Analysis of in vivo functions of Memo in embryonic and mammary gland development

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Patrick Kaeser

aus Fribourg, Schweiz

Basel, 2007

Genehmigt von der Philosophisch-Naturwissenschaftichen Fakultät

Auf Antrag von

Prof. N. Hynes
Prof. P. Matthias
Prof. M. Affolter

Basel, den 08 Oktober 2007

Table of Contents

i) **Summary**

Studies from our lab recently led to the discovery of Memo (mediator of ErbB2-driven cell motility), a novel 297 amino acid protein shown to be required for ErbB2- and other receptor tyrosine kinase-driven cell motility in breast tumor cells. Inhibition of Memo expression had consequences on the microtubule network which could not grow towards the periphery of the cells upon heregulin (a ligand activating ErbB2/ErbB3 and ErbB2/ErB4 heterodimers) stimulation. It also had consequences on the actin cytoskeleton, since more actin stress fibers were seen.

To explore the biological function of Memo, and in order to check if Memo also plays a role in *in vivo* cell migration events, we generated animal models deficient for Memo. We found that Memo is expressed ubiquitously in adult organs as well as in organs of the developing embryo. Unexpectedly, we did not see any defect in migration *in vivo*, despite the presence of a lot of migrating events during development like gastrulation or migration of the neural crest derivatives or of the somitomeres. Instead, we found that Memo seems to play a role in vascular integrity, as demonstrated by the presence of hemorrhages and the dilated small vessels in the Memo deficient embryos. This leads to the death of Memo deficient embryos after 13 days of embryonic development.

To study the *in vivo* role of Memo in the lactating mammary gland, we generated mice deficient for Memo in luminal alveolar epithelial cells (the cells that produce and secrete milk during lactation). We measured a decrease in the weight of pups from Memo deficient mothers, indicating that they were unable to correctly nurse them. The weight of the mammary gland itself was smaller in the Memo deficient females compared to control females. By histological analyis we saw the abnormal presence of shed cells in the lumen of Memo deficient glands in the first days of lactation. We saw a progressive loss of alveoli (formed by epithelium) which were replaced by adipocytes. Increased apoptosis (controlled cell death) was measured in the Memo deficient glands. Consistent with this apoptosis seen at the histological level, we could see an increase in the levels of pro-apoptotic P-Stat3 and Bax at protein level. We also could see improper localization of the adherens junction proteins E-cadherin

and ß-catenin in the Memo deficient mammary glands. We therefore propose that in the mammary gland Memo plays a role in epithelial cell-cell adhesion, and that if this role is not properly achieved, the cells undergo apoptosis and are shed in the lumen of alveoli which progressively disappear. This leads to improper feeding of the pups.

ii) **Abbreviations**

ATP	adenosine triphosphate
Bcl-2	B-cell lymphoma gene-2
BH3	Bcl-2 homologous domain 3
BrdU	bromo deoxy uridine
CD31	cluster of differentiation 31
CEBP	CCAAT/enhancer binding protein
CKO	conditional knockout
CMV	cytomegalovirus
DNA	deoxyribonucleic acid
ECM	extracellular matrix
Edg-1	endothelial differentiation G-protein coupled receptor 1 (= S1P receptor)
EPO	erythropoietin
ErbB2	the name derives from the virus causing erythroblastosis in avian
Erk	extracellular-signal regulated kinase
ES cell	embryonic stem cell
FGF	fibroblast growth factor
Flk-1	fetal liver kinase-1 (also named VEGFR2)
Flt-1	Fms-like tyrosine kinase
HIF	hypoxia inducible factor
IGFBP	insulin-like growth factor binding protein
IL-6	interleukin-6
KO	knockout
LIF	leukemia inducible factor
LYVE	lymphatic vessel endothelial hyaluronan receptor
MEMO	mediator of ErbB2-driven cell motility
MMP	matrix metalloproteinase
MMTV	mouse mammary tumor virus
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PECAM	platelet endothelial cell adhesion molecule
PKB	protein kinase B
RNA	ribonucleic acid
S1P	sphingosine 1 phosphate
Shc	src homology 2 domain-containing (protein)
SMA	smooth muscle actin
Stat	signal transductor and activator of transcription
TGF-β	transforming growth factor-β
Tie-2	tyrosin kinases that contain the Ig and EGF domain

TIMP	tissue inhibitor of metalloproteinase
TNF-α