in adult animals developed from murine blastocysts injected with NSCs (Kirchhof et al 2002; Harder et al 2002). Analysis of the murine E12.5 embryos, injected as blastocysts with human acute myeloid leukaemia (AML) cells, revealed that engraftment of donor cells was the most frequent in the blood tissues (Dürr et al 2003). It would therefore be intriguing to investigate whether cell lines deriving from different haematopoietic regions, with their different natures, or in other words their specific cell surface molecules, will be led to their place of origin after injection into developing blastocysts.

3.7 Questions

All cell types have their cellular identity and function in embryos and adult organisms. These can be described to a certain extent with specific biological markers.

The four cell lines used in this study originate from different murine haematopoietic regions. They are: DAS 104-4 and DAS 104-8, derived from embryonic haematopoietic AGM region; YSE, derived from yolk sac; and FDCP mix, derived from bone marrow, the haematopoietic region of adult mice. The question that is addressed in this thesis:

Do established cell lines following injection into developing murine blastocyst find their way back home and seed preferentially the site of their origin?

In particular, will DAS 104-4 and DAS 104-8 endothelial cell lines home to AGM region, YSE to yolk sac and FDCP mix to bone marrow and haematopoietic tissues?

Further, will they maintain their cellular identity or will they change their fate and become unrelated cell types?

Through the exposure of injected cell lines to the murine blastocyst microenvironment and analysis of murine embryos and adult organisms these questions are addressed.

This study will put a new light and knowledge on the process of homing and differentiation.

3.8 Experimental strategy

In order to answer previously described questions mouse blastocysts have been used as the experimental system (see Figure). Different cell lines derived from yolk sac (yolk sac endoderm cell line), aorta gonad mesonephros (AGM) region (DAS 104-4 and DAS 104-8 cell lines) and bone marrow-BM (FDCP mix cell line) were separately injected into mouse blastocysts. Injected blastocysts were implanted into pseudopregnent foster mothers and the embryos of different developmental stages (E11.5-E16.5) were analysed. Some of the embryos were allowed to develop into adults and were analysed.

The injected cells carry specific markers for recognition in chimaeric embryos and adults. DAS 104-4 and DAS 104-8 cell lines are transformed with polyoma virus middle T antigen, YSE are transformed with Simian virus 40 large T antigen and are male, FDCP mix are male. Male donor cells were traced in the female embryos.

Figure 4. Experimental strategy. Analysis of homing pattern of different cell lines in mouse embryos and adults

Two major analyses were carried out. First, the different parts or the organs of the chimaeric embryos were dissected, genomic DNA isolated and donor specific PCRs were performed. The PCR products were run on agarose gels and donor signals were detected by Southern blot and hybridization. Autoradiograms have been analysed for donor contribution of injected cells in specific tissues. Second, isolated chimaeric embryos were embedded in paraffin or in the freezing medium, embryonic sections were made and DNA-DNA *in situ* hybridization was performed or combined immunostaining for cell-type specific marker with DNA-DNA *in situ* hybridization. Embryonic sections were analysed for the presence of donor cells.

The homing patterns of injected cell lines, as well as their potential to change their fate were analysed.

4 Materials and Methods

4.1 Materials

4.1.1 Cell culture media and growth factors

Name	Producer
M2 medium	Sigma
M16 medium	Sigma
Hepes	Sigma
DMEM high glucose	Sigma
Powdered IMDM	Gibco
Foetal Calf Serum (FCS)	Biochrom KG
Horse serum	Sigma
L-glutamine	Gibco
Penicillin/Streptomycin	Gibco
Benzyl penicillin	Sigma
Streptomycin	Sigma
Non-essential amino acids	Gibco
Bovine serum albumine	Boeringer Mannheim
β mercaptoethanol	Merck
sodium bicarbonate 7.5%	Integra Biosciencies
Trypsin-EDTA	Gibco
IL-3	Stem Cell Techologies Inc
Hank's balanced salt solution	PAA
Alpha modified of Eagles medium	PAA

4.1.2 Antibodies

Anti-mouse primary antibodies

Name	Clone and Source	Cell type specificity	Producer
Anti-ACTR-IB	T-17; goat polyclonal	YSE cells	Santa Cruz

Anti-ICAM-1	KAT-1; rat monoclonal	epithelial cells	Cymbus Biotech
Anti-CD45	30-F11; rat monoclonal	hematopoietic cells (not eritrocytes)	Pharmingen
Anti-NCAM	MAB310; rat monoclonal	neurons and astrocytes	Chemicron
Anti-albumin	rabbit polyclonal	liver cells	Accurate

Secondary antibodies

Name	Source	Producer
Biotinylated anti-rat IgG (H+L)	rabbit	Vector Labs
Biotinylated anti-rabbit Igs	goat	PharMingen
Biotinylated anti-goat IgG	rabbit	Jackson Immunoresearch

Tertiary layer for detection of bound antibodies

Name	Producer
Streptavidine-Red670	Gibco
Streptavidine-FITC	Gibco
Streptavidine-AP	DAKO

For detection of streptavidine-alkaline phosphatase used Vector Red Phosphatase supstrate kit (Vector Labs)

4.1.3 Chemicals

Name	Producer
Agarose NEEO (Ultra Quality)	Roth AG
DAKO fluorescent mounting medium	DAKO corporation
Dextransulphate	Amersham
Dimethylpolysiloxan 50 centistokes	Sigma
EDTA	Applichem
EGTA	Applichem
Ethanol	Applichem
Ficoll	Sigma

Isopropanol	Applichem
Ketanest 25mg/ml (S-ketaminhydrochlorid)	Parke Davis
Mowiol	Calbiochem
NaOH	Applichem
37% HCl	Applichem
Polyvinyl-pyrollidone	Sigma
Phenol-Chlorophorm	Applichem
Potassium-chloride	Applichem
Rompun 2%(xylazinhydrochlorid)	Bayer
Sodium –citrate	Applichem
Soduim-borate	Applichem
Sodium-chloride	Applichem
Water Ultra pure	Merck
Xylene based mounting medium	Merck

4.1.4 Enzymes

Name	Producer	Concentration
Proteinase K	Sigma	10U/µl
RNAse	Sigma	15U/µl
DNAse I	Sigma	15U/µl
Collagenase (type I)	Sigma	100mg

4.1.5 Laboratory equipment

Instrument	Producer
T3 Thermocycler	Biometra Goettingen
Trio Thermoblock	Biometra Goettingen
Uno Thermoblock	Biometra Goettingen
BioPhotometer	Eppendorf
Centrifuge 5417C	Eppendorf
Centrifuge Minifuge RF	Heraeus
Microscope IMDRB	Leitz

Microscope Telofal 31	Zeiss
Cytospin	Thermo Shandon
In situ cycler	Thermo Shandon
Ultraviolet Crosslinker	Amersham Life Sciencies

4.2 Methods

4.2.1 Isolation of DNA from cells and tissues

1. put the tissue or cells in 700µl of lysis buffer with proteinase K
2. incubation over night (o/n) at 56°C in the rotation incubator
3. add 7µl of RNAse to degrade RNA
4. incubation 30 min at 37°C
5. add an equal volume of Phenol-Chlorophorm, shake 5 min
6. centrifuge for 10 min at 20000g (10000rpm)
7. transfer supernatant to another tube; hydrophilic DNA is in aqueous phase of supernatant, while the proteins are in the interphase
8. add an equal volume of phenol/chlorophorm to the water phase, shake 5 min
9. centrifuge for 10 min at 10000rpm
10. transfer the supernatant to a new tube
11. add an equal volume of isopropanol to the water phase, shake
12. incubate for 20 min at -80°C
13. centrifuge for 10 min at 20000g (14000rpm); DNA will be visible as a white pellet at the bottom of the tube
14. remove supernatant and add 700µl of 70% ethanol (washing of DNA)
15. vortex to dissolve the pellet
16. centrifuge for 20 min at 20000g
17. remove supernatant and add 700µl of 70% ethanol
18. centrifugation for 20 min at 20000g
19. remove ethanol and dry the DNA
20. dissolve DNA pellet in ddH_2O

4.2.2 Measurement of DNA concentration

The aromatic rings of purine- and pyrimidine bases of nucleic acids dissolved in water absorb the light at wave length 260nm. The concentration of DNA and optical density (OD) at 260nm are in linear relationship so it is possible to calculate the concentration with very simple formula:

C= OD_{260nm}x 50µg/ml double stranded DNA

C= OD_{260nm}x 40µg/ml single stranded DNA

C= OD_{260nm}x 33µg/ml single stranded RNA

Bio Photometer Eppendorf is used for measuring of DNA concentration.

4.2.3 Polymerase chain reaction (PCR)

The amplification of specific the DNA fragments was performed in Trio Thermoblock, Uno Thermoblock or T3 Thermoblock (Biometra Goettingen). 200ng of genomic DNA was amplified. All the oligonucleotides (primers) were sinthesized by MWG Biotech**.**

Myogenin PCR

PCR reaction:

5µl 10x PCR buffer

2µl $dNTP_s$ (2.5mM each)

120ng sense primer (5'-TTA CGT TCG TGG ACA GC-3')

80ng antisense primer (5'-TGG GCT GGG TGT TAG TCT TA-3')

0.05µl Super Taq Polymerase (5U/µl)

200ng DNA

add till 50µl ddH_2O

Cycles:

95°C	5min	1 cycle
95°C	30 sec	
58°C	30 sec	36 cycles
72°C	90 sec	
72°C	2min	1 cycle

Fragment size: 245 bp

Primer sequences for PCR are taken from literature (Mueller et al. 1994)

YMT PCR

PCR reaction:
5µl 10x PCR buffer
2µl $dNTP_s$ (2.5mM each)
100ng sense primer (5'-CTG GAG CTC TAC AGT GAT GA-3')
100ng antisense primer (5'-CAGTTA CCA ATC AAC ACA TCA-3')
0.05µl Super Taq Polymerase (5U/µl)
200ng DNA
add till 50µl ddH_2O

95°C	5min	1 cycle
95°C	10 sec	
60°C	30 sec	32 cycles
72°C	35 sec	
72°C	2min	1 cycle

Fragment size: 342 bp
Primer sequences for PCR are taken from literature (Medvinsky et al.)

Simian virus 40 large T antigen PCR

PCR reaction:
5µl 10x PCR buffer
2µl $dNTP_s$ (2.5mM each)
100ng sense primer (5'-TCC AAC CTA TGG AAC TGA TG-3')
100ng antisense primer (5'-AGT CAA GGC ACT ATA CAT CA-3')
0.05µl Super Taq Polymerase (5U/µl)
200ng DNA
add till 50µl ddH_2O

95°C 5min 1 cycle
95°C 30 sec
56°C 60 sec 40 cycles
72°C 60 sec
72°C 2min 1 cycle
Fragment size: 563 bp
Primer sequences for PCR are taken from literature (Gendron et al.1996)

Polyoma virus middle T antigen PCR

PCR reaction:
5µl 10x PCR buffer
2µl $dNTP_s$ (2.5mM each)
100ng sense primer (5'-AGT CAC TGC TAC TGC ACC CAG-3')
100ng antisense primer (5'-CTC TCC TCA GTT GTT CGC TCC-3')
0.05µl Super Taq Polymerase (5U/µl)
200ng DNA
add till 50µl ddH_2O

94°C 2min 1 cycle
94°C 40 sec
64°C 40 sec 35 cycles
72°C 60 sec
72°C 5min 1 cycle
Fragment size: 492 bp

4.2.4 Separation of DNA fragments on agarose gel

The amplified DNA fragments are separated on 1.5% agarose gel. 6g of agarose is dissolved in 400ml of TBE-buffer, cooked in microwave and 20µl of 0.5% ethidium bromide solution is added. 10-20µl of PCR product is mixed with 3µl of Bromphenolblue buffer. Electrophoretic separation is dependant on fragment size and is performed in electric field of 5-8V/cm ca. 30-60 min. Ethidium bromide incorporates between nucleic acids and visualisation is possible at 560nm on UV light. Standard size of markers is compered with separated PCR products.

Markers: PTZ plasmid digested with restriction enzyme HinfI and pSM with Hind III. Ptz DNA fragments have size from 44 to 1202 bp, and pSM between 145 and 3440 bp.

4.2.5 Southern blotting

This method is used for detection of DNA fragments transfered to nylon membrane. After separation of DNA fragments on agarose gel, gel was shaken 2 times 20 min in 0.5M NaOH/1.5M NaCl at room temperature. Blotting is set up by placing the gel face down, with Nytran (NY13N) membrane, soaked in water for 5 min, and then in 0.5M NaOH /1.5M NaCl. Dry papers are on the top. Gel is blotted over night.
Next day Nytran filter is rinsed 2 times in 1x 0.5M Tris /1.5M NaCl and 2xSSC, air dry and cross linked 60 sec at 120 mJ/cm^2 UV light.

Hybridization

Wet filters in 2xSSC, separated by meshes, are loaded into hybridization bottles.
Prehybridize at 65°C for 2h in prehybridization solution.
Pehybridization solution contains finally: 3xSSC; 0.1%SDS; 10xDenhardt's;
50µg/ml salmon sperm DNA
100xDenhardt's solution contains: 2%Ficoll; 2% Bovine serum albumin (fraction V); 2% polyvinyl pyrolidone
Hybridize overnight in roller bottles.
Hybridization solution contains: 3xSSC; 0.1%SDS; 10xDenhardt's; 10%(w/v) dextransulfate; 50µg/ml salmon sperm DNA and the radioactive labelled probe*

Washing

Prewarm 0.5 l/per bottle of 2xSSC/ 0.1%SDS and 0.5 l/per bottle of 0.2x SSC /0.1% SDS at 65°C
Wash filters 2 times for 20min in 2xSSC/ 0.1%SDS and 2 times for 20min in 0.2x SSC /0.1% SDS.
Wrap the wet filters in Saran wrap.

Exposure of Roentgen films to radioactive labeled filters

The Kodak Biomax MR-1- Roentgen film is exposed to the radioactive labelled filters in the metal cassettes with reflectant background. Dependants on strength of radioactive labelling, filters are incubated a few hours to one week in freezer at -80°C. Developing of the exposed films was done in Kodak M35 XOmat Processor.

***Preparing of radioactively labelled probe**

DNA fragments for labelling are amplified by PCR, separated on agarose gel, cut from the gel and purified with Quiagen quick kit for isolation of DNA. Purified DNA fragments are radioactively labelled by Nick translation. 40ng of DNA fragment is incubated in mixure of nucleotides dATP, dGTP, dTTP and (α^{32}P)-dCTP and enzymes DNAseI and DNA-polymerase I for 45 min at 15°C. DNAse I cuts the phosphodiester bonds between nucleotides, and DNA polymerase I synthesizing complementary strand and incorporates radioactive labelled (α^{32}P)-dCTP. For purifying of labelled fragments Sephadex G50-columns are used, made of insulin syringes with glass wool and Sephadex. After centrifuging for 2 min at 400g, labelled fragments were purified and free of nucleotides and enzymes. The labelled fragments are mixed with hybridization solution.

4.2.6 DNA *in situ* hybridization using DIG-11-dUTP labelled probe

1. Cut the paraffin sections 5-6µm thick and put them on Super Frost slides. Dry them overnight at 37°C
2. Deparaffinize slides in xylene and hydrate by rinsing in grades alcohols to water (100%; 90%; 70%; ddH_2O; 1xPBS)
3. Pretreat slides in Proteinase K or pepsin
    a) a cytospined cells 2-4 min with 10ηg/µl Proteinase K
    b) a tissues and embryos 5-15 min 0.2% pepsin/0.1M HCl

4. Wash in ddH_2O and fix in 4%PFA / 1xPBS 2 min at 4°C
5. Wash in ddH_2O for 5 min
6. Rinse slide in 2 changes of 95% ethanol and 2 changes of 100% ethanol
7. Allow slides to air dry at least 5 min

8. Prepare a labelled probe by mixing 1:4 or 1:3 with hybridization solution
9. Distribute about 20µl of dilute probe on dry sections, cover slip and seal with photo glue
10. Place the slides in a humid chamber, denature at 95°C for 6 min
11. Cool them for 2 min on ice
12. Incubate them at 42°C overnight
13. The next day, remove the cover slips and wash the sections
    a) 2 x 5 min in 2xSSC at 20°C
    b) 1 x 10 min in 0.1xSSC at 42°C
14. Rinse the slides 2 x 5 min in Buffer #1
15. Block the tissue with following solution 15-30 min at room temperature:
    488.5µl of Buffer#1
    10µl of normal sheep serum
    1.5µl of Triton X-100

16. Wipe away excess blocking solution and cover the slides with enough of following solution:
    489.5µl of Buffer#1
    5µl of sheep anti-digoxigenin-alkaline phosphatase
    5µl of normal sheep serum
    1.5µl of Triton X-100
    Incubate for one hour at room temperature

17. Rinse slides in 2 changes of Buffer#1
18. Rinse slides in 3 changes of Buffer#2
19. Prepare NBT/BCIP
    2.5ml of Buffer#2
    50µl of NBT/BCIP
    Cover section and allow incubate in the dark for 30 min to 1 hour.

20. Rinse slides in ddH_2O
21. Counter stain in Nuclear Fast Red solution for 2 min. Rinse in ddH_2O
22. Mount the slides with Mowiol

Buffer#1 (100mM Tris-HCl, 150mM NaCl)

Buffer#2 (100mM Tris-HCl, 100mM NaCl, 50mM $MgCl_2$)

Sheep-anti-digoxigenin-Alk/phos 83466421 Roche

NBT/BCIP nitrobluetetrazolium/5-brom-4-chlor-3 indolylphosphate

Hybridization solution:

Formamide	5ml
Dextran sulphate 10%	2ml
20xSSC	2.5ml
1M Sodium Phosphate pH 6.5	0.25ml
100xDenhardt's solution	0.10ml
ddH_2O	0.138ml
Sonicated salmon sperm	0.0125ml (Carrier DNA conc.250mg/ml)

Labeling of hybridization probe with DIG-11-dUTP by PCR

PCR reaction:

5µl 10x PCR buffer

5µl $dNTP_s$ (2mM dATP, 2mM dGTP, 2mM dCTP, 1.3mM dTTP, 0.7mM DIG-11-dUTP)

100ng sense primer

100ng antisense primer

0.2µl Super Taq Polymerase (1U/sample)*

20ng DNA templete

add till 50µl ddH_2O

* DIG-11-dUTP slows the Taq polymerase

4.2.7 Combined immunostaining with *in situ* hybridization

1. Cut the paraffin sections 5-6µm thick and put them on Super Frost slides. Dry them overnight at 37°C
2. Dewax for 10 min in Xylene
3. Rinse in 100% methanol for 5 min

4. Block endogenous peroxidase with 5ml of 30% hydrogen peroxide in 200ml methanol for 10 min
5. Rinse sections through graded alcohols to 1xPBS (100%; 90%; 70%; ddH_2O; 1xPBS)
6. Antigen retrieval was done with bovine trypsin for 15 min
7. Block unspecific binding of antibody with a serum from the animal where secondary antibody was made. Dilute 1:25 in 1xPBS, block 15-30 min; Remove excess without washing (After this step in some cases endogenous biotin was blocked with 20µl of avidin in 1000µl ddH_2O, and then saturated with 5g of skimmed milk which contains endogenous biotin in 200ml of ddH_2O)
8. Incubate in primary antibody dilution for 1 h-1h 30 min
9. Wash well in 1xPBS for 10 min; for greater stringency use 1xPBS/0.05%Tween-20
10. Incubate in secondary antibody dilution 1:100 (antibody:1xPBS)
11. Wash well in 1xPBS for 10 min; for greater stringency use 1xPBS/0.05%Tween-20
12. Incubate in streptavidin- alkaline phosphatase dilution 1:50
13. Wash well in 1xPBS for 10 min; for greater stringency use 1xPBS/0.05%Tween-20
14. Incubate in Vector red solution
15. Permeabilise by incubating in 1M sodium-thiocyanate for 10 min at 80°C (freshly prepared)
16. Wash in 1xPBS 2 x 5 min
17. Digest with 0.2% pepsin in 0.1M HCl between 5-15 min at 37°C (freshly prepared)
18. Quench in 0.2% glycine in 2xPBS for 5 min at room temperature
19. Rinse in 1xPBS. Post fix in 4%PFA/1xPBS for 2 min (freshly prepared)
20. Dehydrate through graded alcohols to absolute and air dry
21. 15µl Cambio paint add to each section, cover slip and seal with rubber cement
22. Denature at 60°C for 10 min before hybridising over night at 37°C in humid chamber
23. The next day, remove cover slips and wash for 3 x 5 min in 50% formamide/2xSSC at 42°C
24. Wash in 2xSSC for 3 x 5 min at 42°C
25. Place the slides in 4xSSC/0.05% Tween-20 for 2 min
26. Incubate in blocking solution 4xSSC/ 0.05% Tween-20/ 5% milk powder (freshly prepared)
27. Wash the slides in 1xPBS for 15 min with several changes*
28. Apply 200µl of 1:25 anti-FITC antibody in 1xPBS (Roche 150U/ml) to each section
29. Incubate for 60 min in a humid chamber at room temperature

30. Wash in 1xPBS, 2 x 5 min
31. Colour is developed using DAB. 5mg DAB in 10 ml 1xPBS, mix with 20µl of 30% hydrogen peroxide; apply 100µl of mixture to each section.
32. Wash in 1xPBS, tap water, counter stain and mount.

*For direct in situ hybridization at this step protocol is finished, DAPI stained and mounted with DAKO fluorescent mounting medium

Cambio paint Mouse FITC- Chromosome Y paint Cat.Nr.1189-YMF-01
Avidin ABC staining system Santa Cruz Biotechnology Cat. Nr. sc-2019

4.2.8 Embedding of embryos in paraffin

1. Wash the embryos in ice cold 1xPBS 2-3 times
2. Fix them over night in 4%PFA/1xPBS at 4°C
3. Dehydrate them graded alcohols:

20% Ethanol (EtOH)	ovn,4°C
40% EtOH	40 min,4°C
50% EtOH	40 min,4°C
70% EtOH	40 min,4°C
80% EtOH	40 min,4°C
90% EtOH	40 min,4°C
3x100% EtOH	40 min,4°C
Chloroform	20 min, RT
Chloroform	20 min, 60°C
Paraffin change 2-3x	fast, 60°C
Paraffin	ovn, 60°C

Embed the embryos in paraffin cubes; allow hardening, and keeping them at 4°C

4.2.9 Cytospin

Cells look better if fixed before cytospin in 4%PFA/1xPBS for 20 min

1. Take $2x10^5$ cells in 100µl of 1xPBS

2. Centrifuge at 500rpm-800rpm, 3min on the slides
3. Dry them short

4.2.10 Medium for cultivating DAS 104-4 and DAS104-8 endothelial cell lines

DMEM (high glucose 4500mg/l, with Na-pyruvate)	425ml
FBS (foetal bovine serum) or FCS (foetal calf serum)	50ml
MEM nonessential amino acids	5ml
L-glutamine (200mM)	5ml
Penicillin/Streptomycin	5ml
Fungizon	5ml
β-mercaptoethanol*	5ml

*β-mercaptoethanol made fresh weekly as follows:

7µl of β-mercaptoethanol stock added to 10ml 1xPBS and filtered through 0.2µm filter and store at 4°C

4.2.11 Protocol for cultivating yolk sac endodermal stromal cell line

Yolk sac endodermal (YSE) cell line medium (for 1000ml):

High glucose DMEM (4.5g/l glucose)	760ml
Foetal calf serum (ES)	100ml (screened to support ES cells)
Foetal calf serum (BM)	100ml (screened to support of bone marrow)
Penicillin/streptomycin	10ml
Non-essential amino acids (100x)	10ml
L-glutamine (100x)	10ml
β-mercaptoethanol*	10ml

*β-mercaptoethanol made fresh weekly as follows:

7µl of β-mercaptoethanol stock added to 10ml 1xPBS and filtered through 0.2µm filter and store at 4°C

Passaging of yolk sac endodermal cell line

The yolk sac endodermal (YSE) cell line proliferates very well and must be passaged every 3-4 days. 70-80% confluence is the best, before using 1 ml 1x Trypsin-EDTA (Gibco) to free the cells from the plates. Then add 1ml of the medium and flush the cells in and out of 5ml pipette 3-4 times to ensure the cells are not clumped before plating. Failure to disaggregate the cells results in a rapid overgrowth of the plates with the clones of transformed cells. YSE cell line has strong intracellular interactions and is quite difficult to disaggregate.

Freezing down YSE cell line

Freezing medium:
45% DMEM high glucose
45% FCS
10% DMSO

4.2.12 Preparation of Iscove's medium for FDCP mix (Factor dependant cell line Paterson) cell line

Iscove's medium is made fresh every week. The horse serum and Interleukin3 (IL-3) supplements are added to the required amount of medium just before use. It's very important to adjust the osmolarity of the medium.

FDCP mix cell line medium contains:

Powdered Iscove's medium (Gibco, store at 4°C)	17.6g/l
Penicillin (300mg/ml benzyl penicillin = $0.5x10^6$ units, store at -20°C)	1ml
Streptomycin (200mg/ml streptomycin sulphate, store at -20°C)	0.5ml
Sodium bicarbonate (7.5%, store at 4°C)	40ml
Horse serum (20%, tested for FDCP mix cells)	200ml
IL-3 source (7-10% tested conditioned medium or recombinant IL-3)	70ml

Osmolarity of the medium should be 0.32osmol/l

Freezing down FDCP mix:

1. Spin down the cells at 200xg (RCF) for 10 min, RT
2. Resuspend in 90% horse serum and 10% dimetylsulphoxide (DMSO)
3. Freeze $2x10^6$/ml (min - $1x10^6$/ml; max - $5x10^6$/ml), approximately 1.8ml/vial
4. Freeze in a special box with isopropanol at -70°C overnight
5. Next day put the vials in liquid nitrogen

Thawing FDCP mix cells from liquid nitrogen storage

1. Put the vial into a water bath at 37°C for a short time (1 min)
2. Pour the cells into a 50 ml Falcon tube in the laminar flow hood
3. Dilute slowly by adding 10ml complete medium drop wise over 10 min
4. Leave the cells for 10 min in the laminar flow hood
5. Spin them down at 200xg, 10 min, RT
6. Pour off the supernatant
7. Resuspend the pellet in 5ml complete medium
8. Pour into T25 Falcon culture flask, leave in the incubator (5% CO_2, 98% humidity, 37°C) overnight

Removing of dead FDCP mix cells

1. Count the living cells the day after thawing in the 5 ml culture
2. Pour Histopaque in a sterile tube under the hood
3. Drop 5 ml of cell suspension over Histopaque, carefully, do not disturb the interface
4. Spin down at 400xg (RCF), 30 min, RT, BRAKE OFF!
5. The live cells accumulate at the interface while the dead cells sink to the bottom
6. Collect the cells off the interface (take 5ml of cell suspension plus 1ml interface/Histopaque) into a 15ml Falcon tube which contains 5 ml of medium
7. Spin down again at 200xg (RCF), 10 min, RT, BRAKE ON!
8. Pour off the supernatant and resuspend the pellet at $8x10^4$/ml cells in medium

4.2.13 A single cell suspension of hepatocytes

1. Transfer liver of adult mouse in chilled Hanks solution (Ca^{2+} and Mg^{2+} free) with 0.05 EGTA, pH 7.2 and mince it in pieces
2. Transfer them into 50ml tube, wash them twice with 15ml of the same solution, and incubate them with agitation at 37°C for 10 min to remove as much blood as possible. Repeat incubation step once again.
3. Incubate the suspension twice at 37°C for 10 min in EMEM to fully remove any traces of EGTA
4. For enzymatic digestion, the suspension was incubated with Ca^{2+} containing EMEM and collagenase at a dose of 0.5mg/liver and 100µl DNase (1µg/µl), for 15 min, with agitation, at 37°C
5. The suspension was filtered through a 70µm-pore mesh and the filtrate was centrifuged for 3 min at 1000 rpm
6. The pellet was resuspended in EMEM containing 0.3% of foetal bovine serum (FBS) and stored on ice. This enzymatic digestion was repeated three times using half the amount of DNase
7. Count the number of the cells (should be around $1.5x\ 10^8$)

DNA staining using propidium iodide (PI) and sorting of 2n and 4n cells

1. 1-2 $x10^7$ cells were washed in PBS and resuspended in 3 ml of PBS containing 0.3% of saponin
2. Add 60µl of 10mg/ml RNase and 300µl of 100µg/ml PI and incubate for 20 min at RT.
3. Samples were sorted by FACS for 2n and 4n cells in their staining solution.

4.2.14 Animals

Mouse line	Producer	Use
NMRI	Charles River, Sulzfeld	Blastocysts'donors, Foster mothers

4.2.15 Injection of various cells in blastocysts and reimplantation of blastocysts in foster mothers

For obtaining blastocysts 5 weeks old NMRI females were used. Females were intraperitoneally injected with 10U pregnant mare serum (PMS) 46 hours before mating and with 10U human chorion gonadotropin (HCG) on the day of mating. Females were mated with males, and next day the plugs checked. Females with plugs were killed with cervical dislocation at day 3.5 after plug checking, uteri were isolated, and flushed with M2 medium. Blastocysts were collected in drops of M16 medium and kept in incubator. Inverse Leica microscope with injection and holding needles (Biomedical Instruments) was used for injection of the cells in blastocysts. Injection medium contains the mixture of DMEM (high glucose; 450µl), FCS (40µl) and HEPES (10µl). Injected blastocysts were transferred in the uterus of a foster mother. Foster mothers were mated with vasectomiesed males, and transferred with blastocysts at day 2.5 after plug checking. Foster mothers were anesthetized with mixture of Ketanest (700µl), Rompun (50µl) and 1xPBS (250µl).

4.2.16 Isolation of chimaeric embryonic and adult tissues

Chimeric embryos were isolated at different time points of development E11.5, E12.5, E16.5. Each embryo was isolated in separate 3.5 cm dishes, in ice cold 1xPBS and kept on ice. Carotid veins were punctured for collecting of embryonic blood. The following tissues were isolated: yolk sac, foetal liver, brain, hind limbs, foetal blood, visceral part, AGM, rest of the embryo. Half the tissues were subjected to DNA isolation, and half were embedded in freezing medium (Tissueteck) or in paraffin.

Adult animals were sacrificed by cervical dislocation at the age of 1-1.5 months. They were decapitated and peripheral blood was collected. Various tissues were dissected and kept in ice cold 1xPBS in 3.5 cm dishes on ice, before subjected to DNA isolation. Half the tissues of some animals were embedded in freezing medium (Tissueteck). Isolated adult tissues were: cortex, cerebellum, rest of the brain, hippocampus, spinal cord, *nervus ishiadicus*, liver, lung, gut, heart, muscle, ovary, spleen, thymus, bone marrow, peripheral blood, skin, and kidney.

5 Results

5.1 Injection of murine DAS 104-4 cells into murine blastocysts

It has been previously shown that injection of foetal and adult cells, both primary isolates and established cell lines such as murine HSCs, human cord blood- derived HSCs, murine neural stem cells (NSCs), KG-1 (human myeloid leukaemia cell line) and AML (primary human acute myeloid leukaemia) cells into murine blastocysts led to the generation of chimaeric embryos and adults (Geiger et al 1998, Harder et al 2002, Kirchhof et al 2002, Dürr et al 2003). In order to follow the homing properties of AGM-derived DAS 104-4 and DAS 104-8 cells in developing embryos, between 10 to 30 cells were injected into murine blastocysts. As previously mentioned these cells were transformed with polyoma virus middle T antigen. 7 different parts of E10.5 embryos that developed from injected blastocysts were isolated: yolk sac, head, hind limbs, foetal blood, AGM region, visceral part and rest of the embryo. DNA was isolated, and chimaeric tissues were detected by donor contribution PCRs. For every single embryo at least three independent donor-specific PCRs were done. Serial dilutions of 100%, 20%, 2%, 0.2%, and 0% donor DNA in DNA of control animals were amplified to quantify donor signals. If the signal was positive at least twice, the embryo tissue was considered as positive. Myogenin PCRs were used for normalization of genomic DNA. PCR products were separated on agarose gels, subjected to Southern blotting, and hybridized before autoradiography. After 5 independent injections of DAS 104-4 cells, 16 embryos developed from 118 (16/118) injected blastocysts, and 10 /16 embryos were chimaeric (Table 2). Percentage of developed embryos from injected blastocysts was ~13%. 62.5% of all developed embryos were chimaeric. In previous experiments done with human HSCs and primary AML cell around 50% of injected blastocysts developed into embryos, while KG-1 cell-injected blastocysts resulted in development of 18% of blastocysts (Dürr et al 2003). Comparison with these results suggests that highly proliferative DAS 104-4 cells have negative effect on embryonic development.

Strength of the donor signals in chimaeric embryos was between 0.2-20 percent. A representative Southern blot is shown in Figure 5. Different embryonic tissues contained donor cells with a frequency of 27-56%. No tissue revealed a preferential seeding of donor cells. Final analysis of all embryos developed after injection of DAS104-4 is shown in Table 3.

Table 2. Summary of endothelial DAS104-4 cells injection into murine blastocysts.

Cell line	Nr. of injections	Nr. of injected blastocysts	Nr. of developed embryos	Nr. of adults	Percent of developed embryos	Nr. of chimaeric embryos	Percent of chimaeric embryos
DAS 104-4	5	118	16	0	~13% (16/118)	10	~62% (10/16)

Table 3. The distribution of AGM-derived DAS 104-4 contribution in chimaeric midgestational embryos.

tissue / embryo	Yolk sac	Head	Hind limbs	Foetal blood	Rest of embryo	AGM region	Visceral part
D3	-	+	-	-	-	-	-
D4	-	-	-	-	-	-	-
D5	-	-	-	-	-	-	-
D6	-	-	-	-	-	-	-
D7	-	-	-	-	-	-	-
D8	-	-	-	-	-	-	-
D9	-	+	+	+	+	-	-
D10	+	+	+	-	+	+	+
D11	-	+	-	-	-	-	-
D12	-	-	-	-	+	-	-
D13	+	-	+	+	-	+	-
D14	+	+	na	+	+	+	+
D15	+	+	na	+	+	+	+
D16	+	+	na	+	+	+	+
D17	-	+	na	+	+	+	+
D18	+	+	na	+	+	+	+
Summary	**6/16**	**9/16**	**3/11**	**7/16**	**8/16**	**7/16**	**6/16**
Percent	**37%**	**56%**	**27%**	**44%**	**50%**	**44%**	**37%**

Abbreviations: na, not analysed; (-), no donor signal; (+), positive donor signal; All embryos were E10.5 stage of development. Summary shows how many embryos have positive signals in the specific tissues out of all embryos. The same is shown in percentage.

According to these data DAS104-4 cells do not have preferences in homing. They are found in all tissues investigated at similar percentages.

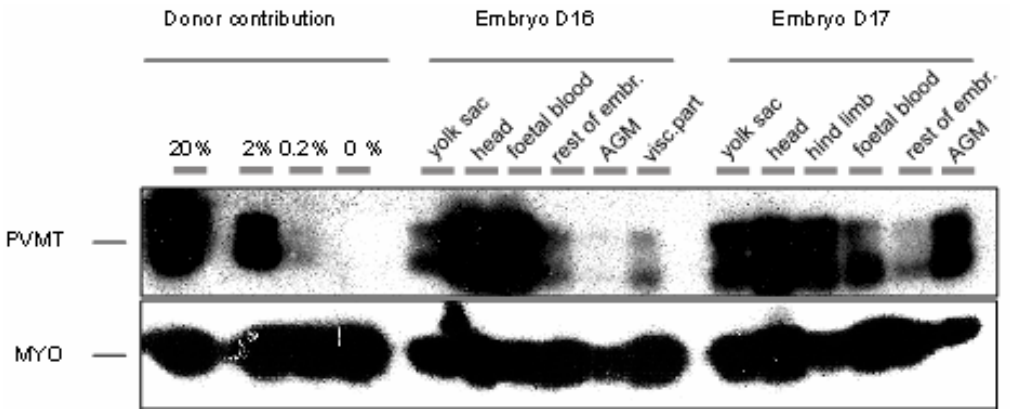


Figure 5. The donor contribution in chimaeric recipients D16 and D17 following injection of DAS 104-4 cells into blastocysts. Analysed embryos D16 and D17 were E10.5 stage of development. Controls are 20%, 2%, 0.2% and 0% genomic DNA from DAS104-4 cells diluted into wild type genomic DNA. Abbreviations: MYO, myogenin; PVMT, polyoma virus middle T antigen; AGM, aorta-gonad-mesonephros region; visc. part, visceral part; rest of embr., rest of the embryo.

5.2 Injection of murine DAS 104-8 cells into murine blastocysts

Similar analysis was done with DAS104-8 cells. Between 10 and 30 DAS104-8 cells were injected into murine blastocysts in 9 independent injections. In total, 145 blastocysts were injected, and 19 animals (embryos and adults) developed, which is ~11%. The tissues of 12 embryos out of 16 were isolated at E11.5, DNA was extracted, and analysed by donor–specific PCRs. 4 embryos were analysed by DNA-DNA *in situ* hybridization using a PVMT DNA fragment labelled with dUTP-digoxigenin (dUTP-DIG). 3 animals were born and analysed as adults by donor contribution PCRs. In total 9/19 animals contained donor cells. 4 embryos were PCR positive, 3 embryos showed donor's signal following *in situ* hybridization and 2 adults were PCR positive. Altogether ~47% of developed animals showed chimaerism (see Table 4).

The chimaerism was in the range of <0.2-2% as estimated by PCRs.

Table 4. Summary of endothelial DAS104-8 cells injection into murine blastocysts.

Cell line	Nr. of injections	Nr. of injected blastocysts	Nr. of developed embryos	Nr. of adults	Percent of developed animals	Nr. of chimaeras	Percent of chimaeras
DAS 104-8	9	145	16	3	~11% (19/145)	9 (7e+2a)	~47% (9/19)

Abbreviations: 7e+2a, 7 embryos and 2 adults

Adult animals were sacrificed at the age of 1 month, 18 different tissues were dissected, and analysed by donor-specific PCRs. Isolated adult tissues were: cortex, cerebellum, rest of the brain, hippocampus, spinal cord, *nervus ishiadicus*, liver, lung, gut, heart, muscle,

ovari, spleen, thymus, bone marrow, peripheral blood, skin, kidney. Two out of three animals were chimaeric, showing donor signals in cortex and cerebellum. Strength of the donor signal in chimaeric adult animals was from 0.2 to more then 2 %. Figure 7 represents a typical Southern blot analysis on 18 adult tissues of animal 1. The analysis revealed donor signals in cortex with more than 2% donor contribution.

Final analysis of all embryos developed after injection of DAS104-4 is shown in Table 5.

Table 5. The donor contribution in different tissues of embryos developed after injection of DAS104-8 cells.

tissue / embryo	Yolk sac	Foetal liver	Head	Hind limbs	Foetal blood	Rest of embryo	AGM region	Visceral Part
D1	-	-	-	-	-	-	-	-
D2	-	-	-	-	-	-	-	-
D19	-	-	-	-	+	+	+	-
D20	-	+	+	+	+	+	+	-
D21	-	na	-	-	+	+	-	+
D22	-	na	-	-	-	-	-	+
D25	-	na	-	-	-	-	-	-
D26	-	na	-	-	-	-	-	-
D27	-	na	-	-	-	-	-	-
D28	-	na	-	-	-	-	-	-
D29	-	-	-	-	-	-	-	-
D30	-	-	-	-	-	-	-	-
Summary	**0/12**	**1/6**	**1/12**	**1/12**	**3/12**	**3/12**	**2/12**	**2/12**
Percent	**0%**	**17%**	**8%**	**8%**	**25%**	**25%**	**17%**	**17%**

All embryos were E11.5 stage of development. Summary shows how many embryos have positive signal in the specific tissue out of all embryos and the same is shown in percentage. Abbreviations: na, not analysed; (-), no donor signal; (+), positive donor signal.

As shown in Table 5, AGM-derived DAS104-8 cells do not have preferential tissues of engraftment. As with DAS 104-4 cells, all analysed tissues from DAS 104-8 injected embryos showed similar percentages of donor cells. Figure 8 shows contribution of DAS 104-8 in chimaeric embryos after DNA-DNA *in situ* hybridization with a PVMT DNA fragment labelled with dUTP-digoxigenin (dUTP-DIG). *In situ* hybridization was done as a positive control on DAS 104-8, and as negative control on the cells that do not carry PVMT antigen. In parallel *in situ* hybridization was performed on sections of injected embryos and on sections of non-injected, wild type embryos. Anti-DIG antibody was coupled with alkaline phosphatase and during enzyme reaction with NBT/BCIP a purple precipitate developed. In Figure 8 panel a) DAS 104-8 cells cytospun and hybridized with PVMT DNA–DIG labelled fragment have been shown. Nuclei of DAS 104-8 cells had purple colour, because they carried PVMT antigen. Panel b) shows a transverse section of the brain of the non-injected embryo hybridized with PVMT DNA–DIG labelled fragment. This was a negative control, none of the cells was coloured purple, because

none of the cells carried PVMT antigen. Panel c) demonstrates the cells that do not carry PVMT antigen, cytospun and hybridized with PVMT DNA–DIG labelled fragment. Nuclei of these cells have not been coloured purple, because did not carry PVMT antigen. Panel d) reveals transverse section of the brain of H14 chimaeric embryo hybridized with PVMT DNA–DIG labelled fragment. One part of mesencephalon contained PVMT antigen positive cells. Panel e) illustrates the part of mesencephalon of H14 chimaeric embryo that contained PVMT antigen positive cells, and it represents higher magnification of panel d).

Figure 6 shows 0.2% donor contribution of DAS104-8 cells in chimaeric embryos in yolk sac, head, hind limbs, peripheral blood in D21 embryo and in yolk sac in embryo D22.

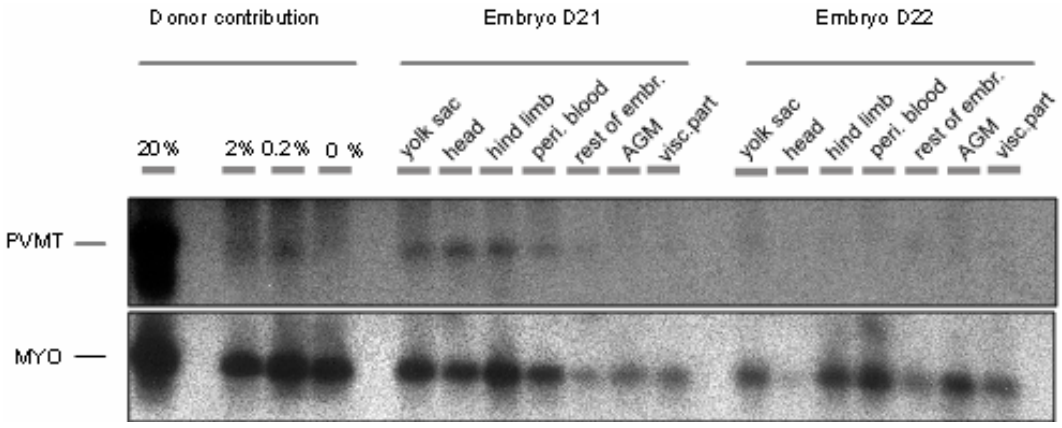


Figure 6. Donor contribution in chimaeric recipients D21 and D22 following injection of DAS 104-8 cells. Analysed embryos D21 and D22 were E11.5. Controls are 20%, 2%, 0.2% and 0% genomic DNA from DAS104-8 cells diluted into wild type genomic DNA. Abbreviations: MYO, myogenin; PVMT, polyoma virus middle T antigen; AGM, aorta-gonad-mesonephros region; visc. part, visceral part; rest of embr., rest of the embryo.

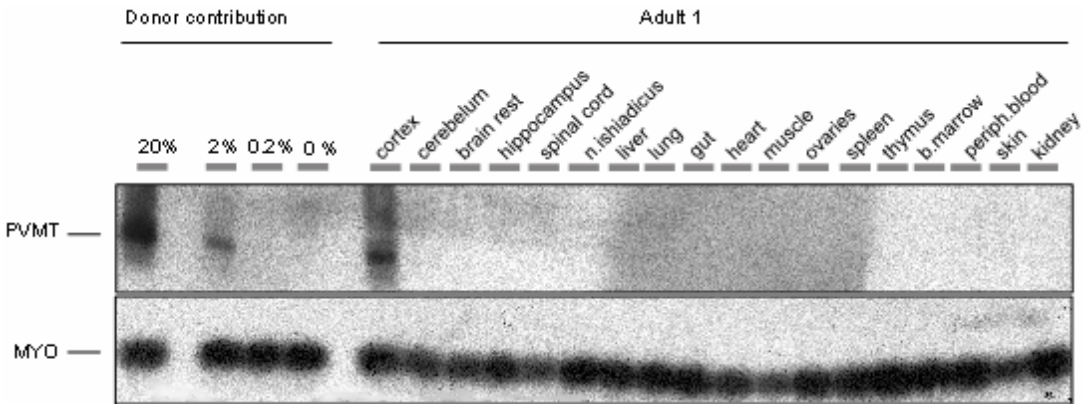


Figure 7. Distribution of donor contribution in chimaeric adult animal 1 developed from a blastocyst injected with DAS 104-8 cells. Chimaeric adult animal 1 was sacrificed at the age of 1 month. Controls are 20%, 2%, 0.2% and 0% genomic DNA from DAS104-8 cells diluted in non transgenic genomic DNA. Abbreviations: MYO,myogenin.; PVMT, polyoma virus middle T antigen; n. ishiadicus, *nervus ishiadicus*; b. marrow, bone marrow; periph. blood, peripheral blood;

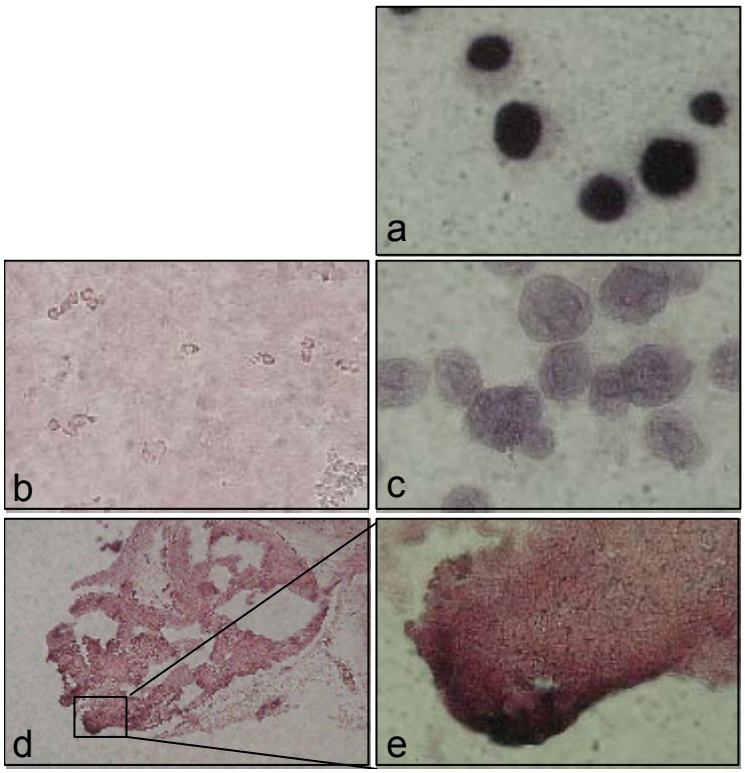


Figure 8. Contribution of DAS 104-8 cells into chimaeric embryos analysed by DNA-DNA *in situ* hybridization. *In situ* hybridization was done on DAS 104-8 and on cells not transformed by PVMT, and on brain tissues isolated from non injected and chimaeric embryos developed from injected blastocysts. Anti-DIG antibody was coupled with alkaline phosphatase and during enzyme reaction with NBT/BCIP purple precipitate developed. a) DAS 104-8 cells cytospun and hybridized (magnification 400x); b) the brain section of the non-injected embryo (200x); c) cells that do not carry PVMT cytospun and hybridized (400x); d) H14 chimaeric embryo (100x); e) magnification of d) (200x).

As mentioned in the Introduction DAS 104-4 and DAS 104-8 are AGM-derived endothelial cell lines and express endothelial markers such as VEGF, CD34, vWF, FLK1, FLT1, CD31 and TIE-2 (Ohneda O et all 1998). The polyoma-middle T antigen has been shown to be a cause of endothelial cell tumours that could be used as a source for endothelial cell lines (Williams R et al 1989). The formation of capillary-like structures when grown on Matrigel also strongly suggests that these cell lines are endothelial. Obviously, DAS 104-4 and DAS 104-8 cells' endothelial characteristics decide which destiny they will have in embryonic environment and not their AGM-origin characteristics.

5.3 Injection of murine YSE (yolk sac endodermal) cells into murine blastocysts

In order to follow the homing properties of a cell line established from yolk sac visceral endoderm, YSE cells were injected into murine blastocysts. This environment is permissive for the development of all three germ layers. Following cell-cell interactions between donor cells and the blastocyst microenvironment, donor cells may show specific homing patterns. In this experiment 612 blastocysts were injected in 44 independent injections with 5-10 YSE cells per blastocyst. This resulted in 55 developed embryos, while 8 animals were born. As previously mentioned these cells were transformed with a recombinant retrovirus containing Simian virus large T (SV40 LT) antigen, therefore donor cells were traced in E12.5 –E16.5 embryos by SV40 LT antigen - specific PCRs. Analysed were: yolk sac, foetal liver, head, hind limb, foetal blood, AGM region, visceral part and rest of the embryo. DNA was isolated, and chimaeric tissues were detected by donor contribution PCRs. For every single embryo at least six independent donor contribution PCRs were done. SV40LT antigen PCRs were done separately from myogenin PCRs because of different PCR conditions. Serial dilutions 100%, 20%, 2%, 0.2%, 0% of YSE cells' DNA in wt DNA were a measure for donor contribution in chimaeric embryos. If the signal was positive at least twice in SV40 LT PCRs, and myogenin were detectable, the embryonic tissue was considered as positive. PCR products were separated on agarose gels, Southern blotted, and hybridized before autoradiography. 18 female mice injected as blastocysts with YSE cells were analysed by DNA-DNA *in situ* hybridization with Y chromosome-FITC labelled probe. Percent of developed animals was around 10%, and of these the percentage of chimaeric animals was around 24% (shown in Table 6). Strength of the donor signal in chimaeric embryos was from 0.2 to 2 % and weaker.

Table 6. Summary of YSE cells injection into murine blastocysts.

Cell line	Nr. of injections	Nr. of injected blastocysts	Nr. of embryos	Nr. of adults	Percent of developed animals	Nr. of chimeras	Percent of chimeras
YSE	44	617	55	8	~10% (63/617)	15	~24% (15/63)

Adult animals were sacrificed at the age of 1-1.5 month, 18 different tissues were dissected, the same as previously mentioned and analysed by donor contribution PCRs. None of the 8 adult animals had a signal in any of the analysed tissues (Figure 10).

Figure 9 is a representative Southern blot analysis illustrating homing of YSE to yolk sac in chimaeric embryo Y4. In Table 7 donor contribution in different tissues of all analysed embryos developed after YSE injection is shown.

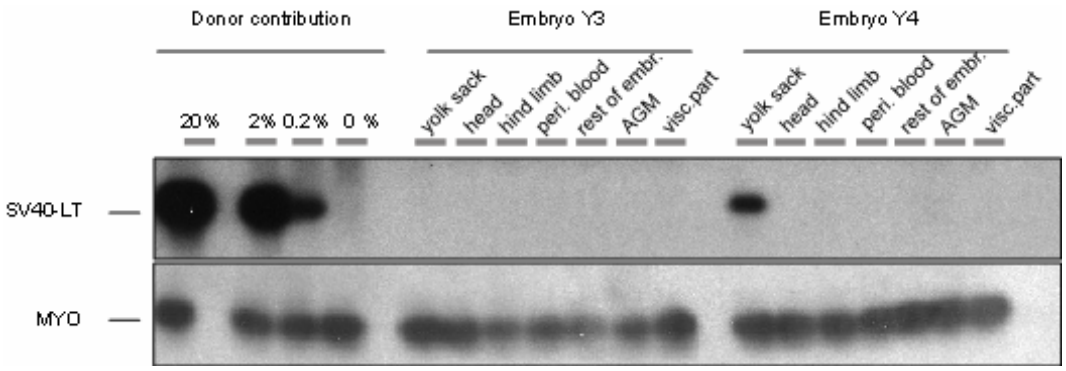


Figure 9. Donor contributions in chimaeric recipients following injection of YSE cells. Embryos Y3 and Y4 were E12.5 stage of development. Controls are 20%, 2%, 0.2% and 0% genomic DNA from YSE cells diluted in wt genomic DNA. Abbreviations: MYO, myogenin; SV40LT, Simian virus large T antigen; AGM, aorta-gonad-mesonephros region; visc. part, visceral part; rest of embr., rest of the embryo.

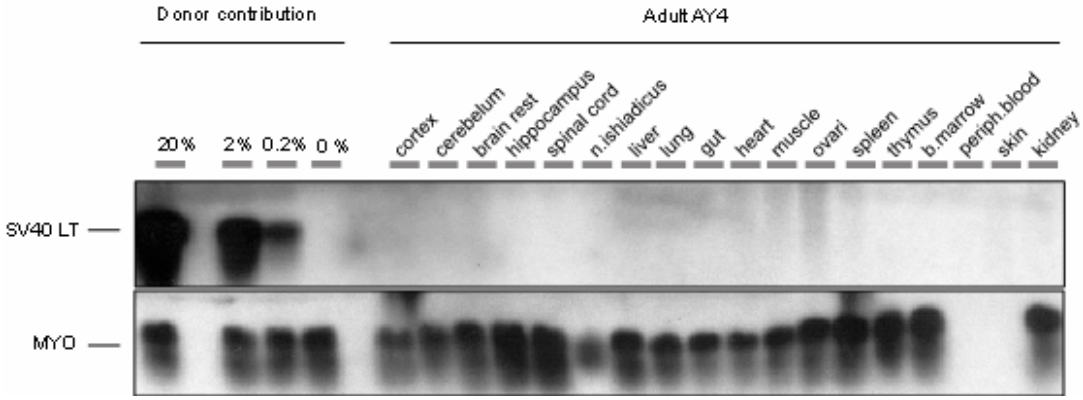


Figure 10. The analysis of donor contribution in the adult animal Y4 that developed from the blastocyst injected with YSE cells. Chimaeric adult animal Y4 was sacrificed at the age of 1 month, 18 different tissues were dissected, DNA was isolated and analysed by donor contribution PCR. Controls are 20%, 2%, 0.2% and 0% genomic DNA isolated from YSE cells diluted in wt genomic DNA. Abbreviations: MYO,myogenin.; SV40LT, Simian virus large T antigen; n. ishiadicus, *nervus ishiadicus*; b. marrow, bone marrow; periph. blood, peripheral blood.

Table 7. The donor contribution in different tissues of the embryos developed after YSE injection.

tissue / embryo	Yolk sac	Foetal liver	Head	Hind limbs	Foetal blood	Rest of embryo	Visceral part
YS1 E15.5	+	na	-	-	-	-	-
YS2 E12.5	-	na	-	-	-	-	-
YS3 E12.5	-	na	-	-	-	-	-
YS4 E12.5	+	na	-	-	-	-	-
YS5 E12.5	-	na	-	-	-	-	-
YS6 E12.5	-	na	-	-	-	-	-
YS7 E12.5	-	na	-	-	-	-	-
YS8 E12.5	-	na	-	-	-	-	-
YS9 E12.5	-	na	-	-	-	-	-
Y10 E13.5	-	+	+	+	-	-	na
Y11 E13.5	-	+	+	+	-	-	na
Y12 E13.5	+	+	+	-	+	+	na
Y34 E16.5	+	-	-	-	-	-	na
Y35 E16.5	+	-	-	-	-	-	na
Y36 E16.5	-	-	-	-	-	-	na
Y37 E16.5	+	-	-	-	-	-	na
Y38 E16.5	-	-	-	-	-	-	na
Y40 E16.5	-	-	-	-	-	-	na
Y41 E16.5	+	+	+	-	-	+	na
Y42 E16.5	+	-	-	-	-	-	na
Y43 E16.5	-	-	-	-	-	-	na
Y44 E16.5	+	+	+	+	-	+	na
Y45 E16.5	-	-	-	-	-	-	na
Y46 E16.5	-	-	-	-	-	-	na
Y47 E16.5	-	-	-	-	-	-	na
Y48 E16.5	-	-	-	-	-	-	na
Y49 E16.5	-	-	-	-	-	-	na
Y50 E16.5	+	+	+	-	-	+	na
Y51 E16.5	-	-	-	-	-	-	na
Y52 E16.5	-	-	-	-	-	-	na
Y53 E16.5	-	-	-	-	-	-	na
Y54 E16.5	-	-	-	-	-	-	na
Y55 E16.5	-	-	-	-	-	-	na
Summary	**10/33**	**6/24**	**6/33**	**3/33**	**1/33**	**4/33**	**0/9**
Percent	**30%**	**25%**	**18%**	**9%**	**3%**	**12%**	**0%**

E12.5-E16.5 stands for embryonic day post coitum. Summary demonstrates how many embryos show donor signals in the specific tissue out of all embryos. The same is shown in percentage. Abbreviations: na, not analysed; (-), no donor signal; (+), positive donor signal.

This study demonstrates that YSE cells preferentially seed yolk sac and foetal liver, while the other tissues are less frequently engrafted. This cell line maintains its yolk sac-derived character. The reason for the high donor contribution in foetal liver may lie in the similarity of visceral endoderm cells of the embryonic yolk sac with foetal liver cells in respect of absorption and secretion of materials from the yolk sac cavity. The yolk sac endoderm cells have endocytic activity including apically located microvilli, coated pits and intracellular tight junctions (Hogan B et al 1986). Due to similar functions of YSE cells and foetal liver cells, through the interaction of blastocyst environment and the cells, similar epitopes could lead YSE cells to seed the foetal liver during mouse development.

In order to identify donor cells on embryonic sections *in situ*, DNA-DNA *in situ* hybridization with Y chromosome FITC-labelled probe was carried out.

Since this study looks for an answer at the question as to whether injected and engrafting cells are able to change their fate in developing embryos, combined immunostaining with DNA-DNA *in situ* hybridization was performed. For yolk sac visceral endoderm, Type I activin receptor (ActRIB) immunostaining was used, which is also expressed on YSE cells before injection. After analysing the DNA-DNA *in situ* data shown in Table 8, many embryos revealed Y chromosome positive cells in the head. In order to determine whether YSE cells had taken on neuronal /glial /neuronal progenitor -like characteristics, immunostaining for the neuronal cell adhesion molecule (NCAM) was done.

Table 8. Distribution of the donor cells in embryonic tissues analysed by DNA-DNA *in situ* hybridization.

tissue / embryo	YS	FL	Head	HL	CP	intestine	lungs	skin
Y25 E11.5	**4/1000**	0/6250	**4/2800**	**4/2000**	0/4500	na	na	**5/1000**
Y26 E15.5	0/3000	0/14500	**5/58000**	na	0/7800	na	na	0/4200
Y28 E16.5	**1/4600**	0/15000	**2/60000**	0/3000	0/6500	0/1200	na	0/1500
Y41 E16.5	na	0/25000	na	na	0/7000	**2/1400**	0/30000	0/1300
Y44 E16.5	0/4000	0/38000	0/20000	0/3400	0/6300	na	0/25000	0/10000
Y49 E16.5	0/3500	0/30000	**3/20000**	0/2750	0/10000	0/1100	0/25000	0/12000
Y51 E16.5	0/4800	0/80000	0/32000	0/4000	0/8000	0/1000	0/50000	**1/6000**
Y52 E16.5	**1/1800**	0/45000	0/38000	0/3500	0/9500	na	na	**1/7000**
Y54 E16.5	0/2000	0/40000	0/30000	0/2800	na	na	0/27000	0/8000
Y55 E16.5	na	0/32000	0/35000	na	na	0/1000	0/22000	0/11000

Embryos numbered Y25-Y55 developed after blastocyst injection with YSE cells. They were E11.5-E16.5 stage of development when analysed. Numbers present number of Y-FITC positive cells found in the total number of analysed cells. Cell number estimation was performed with a microscope ocular that has an engraved square on it. Section thickness' was 5µm. Abbreviations: YS, yolk sac; FL, foetal liver; HL, hind limbs; CP, cartilage primordia; na, not available for analysis.

In Table 8 the contributions of yolk sac-derived YSE cell lines to the developing embryos are described. The 18 female embryos that developed after injection of YSE cells into blastocysts were analysed by DNA-DNA *in situ* hybridization with Y-FITC probe. Table 8 shows only 10 embryos. The other 8 analysed embryos did not contain donor cells. In the chimaeric embryos donor cells were not found in foetal liver, which can be explained

with the fact that only a limited number of cells could be analysed, while in donor contribution PCRs DNA is prepared from the whole foetal liver. Donor contribution PCR is therefore a more sensitive method of analysis.

Since the highest contribution was in head, combined immunostaining for NCAM, a neuronal marker, together with *in situ* hybridization for Y chromosome positive cells was performed. Yolk sac was the most engrafted tissue in donor contribution PCR survey, so combined immunostaining for ActRIB, yolk sac visceral endoderm's marker, with *in situ* hybridization for Y chromosome-FITC was done. Results are illustrated in Table 9.

Table 9. Frequency of donor cells in embryonic tissues analysed by combined immunostaining for markers ActRIB and NCAM, and DNA-DNA *in situ* hybridization with a Y chromosome-specific probe.

tissue / embryo	YS (ActRIB)	Head (NCAM)
Y25 E11.5	**4/1000** ActRIB$^+$, Y$^+$ 0/1000 ActRIB$^-$, Y$^+$	0/2800 NCAM$^+$, Y$^+$ **5/2800** NCAM$^-$, Y$^+$
Y26 E15.5	0/3000 ActRIB$^+$,Y$^+$ 0/3000 ActRIB$^-$, Y$^+$	0/58000 NCAM$^+$, Y$^+$ **7/58000** NCAM$^-$, Y$^+$
Y28 E16.5	0/4600 ActRIB$^+$, Y$^+$ 0/4600 ActRIB$^-$, Y$^+$	0/60000 NCAM$^+$, Y$^+$ **1/60000** NCAM$^-$, Y$^+$
Y41 E16.5	na	na
Y44 E16.5	0/4000 ActRIB$^+$, Y$^+$ 0/4000 ActRIB$^-$, Y$^+$	0/20000 NCAM$^+$, Y$^+$ 0/20000 NCAM$^-$, Y$^+$
Y49 E16.5	0/3500 ActRIB$^+$, Y$^+$ 0/3500 ActRIB$^-$, Y$^+$	0/20000 NCAM$^+$, Y$^+$ **3/20000** NCAM$^-$, Y$^+$
Y51 E16.5	0/4800 ActRIB$^+$, Y$^+$ 0/4800 ActRIB$^-$, Y$^+$	0/32000 NCAM$^+$, Y$^+$ 0/32000 NCAM$^-$, Y$^+$
Y52 E16.5	0/1800 ActRIB$^+$, Y$^+$ 0/1800 ActRIB$^-$, Y$^+$	0/38000 NCAM$^+$, Y$^+$ 0/38000 NCAM$^-$, Y$^+$
Y54 E16.5	0/2000 ActRIB$^+$, Y$^+$ 0/2000 ActRIB$^-$, Y$^+$	0/30000 NCAM$^+$, Y$^+$ 0/30000 NCAM$^-$, Y$^+$
Y55 E16.5	na	0/35000 NCAM$^+$, Y$^+$ 0/35000 NCAM$^-$, Y$^+$

Embryos were developed after blastocyst injection with YSE cells. Numbers present number of the donor cells found in the total number of analysed cells. Estimation of the total cell number was performed with a microscope ocular that has the engraved square on it. Section thickness' was 5µm. Abbreviations: YS, yolk sac; ActRIB, type I activin receptor; NCAM, neural cell adhesion molecule; ActRIB$^+$, Y$^+$ or NCAM$^+$, Y$^+$, frequency of ActRIB positive or NCAM positive and Y chromosome positive cells per analysed cell number; ActRIB$^-$, Y$^+$ or NCAM$^-$, Y$^+$, frequency of ActRIB negative or NCAM negative, but Y chromosome positive cells per analysed cell number; Y25-Y55, numbers of the embryos; E11.5-E16.5, stage of development of analysed embryos.

Table 9 shows that ActRIB and Y-FITC double positive cells are detectable in yolk sac at very low frequencies. Only 4/1000 yolk sac cells were positive for ActRIB and Y-FITC. All the cells that were found in the head of different embryos were positive only for Y-FITC, but none showed expression of NCAM. This suggests that YSE cells show less promiscuity, more inflexibility then DAS 104-4 and DAS 104-8 cells, that they maintain their characteristics and return to their tissue of origin. One of the supportive data would

be that no adult animals show engraftment in any of 18 isolated tissues. Since the yolk sac is an extraembryonic tissue and it is the most frequent seeded, according to donor contribution PCR, there is a high probability that after birth the majority of engrafted YSE cells are lost.

Figure 11, panel a) shows a merge of immunostaining against ActRIB (red), DAPI (blue) nuclear staining, and *in situ* hybridization with Y-FITC probe (green) performed on YSE cytospun cells. Figure 11, panel b) illustrates a female tissue as a negative control, *in situ* hybridized with Y-FITC, and stained with DAPI, but not subjected to immunostaining against ActRIB. Figure 11, panel c) shows cytospun YSE cells which were not subjected to immunostaining or Y-FITC hybridization (further negative control), just stained with DAPI. Figure 11, panel d) shows the male tissue hybridized with Y-FITC, as a positive control, stained with DAPI, but not immunostained against ActRIB.

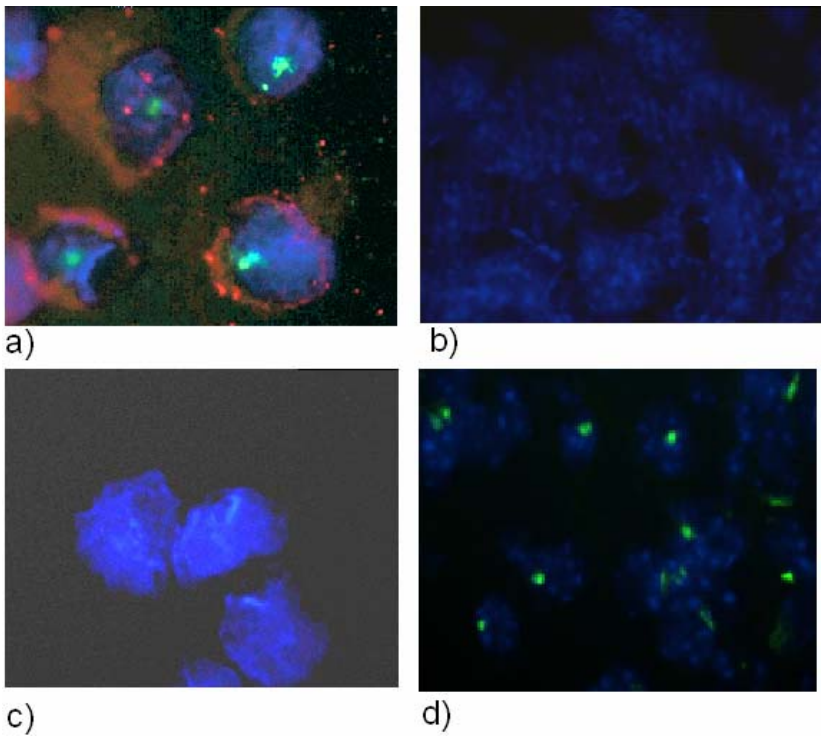


Figure 11. Control immunostaining of YSE cells and *in situ* hybridization with Y-FITC probe. a) cytospun YSE cells, stained with ActRIB (red), *in situ* hybridized with Y-FITC probe (green dot) and stained with DAPI (nuclear stain, blue) magnification 600x; b) female tissue, *in situ* hybridized with Y-FITC probe and stained with DAPI, no immunostaining, magnification 400x; c) cytospun YSE cells, stained with DAPI, no immunostaining and no DNA *in situ* hybridization, magnification 600x; d) male tissue *in situ* hybridized with Y-FITC probe and stained with DAPI, no immunostaining, magnification 400x

Figure 12 illustrates the yolk sac of female embryo Y25, developed after injection of YSE cells, and analysed by combined immunostaining against ActRIB, the yolk sac visceral marker, and Y chromosome specific *in situ* hybridization. In Figure12, panel a) illustrates

donor Y-FITC positive cell, panel b) staining of yolk sac cells against ActRIB and panel c) yolk sac cells stained with nuclear dye DAPI. Merge of all 3 layers revealed the donor Y chromosome FITC positive cell among the recipient female cells in yolk sac of the embryo Y25.

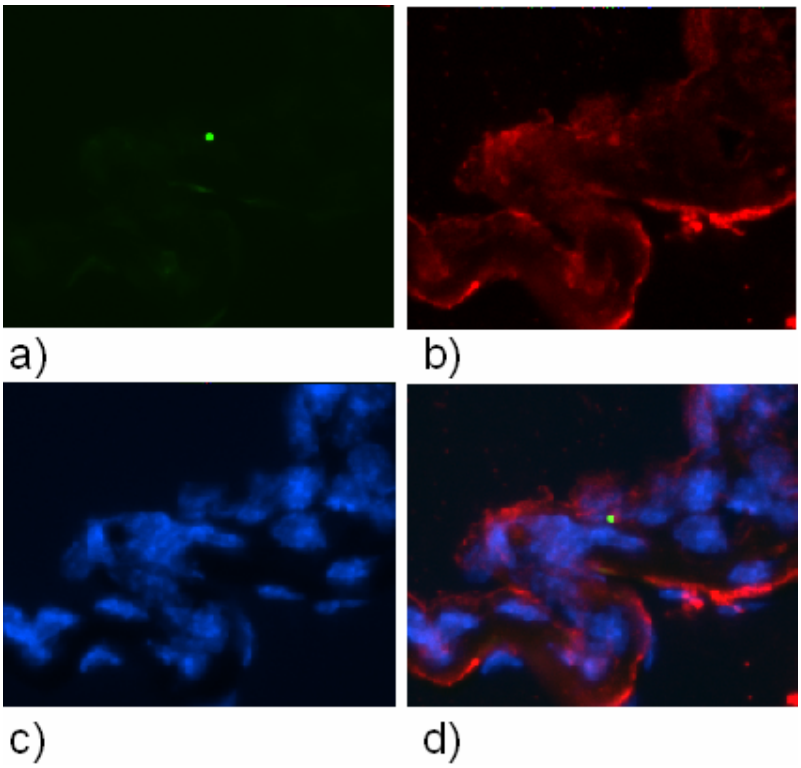


Figure 12. Yolk sac of chimaeric embryo Y25. a) yolk sac hybridized with Y-FITC; b) yolk sac immunostained for ActRIB; c) yolk sac stained for nuclear die DAPI; d) merge; magnification 400x

5.4 Injection of FDCP mix (factor dependant cell Paterson) cells into murine blastocysts

Using the same techniques as described above, cells from the male FDCP mix cell line were injected into murine blastocysts. 10 to 20 cells were injected, during 28 independent injections into a total of 948 blastocysts. 78 embryos and 23 adults developed. Altogether ~10% (101/948) animals developed. 45 /78 embryos were males and 33 /78 embryos were females. 12 /23 adult animals were females and 11/23 were males (Table 10). Since these cells are traced through their male nature, i.e. via the Y chromosome, only female embryos and adults were analysed by Y chromosome specific PCRs. YMT2/B primers for specific sequences on Y chromosome (Medvinsky and Dzierzak, 1996) and primers specific for myogenin gene as internal PCR controls were used (Müller et al 1994). 17 female embryos were analysed by *in situ* hybridization with a Y chromosome FITC labelled probe. 33 female embryos were analysed by Y chromosome specific - donor

contribution PCRs. 12 female adults were analysed by donor contribution PCRs. Altogether 45 female animals were analysed. 33 (26 embryos and 7 adult animals) out of 45 were chimaeric, thus around 73% of analysed animals carried donor cells (Table 10).

Table 10. Summary of FDCPmix cells injection into murine blastocysts.

Cell line	Nr. of injections	Nr. of injected blastocysts	Nr. of developed embryos	Nr. of adults	Percent of developed animals	Nr. of chimeras	Percent of chimeras
FDCP mix	28	948	78 (45m+33f)	23 (12f+11m)	~10% (101/948)	33 (26e+7a)	~73% (33/45)

Abbreviations: m, male; f, female; e, embryo; a, adult;

8 different parts of developing E11.5-E16.5 embryos were isolated: yolk sac, foetal liver, head, hind limb, foetal blood, AGM region, - in embryos E11.5 and E12.5, visceral part, and rest of the embryo and analysed. DNA was isolated, and chimaeric tissues were detected by donor contribution PCRs. For each embryo at least three independent donor contribution PCRs were done. Serial dilutions 100%, 20%, 2%, 0.2%, 0% of male DNA in female DNA were a measure for donor contribution in chimaeric embryos. If the signal was positive at least twice, the embryo tissue was considered as positive. PCR products were preceded as previously described and autoradiographs were analysed.

Table 11 illustrates the distribution of male FDCP mix donor cells in E11.5-E16.5 embryos. The highest donor contribution was found in yolk sac 73%, AGM 50%, foetal blood 47% and foetal liver 37%, and the contribution in visceral part in E11.5-E12.5 embryos is 37%. One can say that these cells preferentially seed haematopoietic tissues, which would be expected because they are a haematopoietic progenitor cell line. Strength of the donor signals in chimaeric embryos was from <0.2->2%.

Table 11. The distribution of bone marrow derived -male FDCP mix donor contribution in embryos developed after FDCP mix cell injection into murine blastocysts.

tissue / embryo	Yolk sac	Foetal liver	Head	Hind limbs	Foetal blood	Rest of embryo	AGM region	Visceral part
F1 E11.5	-	na	-	-	-	-	-	-
F16 E11.5	-	na	-	-	-	-	-	-
F17 E11.5	-	na	-	-	+	-	-	-
F19 E11.5	+	na	+	+	-	-	-	-
F21 E11.5	-	na	-	-	+	-	+	+
F22 E11.5	+	na	-	-	-	-	+	+
F24 E12.5	+	na	-	+	-	-	-	-
F25 E12.5	-	na	-	-	-	-	-	-
F26 E12.5	-	na	-	-	-	-	-	-
F31 E12.5	+	na	-	-	-	-	-	+
F32 E12.5	+	na	-	-	+	-	+	-
F34 E12.5	+	na	-	-	-	-	+	-
F35 E12.5	+	na	+	+	+	+	+	+
F37 E12.5	+	na	-	+	+	+	+	-
F40 E12.5	+	+	-	+	-	+	+	+
F42 E12.5	+	+	+	-	-	+	+	+
F56 E16.5	+	-	-	-	+	-	na	na
F57 E16.5	+	-	-	-	+	-	na	na
F59 E16.5	+	-	-	-	+	-	na	na
F60 E16.5	+	-	-	+	+	-	na	na
F63 E14.5	+	+	-	+	-	-	na	na
F70 E16.5	+	+	-	-	+	+	na	na
F73 E16.5	-	-	-	+	-	+	na	na
F74 E16.5	+	+	-	-	+	-	na	na
F77 E16.5	+	-	-	-	+	+	na	na
F79 E16.5	+	-	-	-	-	-	na	na
F82 E16.5	+	+	+	+	-	+	na	na
F84 E16.5	-	-	-	-	-	-	na	na
F86 E16.5	+	-	-	-	+	-	na	na
F93 E16.5	+	-	-	+	+	-	na	na
Summary	**22/30**	**6/16**	**4/30**	**10/30**	**14/30**	**8/30**	**8/16**	**6/16**
Percent	**73%**	**37%**	**13%**	**33%**	**47%**	**27%**	**50%**	**37%**

Abbreviations: na, not analysed; (-), no donor signal; (+), positive donor signal. Summary shows how many embryos have positive signal in the specific tissue out of all embryos, and the same in percentage. E11.5-E16.5 stands for embryonic stage of development.

Adult animals were sacrificed at age of 1-1.5 month, 18 different tissues were dissected, the DNA was isolated and analysed by Y chromosome specific PCRs. Isolated adult tissues were the same as for the previously analysed adult animals developing after blastocysts injection of YSE and DAS 104-8 cells. Table 12 describes the distribution of FDCP mix contribution in adult animals. Adult animals are labelled with numbers A1-A13 and the isolated tissues are numerated as formerly shown. If there was twice repeated donor signal in the adult tissue after at least three independent YMT2/B PCRs, tissue is labelled as positive (+). If there was no donor contribution in specific tissue, it was considered as negative (-). Strength of the donor signal in chimaeric adults was from <0.2->2%.

According to the data shown in table 12, the most seeded tissues with 33% are neuronal (rest of the brain and hippocampus) and muscle and ovary, then adult liver 25%, n. ishiadicus, spinal cord, lung, gut and thymus 17%, while in bone marrow and peripheral blood these cell progeny was found in 8%. Another haematopoietic tissue, spleen, was not found to be chimaeric at all.

Table 12. Distribution of bone marrow derived -male FDCP mix donor contribution in the adult animals developed after FDCP mix cell injection into murine blastocysts.

adult / tissue	A1	A2	A3	A4	A6	A7	A8	A9	A10	A11	A12	A13	summary	percent
1	+	-	-	-	-	-	-	-	-	-	-	-	1/12	8%
2	-	-	-	-	-	-	-	-	-	-	-	-	0/12	0%
3	+	+	+	-	-	-	-	-	-	-	-	+	4/12	33%
4	-	+	-	+	-	-	+	-	+	-	-	-	4/12	33%
5	-	-	-	-	-	-	+	-	+	-	-	-	2/12	17%
6	-	-	-	-	-	-	+	-	+	-	-	-	2/12	17%
7	-	+	-	-	+	-	+	-	-	-	-	-	3/12	25%
8	+	+	-	-	-	-	-	-	-	-	-	-	2/12	17%
9	-	+	-	-	-	-	+	-	-	-	-	-	2/12	17%
10	-	-	-	-	-	-	-	-	-	-	-	-	0/12	0%
11	+	+	+	-	-	-	-	-	+	-	-	-	4/12	33%
12	-	+	+	-	-	-	+	-	+	-	-	-	4/12	33%
13	-	-	-	-	-	-	-	-	-	-	-	-	0/12	0%
14	-	+	-	-	-	-	+	-	-	-	-	-	2/12	17%
15	-	-	-	-	-	-	-	-	-	-	-	+	1/12	8%
16	-	+	-	-	-	-	-	-	-	-	-	-	1/12	8%
17	-	-	-	-	-	-	+	-	-	-	-	-	1/12	8%
18	-	-	-	-	-	-	+	-	-	-	-	-	1/12	8%

Adult animals were 1-1.5 months old when analysed. Summary shows how many adult animals have positive signals in specific tissues out of all analysed adults; the same is shown in percentage. Abbreviations: na , not analysed; (-), no donor signal; (+), positive donor signal ; A1-A13 - numbers of adult animals; analysed tissues: 1.cortex; 2.cerebelum; 3.rest of the brain; 4.hippocampus; 5.spinal cord; 6.n.ishiadicus; 7.liver; 8.lung; 9.gut; 10.heart; 11.muscle; 12.ovary; 13.spleen; 14.thymus; 15.bone marrow; 16.peripheral blood; 17.skin; 18.kidney

A representative Southern blot of analysed embryos is shown in Figure 13. Embryos F82, F84, F86 were analysed at E16.5. Embryo F84 shows representative distribution of donor contribution, the strongest donor signals in yolk sac, foetal liver and foetal blood and less in hind limbs and the rest of the embryo. Embryo F86 has the strongest male signal in foetal blood, yolk sac and hind limbs, while embryo F82 shows a signal in the hind limbs.

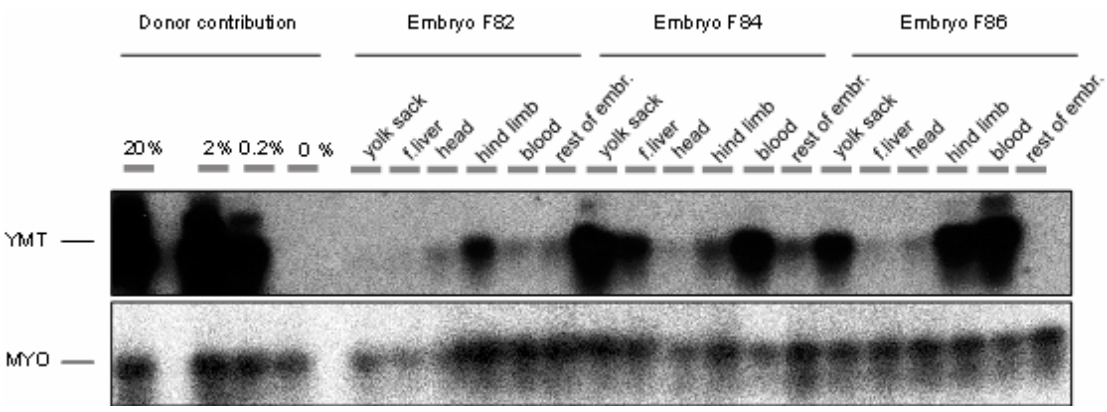


Figure 13. The donor contributions in chimaeric recipients following injection of FDCP mix cells into blastocysts. Analysed embryos F82, F84 and F86 were E16.5. Controls are 20%, 2%, 0.2% and 0% genomic DNA from wt male tissue diluted in wt female genomic DNA. Abbreviations: MYO, myogenin; YMT, sequence on murine Y chromosome; f. liver, foetal liver; rest of embr, rest of the embryo.

A representative Southern blot analysis of chimaeric adult animal AF10 is shown in figure 14. Adult 10 has more then 2% donor contribution in hippocampus, less then 0.2% in spinal cord and *nervus ishiadicus* and weak donor signal in ovaries and muscle.

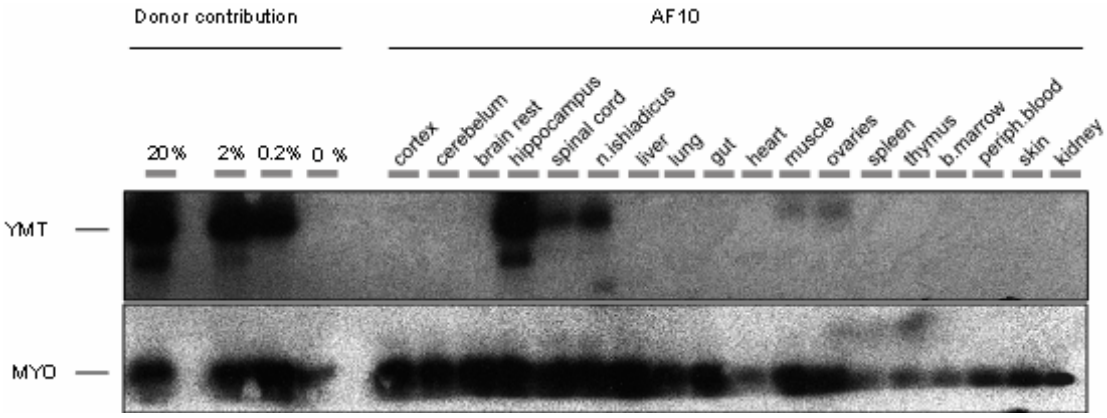


Figure 14. The donor contribution in the chimaeric adult animal AF10 developed from blastocysts injected with FDCP mix cells. Chimaeric adult animal AF10 was sacrificed at age of 1 month, 18 different tissues analysed. Controls are 20%, 2%, 0.2% and 0% genomic DNA from wt male diluted in wt female genomic DNA. Abbreviations: MYO myogenin; YMT, sequence on murine Y chromosome; n. ishiadicus, *nervus ishiadicus*; b. marrow, bone marrow; periph. blood, peripheral blood.

In order to find out whether the FDCP mix cells detected in chimaeric embryos maintained their haematopoietic character, combined immunostaining for the pan-haematopoietic marker CD45 and DNA-DNA *in situ* hybridization with Y chromosome FITC labelled probe was carried out on sections of embryos. The results are summarized in Table 13. 17 female embryos were analysed by combined immunostaining with *in situ* hybridization (Y chromosome FITC labelled probe). In Table 13 only 12 embryos are shown. The embryos F46, F47, F63, and F79 were negative and embryo F49 had positive

Y-chromosome signal in cartilage primordia (Figure 15). The majority of the Y chromosome–FITC positive cells were CD45 positive, and the tissues with the highest repopulation frequency were cartilage primordia (future vertebra and site of E15-E16.5 and adult haematopoiesis), head, hind limbs (also future bones and site of haematopoiesis) and skin with a lower frequency. The foetal liver and yolk sac were also highly repopulated, while CD45 positive Y chromosome–FITC positive cells were found in the intestine and the lungs to less extent. Since some of the cells were CD45 negative Y chromosome–FITC positive, it was interesting to investigate whether these cells where still haematopoietic or they had changed their nature.

Most of the CD45 negative Y chromosome–FITC positive cells were found in the foetal liver and in the head. Therefore, in the following investigation, combined immunostaining for albumin (a marker for foetal liver cells) with *in situ* hybridization with Y chromosome FITC probe was performed. Additionally, combined immunostaining for neuronal cell adhesion molecule -NCAM (a marker for the neuronal and glial cells) with *in situ* hybridization with Y chromosome FITC probe was carried out. The results are shown in Table 14. In the foetal liver of analysed embryos donor (Y chromosome positive) cells were found, but none were albumin positive. Some donor (Y chromosome positive), NCAM positive cells were found in the foetal brain at a very low percentage.

Table 13. Distribution of donor cells in embryonic tissues after combined immunostaining for the pan- haematopoietic marker CD45 and DNA-DNA *in situ* hybridization with a Y chromosome FITC labelled probe.

tissue / emb	YS	FL	Head	HL	CP	intestine	skin	lungs
F56	**6/6900** CD45$^+$, Y$^+$ 0/6900 CD45$^-$, Y$^+$	0/17500 CD45$^+$, Y$^+$ 0/17500 CD45$^-$, Y$^+$	**5/6250** CD45$^+$, Y$^+$ **5/6250** CD45$^-$, Y$^+$	**1/9000** CD45$^+$, Y$^+$ 0/9000 CD45$^-$, Y$^+$	0/2000 CD45$^+$, Y$^+$ 0/2000 CD45$^-$, Y$^+$	na	**1/7050** CD45$^+$, Y$^+$ 0/7050 CD45$^-$, Y$^+$	0/15000 CD45$^+$, Y$^+$ 0/15000 CD45$^-$. Y$^+$
F57	0/5600 CD45$^+$, Y$^+$ 0/5600 CD45$^-$, Y$^+$	0/22000 CD45$^+$, Y$^+$ 0/22000 CD45$^-$, Y$^+$	**1/10500** CD45$^+$, Y$^+$ 0/10500 CD45$^-$, Y$^+$	0/8000 CD45$^+$, Y$^+$ 0/8000 CD45$^-$, Y$^+$	na	na	**4/10500** CD45$^+$, Y$^+$ **2/10500** CD45$^-$, Y$^+$	na
F59	0/4800 CD45$^+$, Y$^+$ 0/4800 CD45$^-$, Y$^+$	**5/15360** CD45$^+$, Y$^+$ 0/15360 CD45$^-$, Y$^+$	0/30000 CD45$^+$, Y$^+$ 0/30000 CD45$^-$, Y$^+$	0/8500 CD45$^+$, Y$^+$ 0/8500 CD45$^-$, Y$^+$	0/1800 CD45$^+$, Y$^+$ 0/1800 CD45$^-$, Y$^+$	**10/3000** CD45$^+$, Y$^+$ 0/3000 CD45$^-$, Y$^+$	0/10000 CD45$^+$, Y$^+$ 0/10000 CD45$^-$, Y$^+$	0/10000 CD45$^+$, Y$^+$ 0/10000 CD45$^-$, Y$^+$
F60	na	**2/10000** CD45$^+$, Y$^+$ **2/10000** CD45$^-$, Y$^+$	**3/35000** CD45$^+$, Y$^+$ 0/35000 CD45$^-$, Y$^+$	0/6500 CD45$^+$, Y$^+$ 0/6500 CD45$^-$, Y$^+$	**5/2500** CD45$^+$, Y$^+$ 0/2500 CD45$^-$, Y$^+$	na	**1/9800** CD45$^+$, Y$^+$ 0/9800 CD45$^-$, Y$^+$	0/9000 CD45$^+$, Y$^+$ 0/9000 CD45$^-$, Y$^+$
F70	**2/4300** CD45$^+$, Y$^+$ **2/4300** CD45$^-$, Y$^+$	0/12000 CD45$^+$, Y$^+$ 0/12000 CD45$^-$, Y$^+$	0/32000 CD45$^+$, Y$^+$ 0/32000 CD45$^-$, Y$^+$	**4/7000** CD45$^+$, Y$^+$ 0/7000 CD45$^-$, Y$^+$	**5/2000** CD45$^+$, Y$^+$ 0/2000 CD45$^-$, Y$^+$	na	0/10000 CD45$^+$, Y$^+$ 0/10000 CD45$^-$, Y$^+$	0/7500 CD45$^+$, Y$^+$ 0/7500 CD45$^-$, Y$^+$
F73	**2/3000** CD45$^+$, Y$^+$ 0/3000 CD45$^-$ Y$^+$	0/15000 CD45$^+$, Y$^+$ 0/15000 CD45$^-$, Y$^+$	**3/30000** CD45$^+$, Y$^+$ 0/30000 CD45$^-$, Y$^+$	**3/7000** CD45$^+$, Y$^+$ 0/7000 CD45$^-$, Y$^+$	**7/7520** CD45$^+$, Y$^+$ 0/7520 CD45$^-$, Y$^+$	na	0/15000 CD45$^+$, Y$^+$ 0/15000 CD45$^-$, Y$^+$	0/8000 CD45$^+$, Y$^+$ 0/8000 CD45$^-$, Y$^+$
F74	0/4600 CD45$^+$, Y$^+$ 0/4600 CD45$^-$, Y$^+$	0/20000 CD45$^+$, Y$^+$ 0/20000 CD45$^-$, Y$^+$	0/38000 CD45$^+$, Y$^+$ 0/38000 CD45$^-$, Y$^+$	**7/6000** CD45$^+$, Y$^+$ 0/6000 CD45$^-$, Y$^+$	**3/1500** CD45$^+$, Y$^+$ 0/1500 CD45$^+$, Y$^+$	0/4500 CD45$^+$, Y$^+$ 0/4500 CD45$^-$, Y$^+$	**7/8000** CD45$^+$, Y$^+$ 0/8000 CD45$^-$, Y$^+$	0/5500 CD45$^+$, Y$^+$ 0/5500 CD45$^-$, Y$^+$
F77	na	0/16000 CD45$^+$, Y$^+$ 0/16000 CD45$^-$, Y$^+$	**4/40000** CD45$^+$, Y$^+$ 0/40000 CD45$^-$, Y$^+$	**7/11000** CD45$^+$, Y$^+$ 0/11000 CD45$^-$, Y$^+$	0/2400 CD45$^+$, Y$^+$ 0/2400 CD45$^-$, Y$^+$	0/3600 CD45$^+$, Y$^+$ 0/3600 CD45$^-$, Y$^+$	**2/9000** CD45$^+$, Y$^+$ 0/9000 CD45$^-$, Y$^+$	**10/6000** CD45$^+$, Y$^+$ 0/6000 CD45$^-$, Y$^+$
F82	**3/9400** CD45$^+$, Y$^+$ **6/9400** CD45$^-$, Y$^+$	0/18000 CD45$^+$, Y$^+$ 0/18000 CD45$^-$, Y$^+$	**4/30000** CD45$^+$, Y$^+$ 0/30000 CD45$^-$, Y$^+$	0/7000 CD45$^+$, Y$^+$ 0/7000 CD45$^-$, Y$^+$	0/2800 CD45$^+$, Y$^+$ 0/2800 CD45$^-$, Y$^+$	na	0/7500 CD45$^+$, Y$^+$ 0/7500 CD45$^-$, Y$^+$	0/8000 CD45$^+$, Y$^+$ 0/8000 CD45$^-$, Y$^+$
F84	0/7600 CD45$^+$, Y$^+$ 0/7600 CD45$^-$, Y$^+$	**2/15000** CD45$^+$, Y$^+$ **1/15000** CD45$^-$, Y$^+$	**10/35000** CD45$^+$, Y$^+$ 0/35000 CD45$^-$, Y$^+$	0/7500 CD45$^+$, Y$^+$ 0/7500 CD45$^-$, Y$^+$	**8/4500** CD45$^+$, Y$^+$ **2/4500** CD45$^-$, Y$^+$	**1/7500** CD45$^+$, Y$^+$ **2/7500** CD45$^-$, Y$^+$	**2/10000** CD45$^+$, Y$^+$ 0/10000 CD45$^-$, Y$^+$	0/6200 CD45$^+$, Y$^+$ 0/6200 CD45$^-$, Y$^+$
F86	**4/6000** CD45$^+$, Y$^+$ 0/6000 CD45$^-$, Y$^+$	**7/32000** CD45$^+$, Y$^+$ 0/32000 CD45$^-$, Y$^+$	0/30000 CD45$^+$, Y$^+$ 0/30000 CD45$^-$, Y$^+$	**4/12000** CD45$^+$, Y$^+$ 0/12000 CD45$^-$, Y$^+$	**6/3200** CD45$^+$, Y$^+$ **3/3200** CD45$^-$, Y$^+$	na	**2/10000** CD45$^+$, Y$^+$ 0/10000 CD45$^-$, Y$^+$	**1/30000** CD45$^+$, Y$^+$ 0/30000 CD45$^-$, Y$^+$
F93	0/6300 CD45$^+$, Y$^+$ 0/6300 CD45$^-$, Y$^+$	0/22000 CD45$^+$, Y$^+$ 0/22000 CD45$^-$, Y$^+$	0/40000 CD45$^+$, Y$^+$ 0/40000 CD45$^-$, Y$^+$	0/9000 CD45$^+$, Y$^+$ 0/9000 CD45$^-$, Y$^+$	0/2600 CD45$^+$, Y$^+$ 0/2600 CD45$^-$, Y$^+$	0/5000 CD45$^+$, Y$^+$ 0/5000 CD45$^-$, Y$^+$	0/9200 CD45$^+$, Y$^+$ 0/9200 CD45$^-$, Y$^+$	0/9000 CD45$^+$, Y$^+$ 0/9000 CD45$^-$, Y$^+$

Embryos were E16.5 stage of development when analysed. Numbers present a number of Y-FITC positive cells found in the total number of analysed cells. Estimations of the cell numbers were performed with a microscope ocular that has the engraved square on it. Section thickness' was 5µm. Abbreviations: emb, embryo; YS, yolk sac; FL, foetal liver; HL, hind limbs; CP, cartilage primordia; na, not available for analysis; F56-F93, numbers of the embryos; CD45$^+$, pan-haematopoietic marker; CD45$^+$, Y$^+$, frequency of CD45 positive and Y chromosome positive cells per analysed cell number; CD45$^-$, Y$^+$, frequency of CD45 negative but Y chromosome positive cells per analysed cell number.

Table 14. Distribution of donor cells in embryonic tissues analysed by combined immunostaining for markers NCAM and albumin, and DNA-DNA *in situ* hybridization with a Y chromosome FITC labelled probe.

tissue / embryo	Head (NCAM)	FL (albumin)
F56	0/6250 NCAM$^+$,Y$^+$ **10/6250** NCAM$^-$, Y$^+$	na
F57	**1/10500** NCAM$^+$,Y$^+$ **1/10500** NCAM$^-$, Y$^+$	na
F59	na	0/15360 albumin$^+$, Y$^+$ **2/15360** albumin$^-$, Y$^+$
F60	0/35000 NCAM$^+$,Y$^+$ 0/35000 NCAM$^-$, Y$^+$	0/10000 albumin$^+$, Y$^+$ **1/10000** albumin$^-$, Y$^+$
F70	na	na
F73	**6/30000** NCAM$^+$, Y$^+$ **4/30000** NCAM$^-$, Y$^+$	na
F74	na	na
F77	**1/40000** NCAM$^+$, Y$^+$ 0/40000 NCAM$^-$, Y$^+$	na
F82	0/30000 NCAM$^+$, Y$^+$ 0/30000 NCAM$^-$, Y$^+$	na
F84	0/35000 NCAM$^+$, Y$^+$ **1/35000** NCAM$^-$, Y$^+$	0/15000 albumin$^+$, Y$^+$ 0/15000 albumin$^-$, Y$^+$
F86	na	0/32000 albumin$^+$, Y$^+$ 0/32000 albumin$^-$, Y$^+$
F93	na	na

Estimations of the cell numbers were performed with a microscope ocular that has the engraved square on it. The section thickness' was 5µm. Numbers present the number of Y-FITC positive cells found in the total number of analysed cells. Abbreviations: NCAM, neural cell adhesion molecule; albumin, foetal liver marker; FL, foetal liver; F56-F93, numbers of the embryos; na, not analysed; NCAM$^+$, Y$^+$, frequency of NCAM positive and Y chromosome positive cells per analysed cell number; NCAM$^-$, Y$^+$, frequency of NCAM negative, but Y chromosome positive cells per analysed cell number.

In Figure15 indirect Y chromosome–FITC DNA-DNA *in situ* hybridization on 5µm thick section of female chimaeric embryo F49 has been shown. Anti-FITC antibody coupled with peroxidase (POD) and its substrate diaminobenzidine (DAB) develop a brown colour in the spot where the Y chromosome is present. Panel a) shows a male tissue hybridized with Y chromosome-FITC probe as a positive control. Panel b) illustrates female tissue hybridized with Y chromosome-FITC probe as a negative control. The Y chromosome signals were found in cartilage primordia of embryo F49 after DNA-DNA *in situ* hybridization, which is shown in panel c) and d). Cartilage primordia will develop to vertebrae, one of the future haematopoietic sites.

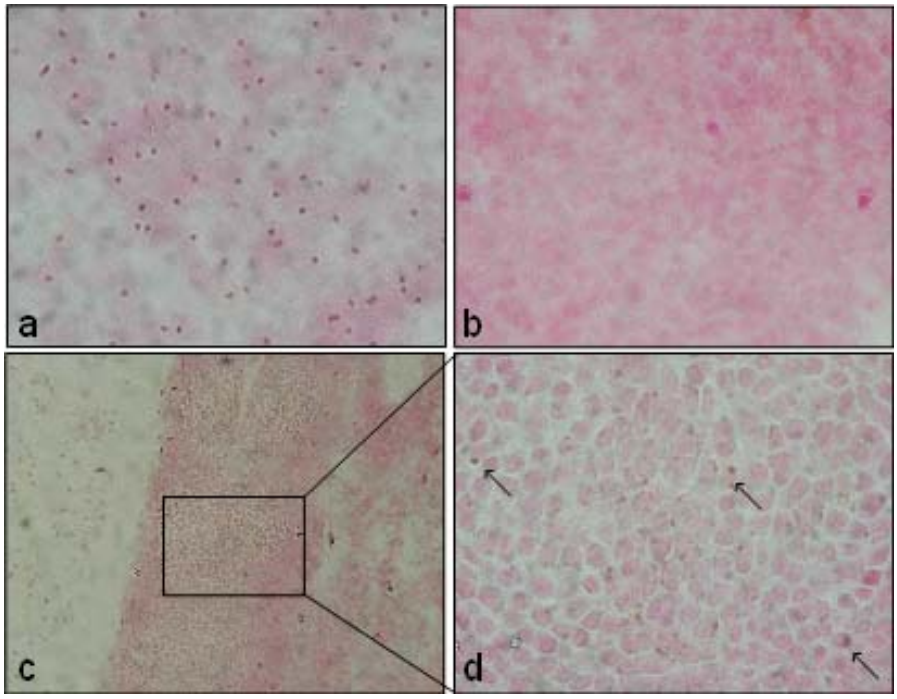


Figure 15. Indirect Y chromosome FITC-specific DNA-DNA *in situ* hybridization. Anti FITC peroxidase antibody and diaminobenzidine (DAB) develops a brown colour on the spot where the Y chromosome is present. a) male tissue, positive control; magnification 200x; b) female tissue, negative control; 200x; c) positive Y chromosome signal in cartilage primordium of the chimaeric embryo F49; 100x; d) positive Y chromosome signal in cartilage primordium of the chimaeric embryo F49; magnification 200x. The sections were 5µm thick. The arrows show the Y chromosome signal. Box shows picture d) in smaller magnification. Nuclear fast red stained nuclei.

In order to examine whether the Y chromosome positive cells in female chimaeric embryos are haematopoietic, staining for CD45 pan-haematopoietic marker was performed. In Figure 16, panel b) merge of immunostaining against CD45 marker combined with Y chromosome–FITC *in situ* hybridization and DAPI nuclear staining on FDCP mix cells is shown as positive control. As negative control cytospun FDCP mix cells were not immunostained and not hybridized with Y chromosome specific probe which is illustrated in Figure 16, panel d). Female and male tissues of non-injected animals, subjected to Y chromosome–FITC *in situ* hybridization, not immunostained against CD45 and stained with DAPI are shown in panel a) and c), respectively and represent negative and positive control.

Figure 17 shows a cartilage primordium of the chimaeric female embryo F70, developed after injection of FDCP mix cells. A representative positive cell found in the cartilage primordium of female embryo F70 is shown in panel a). Panel b) represents the cartilage primordium cells stained with DAPI, nuclear stain. In panel c) CD45 positive staining is shown. Merged layers revealed Y chromosome positive cell among recipient cells and that is shown in panel d).

Not all the donor cells in chimaeric embryos developed after injection of FDCP mix were CD45 positive. Figure 18, panel d) represents CD45 negative and Y chromosome -FITC positive cell in yolk sac of female embryo F70. Panel a) represents positive donor cell, panel b) shows nuclei of yolk sac cells in embryo F70, stained with DAPI and panel c) CD45 positive staining of yolk sac.

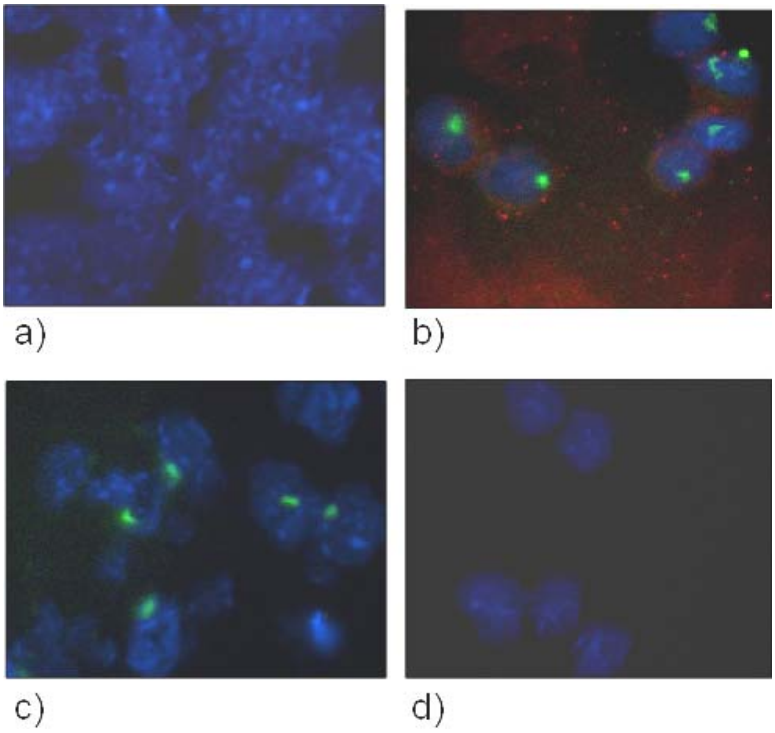


Figure 16. Control immunostaining and *in situ* hybrydization with Y-FITC probe. a) female tissue, *in situ* hybridized with Y-FITC probe and stained with DAPI, not immunostained against CD45 marker, magnification 400x; b) FDCP mix cytospun, stained with CD45 (red), *in situ* hybridized with Y-FITC probe (green dot) and stained with DAPI (nuclear stain, blue) magnification 400x, merged; c) male tissue *in situ* hybridized with Y-FITC probe, stained with DAPI and not immunostained against CD45, magnification 400x;d) FDCP mix cytospun, stained with DAPI, not hybridized or stained against CD45 marker magnification 400x;

As previously mentioned, FDCP mix-derived NCAM positive cells were detected in the foetal brain at a very low frequency after analysis by combined immunostaining and Y-chromosome specific *in situ* hybridization. The highest frequency was 6 NCAM positive cells out of 30000 cells in the whole brain (Table 14) of chimaeric embryo F73. Figure 19, panel a) represents cells in the brain stained with DAPI, panel b) shows the same cells immunostained against neural/glial marker NCAM, and panel c) represents donor Y chromosome FITC positive cell. In panel d) a representative NCAM and Y chromosome -FITC positive cell in chimaeric brain of embryo F73 is shown.

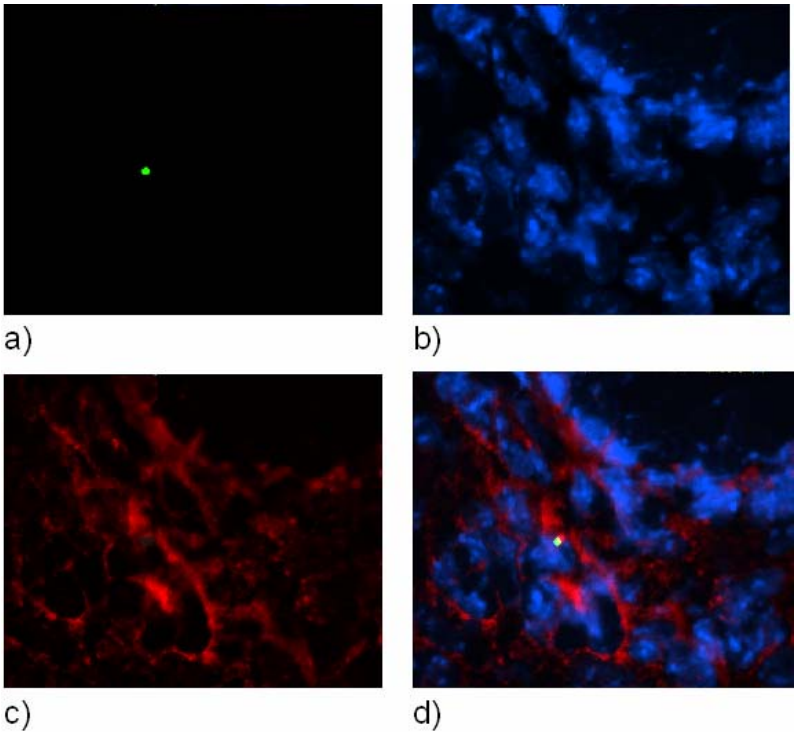


Figure 17. The cartilage primordium of chimaeric embryo F70; a) cartilage primordium hybridized with Y-FITC (green dot); b) cartilage primordium stained for nuclear die DAPI (blue); c) cartilage primordium stained for CD45 (red); d) merge; magnification 400x

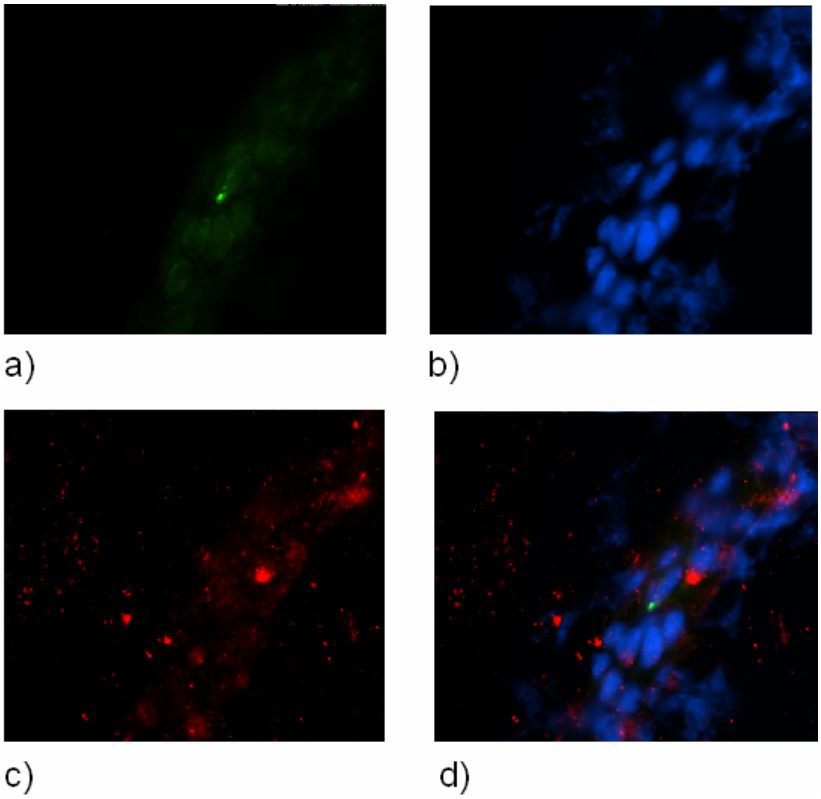


Figure 18. The yolk sac of chimaeric embryo F70; a) yolk sac hybridized with Y-FITC (green dot); b) yolk sac stained for nuclear dye DAPI (blue); c) yolk sac stained for CD45 pan haematopoietic marker (red); d) merge; magnification 400x

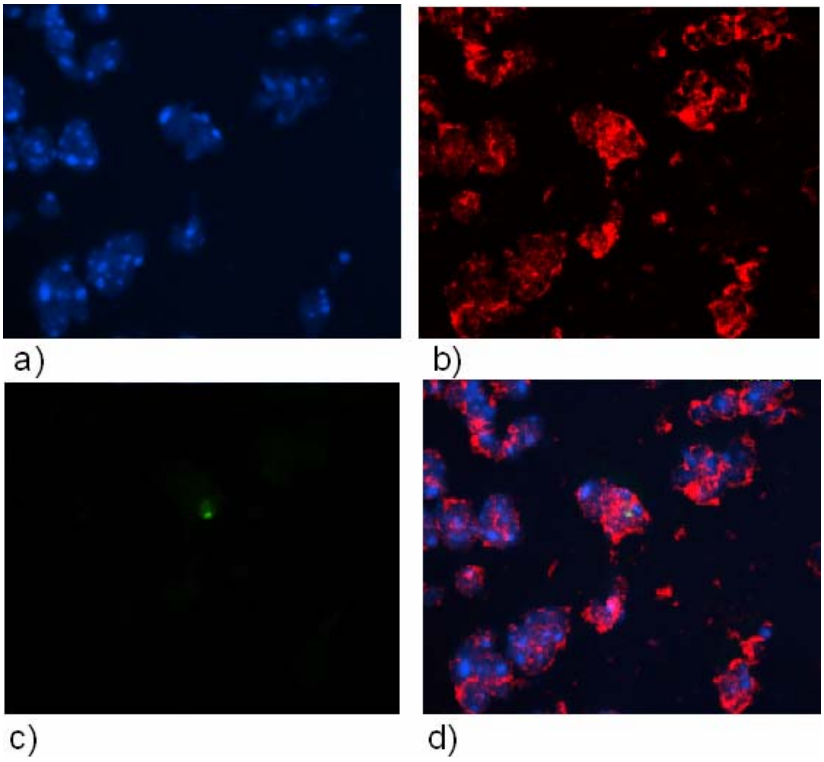


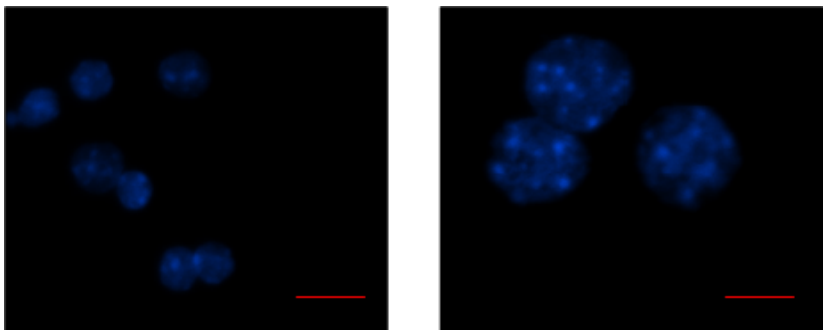
Figure 19. The brain cells of female chimaeric embryo F73; a) the brain cells stained for nuclear dye DAPI (blue); b) the brain cells immunostained for NCAM (red); c) hybridized with Y-FITC (green dot); d) merge; magnification 400x

5.5 Transdifferentiation and cell fusion

A spontaneous fusion of cells is a possible event that can occur in cell culture. It has been reported that mouse bone marrow cells can fuse spontaneously with embryonic stem cells in cultures that contain interleukin 3 (Terada et al 2002). Also, neuronal progenitor cells can fuse with embryonic stem cells when co-cultured and give tetraploid hybrids (Ying et al 2002). These facts have aroused a new controversy as to whether transdifferentiation of somatic stem cells to unrelated cell types exists at all or whether it is just a matter of cell fusion. Since fused cells, and the cells that are tetraploid have larger nuclei, the cell diameter has been used as an indicator of whether or not cells are fusion products. As a control, single cell suspensions of hepatocytes were sorted by FACS for 2n and 4n cells, and the nuclear diameter was determined using Open Lab 3.1.7.software. Figure 20 visualizes the sizes of 2n and 4n nuclei of hepatocytes, and in the Table 15 are their sizes are calculated. Altogether, the diameters of 15 2n, and 17 4n cells were measured. The diameter of 2n cells was 7.52 $\pm$ 0.51µm, and 4n cells had a diameter of 15.00 $\pm$ 0.65µm. This control result confirmed that nuclei of tetrapliod cells have double size of nuclei of 2n cells. After measuring the nuclei sizes of 22 FDCP mix and of 16 YSE cells, their

average nuclei sizes have been determined: 10.60 ± 1.20µm and 11.56 ± 0.54µm, respectively (Table 15).

In order to exclude cell fusion as the event that led to alleged transdifferentiation, the nuclei sizes of the donor and the recipient cells in the same picture were measured. These results have been shown in Table 16. In Figure 12 chimaeric female embryo Y25, developed after injection of YSE cells, was analysed by Y chromosome specific *in situ* hybridization and it was revealed that yolk sac of the embryo contains donor cells. The nuclear diameter of donor cell was 8.19µm and recipient cells average nuclei diameter was 8.68 ± 0.83µm. The diameters of nuclei of donor and recipient cells are similar. The same was revealed after comparison of nuclei diameters of donor and recipient cells in Figure 17 (10.25µm versus 10.49 ± 0.48µm, donor versus recipient cells, respectively), in Figure 18 (11.88µm versus 10.78 ± 0.79µm), and in Figure 19 (11.75µm versus 11.95 ± 0.38µm). Compared to each other these diameters suggest that cell fusion did not occur.



a) b)

Figure 20. Cytospun 2n and 4n hepatocytes. A single cell suspensions of hepatocytes were sorted by FACS for 2n and 4n cells and they were cytospun. a) 2n hepatocytes; b) 4n hepatocytes; red bar represents 10µm

Table 15. The nuclei sizes of 2n and 4n hepatocytes and of FDCP mix cells and YSE cells.

Cytospun cells	Nuclei diametar (µm)
2n hepatocytes	7.52 ± 0.51 (n =15)
4n hepatocytes	15.00 ± 0.65 (n =17)
FDCPmix	10.60 ± 1.2 (n =22)
YSE cells	11.56 ± 0.54 (n =16)

Abbreviations: n, number of cells which nuclear diameters were measured;

Table 16. The diameter of nuclei of the donor and the recipient cells

Diameter / Figure/tissue	Diameter of recipient cells' nuclei (μm)	Diameter of donor cells' nuclei (μm)
Figure 12/YS	8.68 ± 0.83 (n =15)	8.19 (n =1)
Figure 17/CP	10.49 ± 0.48 (n = 20)	10.25 (n =1)
Figure 18/YS	10.78 ± 0.79 (n = 18)	11.88 (n =1)
Figure 19/brain	11.95 ± 0.38 (n = 16)	11.75 (n =1)

Nuclear diameter was measured on cells shown in Figure 12-19 in specific tissues. Abbreviations: YS, yolk sac; CP, cartilage primordia; n, number of cells which nuclear diameters were measured;

6 Discussion

6.1 Using the murine blastocyst system as an experimental strategy

The injection of embryonic stem cells, with a gene of interest deleted via homologous recombination, into murine blastocysts is commonly used as an experimental paradigm to generate gene knock-out mice. After transplantation of injected blastocysts into pseudopregnant foster mothers, chimaeric animals are born. By mating chimaeras and wt mice, heterozygous knockout mice are produced, and by crossing these chimaeric heterozygotes, homozygous knockout mice can be obtained. This technique is also applied as a tool for making chimaeric mice, and our research group as well as the others used this experimental strategy as a tool for observing potential of somatic stem cells (SSCs) (Geiger et al 1998; Clarke et al 2000; Harder et al 2002; Kirchhof et al 2002; Jiang et al 2002; etc). After injection of teratocarcinoma cells into murine blastocysts, the embryonic environment controls the proliferation of tumorous cells, and they can participate in normal murine organogenesis, i.e. differentiate and contribute normal chimaeric tissues (Papaioannou et al 1975). The same could be observed after injection of human acute myeloid leukaemia cells into murine blastocysts (Dürr et al 2003). Blastocyst injection was also used to follow IEM endothelial cell line destiny (Gendron et al 1996).

The murine blastocyst environment is permissive for the development of all three germ layers ectoderm, endoderm and mesoderm of a future adult organism. A highly organized hierarchy of growth factors, chemokines, and ligand impulses in the blastocyst environment leads to the development of the whole mouse organism. The above mentioned investigations showed that SSCs, tumour cells and an endothelial cell line can contribute to chimaeric embryos, and that they seed preferentially the tissues that they derive from. NSCs seed neural tissues, HSCs preferentially seed haematopoietic tissues; the IEM endothelial cell line contributes to microvascular endothelium. These observations led to the idea of using blastocysts injection as an experimental strategy for the examination of the homing potential of DAS 104-4, DAS104-8 AGM deriving cell lines, YSE-yolk sac deriving cell line and FDCP mix bone marrow deriving cell line. It was intriguing to investigate if these cell lines are able to return to their organ of origin, as well as if they have other potentials.

6.2 Homing pattern of AGM-derived DAS 104-4 and DAS 104-8 cells in developing embryos and adults

DAS 104-4 and DAS 104-8 were originally created as cell lines for the support and differentiation of haematopoietic stem cells (HSCs). The DAS 104-4 cell line provoked the expansion of early HSCs of the Lin^- $CD34^+$ $Sca\text{-}1^+$ $c\text{-}kit^+$ phenotype, and was proficient at maintaining foetal liver-deriving HSCs able to repopulate the bone marrow of lethally irradiated mice, but was inefficient at the induction of haematopoietic differentiation.
DAS 104-8 cells induced foetal liver HSCs to differentiate down the myeloid, erythroid and B-lymphoid pathways, but did not support self renewal of HSCs (Ohneda et al 1998).
In this study they were used for the investigation of their homing pattern in midgestational chimaeric embryos developed after injection of these cell lines into murine blastocysts.
The assumption was that AGM-deriving DAS 104-4 and DAS 104-8 cell lines, express AGM region specific markers which would preferentially lead them to the organ of origin, in the embryos which developed following injection.
The experiments with DAS 104-4 and DAS 104-8 cell lines demonstrate that after injection of these cells into murine blastocysts it was possible to obtain chimaeric embryos and adults. Both the blastocysts and developing embryos are able to provide a suitable environment for survival of DAS 104-4 and DAS 104-8 cells. DAS 104-4 cells contributed to all isolated tissues of chimaeric embryos. Adults were not analysed. DAS 104-8 cells seeded all isolated tissues, except yolk sac in analysed embryos, and in the adults they engrafted brain. Interestingly, as with the other cells injected into blastocysts in this survey, they had an adverse effect on the development of the embryos, such that just 13% DAS 104-4, and 11 % DAS 104-8 of the all injected blastocysts developed. As previously mentioned, these cell lines are endothelial cell lines (although they derive from AGM region) that produce VEGF, and express endothelial markers including CD34, vWF, FLK-1 (receptor tyrosine kinase for VEGF), FLT-1 (second receptor tyrosine kinase for VEGF), PECAM or CD31 and TIE-2. They were transformed using a polyoma virus middle-T expressing retrovirus. It has been previously shown that this oncogene transforms endothelial cells and maintains them in an endothelial-like state (Bautch et al 1987; Williams et al 1989). Expression of the polyoma virus middle-T antigen in vivo seems to be sufficient to induce the development of endothelial tumours diagnosed as haemangioma (a variety of vascular tumours or lesions that consist of endothelial cells, pericytes - smooth muscle like-cells, and stromal cells and look like blood filled cysts). Haemangiomas could rapidly be induced by injection of transformed

endothelioma cells that carry polyoma virus middle-T antigen into syngeneic, nonsyngeneic mice, and rats or by direct injection of retrovirus vectors carrying polyoma virus middle-T antigen in mice and chicks (Williams et al 1989). The transgenic mice (made by injection of polyoma virus middle-T antigen DNA construct into the fertilized eggs) develop vascular lesions within 2 weeks to one year. The mice that carry haemangiomas have anaemia and enlarged spleens and usually die from internal haemorrhage because of rupture of a haemangioma. The chimaeric embryos developed multiple haemangiomas at midgestation, immediately after the appearance of endothelial cells. Interestingly, we did not notice any haemangioma in our analysed embryos or adult mice, which again confirms the observation that blastocyst environment controls tumourigenicity (Dürr et al 2003).

The distribution of the AGM-derived DAS 104-4 cells donor contribution in chimaeric embryos shows promiscuity. They are found in all isolated tissues of the chimaeric embryos with a similar contribution frequency. AGM-derived DAS 104-8 cells show a similar donor contribution distribution in the chimaeric embryos. In 3 adults they were found in cortex and cerebellum (parts of the brain). Thus, since these cells do not show the ability to home preferentially to the AGM region of the chimaeric embryos or the chimaeric adults, although they originate from it, it could be suggested that in this a complicated, and still unknown interaction between blastocyst environment and DAS cell lines, their strong endothelial nature leading them to the endothelium of different organs. This could explain why their distribution is so diverse. Support for this hypothesis lies in the fact that DAS cell lines produce VEGF, a very important protein involved in vasculogenesis that enables differentiation of the angioblasts and their multiplication to form the endothelial tube. DAS cell lines also express the VEGF receptors FLK-1 and FLT-1, important for the appearance of blood islands and vasculogenesis, or the organization of endothelial cells into blood vessels, respectively (Ferera et al 1996; Fong et al 1995; Shalaby et al 1995). Expression of TIE-2, a receptor for angiopoietins, which mediate the interaction between the endothelial cells and the pericytes, and whose deficiency leads to malformed smooth muscles of blood vessels (Vikkula et al 1996; Suri et al 1996), would be a further argument for DAS cells' endothelial homing pattern. Based on the embryonic expression pattern, it would be expected to find DAS cells in various tissues of the adult animals. This is however not the case. The explanation could be that these cell lines incorporate in the endothelium or different parts of the embryos but due to terminal differentiation are eliminated in normal processes of the cell death, or that they were simply not engrafted in the other tissues.

6.3 Homing pattern of yolk sac endoderm-derived YSE cells in developing embryos and adults

The YSE (yolk sac endoderm) cell line was originally created to define the role of the murine yolk sac endoderm in supporting haematopoiesis. It was made from a E9.5 murine yolk sac visceral endoderm layer using a recombinant retrovirus vector carrying Simian virus 40 large T antigen (SV40 LT antigen). The immortalized YSE cell line expressed almost all morphological characteristics of freshly isolated yolk sac endoderm. This cell line stimulated the proliferation of haematopoietic progenitors and showed, similarly to the differentiation of blood island haematopoietic progenitor cells, their differentiation to macrophages and restricted differentiation to neutrophils (Yoder M et al 1994).

The experiments with the YSE cell line demonstrate that after injection of these cells into murine blastocysts it was possible to obtain chimaeric embryos but not adults. Both blastocysts and developing embryos are able to provide a suitable environment for survival of YSE cell line cells.

The YSE cell line had a negative effect on the development of embryos, such that just 10% the injected blastocysts developed into embryos and adults. The number of cells injected into blastocysts had to be lowered (5 to 10 cells maximum) compared with the other cells used in this survey, because the embryos were dying with higher numbers. The fact that they were the most injected cells (44 independent injections into blastocysts), and that just 24% of the developed embryos were chimaeras, argues for their harmful effect on embryonic development. Since YSE cells grow much faster than non-transformed cells in the culture it seems that they could not be controlled by the embryonic environment all the time, and that they were killing the embryos. In cases when they were under the control of the embryonic microenvironment, they would normally contribute to the embryonic tissues. No tumour formations were observed, despite the fact that YSE cells were immortalized with the SV40 Large T antigen.

Simian virus 40 is DNA virus and belongs to Polyomaviridae family. The SV40 LT antigen is a potent oncogene, and plays several roles in viral infection and tumourigenesis. It is known that SV 40 LT antigen induces tumours in mice, hamsters and humans via inactivation of the tumour-suppressor proteins p53 and pRB family members (Sullivan and Pipas 2002).

The generation of chimaeras is further proof that transformed cells can lose their neoplastic properties under the appropriate environmental conditions.

The distribution of YSE cells donor contribution in developed embryos showed that these cells preferentially (30%) return to yolk sac, their organ of origin. The second most repopulated organ was the foetal liver. The reason for this could lie in the similar functions and morphology of yolk sac endoderm cells and liver cells. The yolk sac endoderm and the liver have different origins, but similar function. The liver is developed from the definitive endoderm, which derives from the primitive ectoderm, and the yolk sac is developed from primitive endoderm. The most important function of the yolk sac visceral endoderm is absorption- uptake of substances from the maternal circulation, and secretion of serum proteins required by the foetus such as alfa foeto-protein (AFP), transferrin, high- and low-density apolipoproteins and α_1-antitripsin. The visceral endoderm therefore performs the same function as both the large intestine and the foetal liver. The morphology of visceral endoderm cells is highly specialized for absorption; they have apically located microvilli and coated pits as well as lysosomes and intracellular tight junctions (Hogan et al 1986).

The immortalized yolk sac cell line (YSE) used in this survey displayed most of the morphologic, physiologic and biosynthetic features of visceral yolk sac endoderm cells such as a polarized morphology with tight junctions, uptake and secretions of proteins, expression of Endo B mRNA and cytokeratins. YSE cells expressed laminin and collagen IV and did not express AFP, which is not a common description of visceral yolk sac endodermal cells. Therefore, YSE cells have an intermediate phenotype between visceral and parietal endoderm (Yoder et al 1994).

The conclusion that could be drawn is that due to the similar features and functions of foetal liver and yolk sac visceral endoderm, and therefore similar cell type phenotype and expressing markers, this immortalized cell line preferentially seeds yolk sac, with foetal liver as the next favoured organ.

It was not possible to detect chimaeric tissue in the 18 isolated tissues from each adult animal developed from these injected blastocysts. Altogether 8 animals were analysed. One of the reasons could be that if the yolk sac is the preferentially seeded tissue, it was not possible to detect any adult chimaeric tissue, since it is an extraembryonic tissue and it is rejected at birth. A second reason could be that these cells become part of normal tissue upon embryonic environmental control, terminally differentiate, and are eliminated in the normal process of apoptosis. A third reason could be that these cells never engrafted after injection into the blastocysts, or contributed to the tissues of the developed embryos, so were not present in chimaeras.

Y chromosome-specific in situ hybridization showed that these cells mostly contributed to the head, the yolk sac and the skin, and less to the intestine and the hind limbs of the analysed embryos, but none contributed to the foetal liver. Discrepancies between donor contribution PCRs and in situ hybridization data could be explained with different sensitivity of these two methods. In donor-specific PCRs the whole livers were analysed, while just limited number of liver cells was present on the sections analysed by in situ hybridization. It could be that donor cells were simply not present on the analysed sections. It is also possible that these embryos did not have chimaeric livers.

Since YSE cells were mostly repopulating the head of the chimaeric embryos, combined immunostaining for neuronal cell adhesion molecule (NCAM) with Y chromosome-specific in situ hybridization was carried out. Although the Y chromosome positive cells were detected in the heads of the analysed embryos, none was NCAM positive. This suggests that these Y chromosome positive cells, i.e. donor cells, do not have a function as neurons or astrocytes. Their function in the brain remains unidentified.

YSE cells progeny was found in the yolk sac of the analysed embryos by Y chromosome-specific in situ hybridization. In order to reveal if they are expressing the marker of the visceral yolk sac endoderm activin receptor type I (Act RIB), combined immunostaining for ActRIB with Y chromosome-specific in situ hybridization was done. The results showed that it is possible to find double positive cells (ActRIB^{+}, Y chromosome^{+}) although at a very low frequency (4 double positive cells out of 1000 in the whole yolk sac). So, donor YSE-deriving cells reside in the yolk sac and they probably took over a normal function as recipient's cells.

Overall it seems that yolk sac endoderm cells show less flexibility in their homing pattern than the DAS 104-4 and DAS 104-8 cells, and a greater preference to seed the organ of origin (yolk sac) and an organ with similar function - the foetal liver. These cells are more rigid, and more difficult to place under the control by blastocyst environment, which would be a further argument to the idea that every single cell line has its own homing pattern dependent on the cell type specific properties.

6.4 Homing pattern of bone marrow-derived FDCP mix cells in developing embryos and adults and their changed potential

FDCP mix cells are a multipotent progenitor cell line, that have been established in long-term bone marrow cultures and maintained as proliferating, undifferentiated cells by the growth factor interleukin 3 (IL-3). As previously mentioned they are neither immortalized nor transformed, and have no detectable karyotypic abnormalities, and are not leukaemic in vivo. If provided with appropriate stromal cells and/or growth factors they will undergo multilineage myeloid/erythroid or osteoclast differentiation, but it has been not possible to induce lymphocyte differentiation in vitro (Ford AM et al 1992). Early after isolation this cell line was able to produce spleen colonies in irradiated mice. After 3 months of FDCP mix cell line in culture, the spleen colony-forming ability was lost, but if they were directly injected into the spleen, they were able to establish haematopoiesis, suggesting that they are defective in spleen-homing capacity when injected intravenously (Spooncer E et al 1986).

In this study FDCP mix cells were injected into murine blastocysts, and it was possible to obtain chimaeric embryos and adults. Both blastocysts and developing embryos are able to provide a suitable environment for survival of FDCP mix cells.

FDCP mix cells also had a negative effect on the development of the embryos, such that just 10% the injected blastocysts developed into embryos and adults. In comparison to previously described injections of DAS 104-4, DAS 104-8 and YSE cells, after injection of FDCP mix cells into blastocysts the highest percent of developed animals were chimaeric (73%), which would suggest that these cells went under control of the blastocysts environment and took part in normal development of the embryos more easily than the other cell types. One of the reasons for this could be that they are not transformed, and do not proliferate faster than cells of ICM, so blastocyst environment controls their proliferation and they take part in normal embryogenesis.

The distribution of FDCP mix cells' donor contribution in developed embryos showed that they were present in all examined tissues, with a preference for the haematopoietic tissues. The most seeded tissues were yolk sac (73%) and AGM region (50%), the first extraembryonic and intraembryonic haematopoietic sites. The reason could be that these sites are the first homing targets for FDCP mix haematopoietic progenitors, due to existence of the specific chemoattractants, chemokines, growth factors and microenvironment able to maintain their survival. In developing embryos the progeny of FDCP mix cells was also found at a high frequency in foetal blood (47%) which confirms the idea that these cells took part in normal

embryonic haematopoiesis and differentiated down the specific lineages. The foetal livers were seeded with the same frequency (37%) as visceral part (37%), while donor contribution in the head of the embryos was relatively low -13 %.

In the developed embryos analysed by combined immunostaining against CD45 pan-haematopoietic marker with Y chromosome-specific in situ hybridization, the most engrafted organs, with the highest contribution were cartilage primordia, future vertebrae or the future bones of the hind limbs. The explanations could be that the haematopoietic progenitors are homing to bone marrow, that they are differentiated haematopoietic cells or that they are preosteoclasts. Osteoclasts have haematopoietic origin and are important in the formation of bone by endochondral ossification during embryogenesis, and for remodelling the bones in adults. They are multinucleated cells, but their preosteoclasts are not. Multinucleated cells were never found in the cartilage primordia, therefore the Y chromosome-FITC positive cells could be preosteoclasts.

As described in Table13 Y chromosome positive cells were found in the majority of the analysed tissues (head, skin, yolk sac, and foetal liver) and they were mostly CD45 positive. This confirms that FDCP mix haematopoietic progenitors undergo normal differentiation. Since some of the Y chromosome positive cells in the foetal livers of the analysed embryos were CD45 negative, combined immunostaining against albumin, the liver marker, with Y chromosome-specific in situ hybridization was carried out. None of the Y chromosome positive cells was positive for albumin. Therefore, FDCP mix cells did not take part in the formation of hepatocytes. They were not able to change their fate in the endodermal direction.

Similarly, since some of the Y chromosome positive cells in the heads of the analysed embryos were CD45 negative, combined immunostaining against NCAM marker and Y chromosome -specific in situ hybridization was completed. It was intriguing to discover whether FDCP mix cells were able to broaden their potential after injection into murine blastocysts. Surprisingly, it was revealed that some of the Y chromosome positive cells express the NCAM marker, a marker of neuronal progenitors, neurons, astrocytes and Schwann cells (Paratcha et al 2003), at very low frequency (the highest 0.02%). This could be due to transdifferentiation of FDCP mix cells from haematopoietic (mesodermal origin) to neuronal (ectodermal origin) destiny.

It has been previously described that bone marrow cells that had been genetically marked with a retroviral tag, or by using male cells transplanted into adult female mice, could contribute to the central nervous system. The same group argue that these BM cells additionally formed microglia, however their origin is still in discussion (Eglitis and Mezey 1997). Some research

groups claim that microglia have mesodermal origins, as well as some that have neuroectodermal, and some with monocytic origin (Kaur C et al 2001). Microglial cells are activated phagocytic cells inside the brain parenchyma, and are actively involved in the removal of neurons undergoing apoptosis, and in some diseases such as multiple sclerosis can even engage in the active killing of neurons. Activated microglial cells express proinflamatory cytokines, analogous to activated macrophages outside the brain (Cotman et al 1998). Microglial cells do not express NCAM, so it is not likely that the cells found in the brain of the chimaeric embryos Y chromosome FITC positive cells and NCAM positive are microglia. It is more likely that FDCP mix cells are able to change their destiny into neuronal cells, when exposed to the permissive blastocysts environment.

In the adult animals developed from the blastocysts injected with FDCP mix haematopoietic progenitors, the distribution of donor contribution to the adult organs was unexpected. These cells repopulated preferentially the rest of the brain, hippocampus, muscles and ovary (33%), while the most repopulated haematopoietic tissue was liver (25%), then thymus (17%) and then bone marrow (8%). The spleens of the chimaeric adults were not engrafted at all. The reason for this could be that these cells simply lost their ability to home to spleen. Support for this assumption could be a repopulation experiment (data not shown) where injection of FDCP mix cell into irradiated C57BL/6 and BDF-1 mice, failed to repopulate them.

The preference for the hippocampus and the rest of the brain could lie in the fact that these cells are able to transdifferentiate, and change their fate to neuronal progenitors as had been previously shown with combined NCAM immunostaining and Y chromosome-specific in situ hybridization on the slides of the embryos. There is a possibility that they are found in hippocampus of adults, because dental gyrus of hippocampus is one of the sites in the adult brain where neuronal stem and progenitor cells reside. They could be present in the rest of the brain of the adults because they transdifferentiate to neurons, astrocytes and Schwann cells. It could also be that they simply differentiate to blood cells and microglia and, that this is why they are found in the brain. Combined immunostaining for neuronal markers with Y chromosome-specific in situ hybridization could be performed to explain this mystery. It would be interesting to carry out combined immunostaining for T cell markers with Y chromosome-specific in situ hybridization to reveal whether FDCP mix haematopoietic progenitor cells are able to seed the T lymphoid lineage after exposure to the blastocyst environment.

6.5 Transdifferentiation and cell fusion

If the process of transdifferentiation really exists, it still remains the most intriguing question in stem cell and developmental biology. The pompous titles: Turning brain into blood; From marrow to brain; (Bjornson et al 1999; Brazelton et al 2000); Turning blood into brain (Mezey et al 2000) shook the main principles of developmental biology and opened many new questions. The fact that bone marrow cells can have a broader potential, and that they can give rise to microglia and macroglia (Eglitis and Mezey 1997) hepatocytes (Lagase et al 2000; Petersen et al 1999; Alison et al 2000), cardiac muscle (Orlic et al 2001a; Orlic et al 2001b; Jackson et al 2001), skeletal muscle (Gussoni et al 1999; Ferari et al 1998; Bittner et al 1999), neural tissues (Brazelton et al 2000; Eglitis and Mezey 1997; Mezey et al 2000), prompted the idea that FDCP mix cells could have a broader potential than previously thought.

In this study a broader potential of FDCP mix cells has indeed been shown, but at very low frequency.

Recent reports described cell fusion as a mechanism that happens in vitro and in vivo and can lead to false interpretation of stem cell plasticity (Terada et al 2002; Ying et al 2002; Vassilopoulous et al 2003; Wang et al 2003). In order to exclude a possible cell fusion, the nuclei size of the donor's and the recipient's cells were determined on the same slide. The fused cells are supposed to have the larger nuclei. A cell suspension of the adult mice hepatocytes sorted for 2n and 4n cells showed that these cytospun cells have a different size of the nuclei. Nuclear diameter of sorted 4n cells was double (15.00 $\pm$ 0.65 µm) compared to nuclear diameter of 2n cells (7.52 $\pm$ 0.51µm). When the size of the donor's and the recipient's nuclei were measured on the same slide, they were similar (8.19µm versus 8.68 $\pm$ 0.83µm; 10.25µm versus 10.49 $\pm$ 0.48µm; 11.88µm versus 10.78 $\pm$ 0.79µm; 11.75µm versus 11.95 $\pm$ 0.38µm for donor's versus recipient's nuclei, on the Figure 12, Figure 17, Figure 18, Figure 19, respectively). Since the nuclear diameters of recipient and donor cells were similar, this result would suggest that donor cells were not fused with recipient's cells. But this still does not completely exclude the cell fusion.

It has been recently shown that a single haematopoietic stem cell, when injected into adult muscle damaged mice models can contribute to muscle regeneration, at a low frequency and it increases with muscle damage. It has been also proposed that HSCs do not directly transdifferentiate to skeletal muscle cells but that inflammatory cells like monocytes, macrophages or neutrophils spontaneously, fuse to the muscle cells at very low frequency (Corbel et al 2003; Camargo et al 2003). This model is consistent with recent observations

that the mechanism of transdifferentiation in liver, and brain is actually cell fusion (Vassilopoulous et al 2003; Wang et al 2003; Alvarez-Dolado et al 2003). But the findings by Camargo et al are questioned by recent work that illustrates that HSCs express lineage affiliated genes such as lysozyme M and retain long-term multilineage repopulation potential (Ye M et al 2003).

In this study FDCP mix cells were injected into blastocysts, a completely different system to the adult environment, but it would be possible that FDCP mix cells differentiate into macrophages which fuse at a very low frequency to neurons. It would be also possible that the mechanism of transdifferentiation differs from tissue to tissue and that not every noticed event of transdifferentiation can be explained by cell fusion. New findings open new questions. Although tetraploid mice and humans have been described and tetraploid cells can form all cell types in the body and form the organs (Snow 1973; Scarbrough 1984) why would such a mechanism like cell fusion exist in normal organisms. The explanation could be that it is a very rare event. The fused cells can fail to proliferate further and tetraploidy can induce cell cycle arrest in - G1 in non - transformed cells (Andreassen et al 2001) or cell fusion can lead to genetic instability and neoplastic transformation which is incompatible with life. This does not really explain the regenerative capacity of the bone marrow cells or the existence of MAPC cells. Nobody noticed tumour formation in mice either. One should not easily exclude both cell fusion and cell plasticity possibilities, the truth could lie somewhere in between.

The question of whether the changing of the fate of FDCP mix is transdifferentiation or cell fusion event remains to be fully answered.

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8 Abbreviations

A	adenine
ActRIB	type I activin receptor
AGM	aorta-gonad-mesonephros
AML	acute myeloid leukaemia
$\alpha^{32}P$	radioactive phosphorus
AP	alkaline phosphatase
bFGF	basic fibroblast growth factor
BM	bone marrow
bp	base pairs
C	cytosine
CFU-S	colony-forming-unit of the spleen
CLPs	common lymphoid progenitors
CMPs	common myeloid progenitors
CNS	central nervous system
dATP	deoxi-adenosine-triphosphate
dCTP	deoxi-cytosine-triphosphate
dGTP	deoxi-guanosine-triphosphate
dTTP	deoxi-thymidine-triphosphate
dUTP	deoxi-uridine-triphosphate
dUTP-DIG	deoxi-uridine-triphosphate, digoxigenin labelled
DAPI	4’, 6-diamidino-2-phenylindol
DAS	dorsal- aorta-stromal
DNA	deoxiribonucleic acid
dNTPs	deoxi-nucleotide-triphosphates
DNase	deoxyribonuclease
DMSO	dimethylsulphoxid
DMEM	Dulbeco’s modified Eagle’s medium
E	embryonic day
ECM	extracellular matrix
EDTA	ethylendiaminotetraacetic acid
EGTA	ethylenglycol bis (β-aminoethylenether)-N, N’-tetraacetic acid
EMEM	Eagle’s minimum essential medium

EtOH	ethanol
ES cells	embryonic stem cells
FACS	fluorescence-activated cell sorter
FBS	foetal bovine serum
FCS	foetal calf serum
FDCP	factor dependant cell Paterson
FITC	fluorescein-5-isothiocyanate
G	guanine
g	gravitation: 9,81 m/s^2
HEPES	N-2-hydroxyethylpiperazin-N'-2-ethansulphonacid
HCG	human chorion gonadotropin
HSCs	haematopoietic stem cells
HPP-CFC	high proliferative potential colony forming cells
ICM	inner cell mass
kb	kilobases
KG-1	human myeloid leukaemia cell line
IL-3	interleukin 3
LIF	leukaemia inhibitory factor
M	molar
MAPC	multipotent adult progenitor cell
MEPs	megakaryotic/erythroid progenitors
min	minutes
ml	mililitre
mM	milimolar
µm	micrometer
µl	microliter
µg	microgram
MPPs	multipotent progenitors
nm	nanometer
ng	nanogram
NBT/BCIP	nitrobluetetrazolium/5-brom-4-chlor-3 indolylphosphate
NCAM	neural cell adhesion molecule
NCCs	neural crest cells
NSCs	neural stem cells

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGCs	primordial germ cells
PFA	paraformaldehyd
PI	propidium iodide
PMS	pregnant mare serum
PNS	peripheral nervous system
PVMT	polyoma virus middle T antigen
RCF	relative centrifugal force
RNase	ribonuclease
Rpm	rounds per minute
RT	room temperature
sec	seconds
SDS	sodium dodecyl sulphate
SSC	sodium salt citrate
SV40LT	Simian virus 40 large T antigen
TBE	tris-borate-EDTA
T	thymine
U	units
UV	ultraviolet light
V	volt
YSE	yolk sac endoderm

9 *Curriculum vitae*

PERSONAL DETAILS

Date of Birth: October 18th, 1971
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EDUCATION

9'1978 – 7'1986 **Primary education** at *Aca Aleksic* school, Aleksandrovac, Serbia.

9'1986 – 7'1990 **Secondary education** at the *Education Centre 9th May*, in Aleksandrovac, Serbia.

9'1990 – 2'1998 **Diploma in Molecular Biology and physiology,** at the Department of Molecular Biology and Physiology, Faculty of Biology, University of Belgrade, Serbia.

Diploma thesis. Title; "Supernumerary Marker Chromosome", at the Human Genetics Laboratory, Institute of Mother and Child, Belgrade, Yugoslavia.

3'1998 – 6'2000 **Research Assistant,** at the Radiobiology and Molecular Genetics Laboratory, at The Institute of Nuclear Sciences "Boris Kidric", Vinca, Belgrade**,** Serbia**.**

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6'2000 – 4'2004 **Research Doctorate**
In Stem Cell Research Lab led by Prof. Dr. Albrecht Müller, at the *Institut für Medizinische Strahlenkunde und Zellforschung (MSZ)*, *Bayerische Julius-Maximilians-Universität Würzburg*, Germany.
Title: *In vivo* analysis of homing pattern and differentiation potential of cells deriving from embryonic and adult haematopoietic regions

10 Publication list

Petrovic S, Cross M, Müller AM.
Differentiation potential of FDCP mix cells in embryonic environment (*in preparation*)

Harder F, Kirchhof N, **Petrovic S**, Wiese S, Müller AM. Erythroid-like cells from neural stem cells injected into blastocysts. (*submitted*)

Kirchhof N, Harder F, **Petrovic S**, Kreutzfeld S, Schmittwolf C, Dürr M, Kirsten J, Mühl B, Merkel A, Müller AM. Developmental potential of hematopoietic and neural stem cells: unique or all the same? Cells Tissues Organs 2002; 171:77-89

Avots A, Harder F, Schmittwolf C, **Petrovic S**, Müller AM. Plasticity of hematopoietic stem cells and cellular memory. Immunological Reviews 2002;Vol 187: 9-21

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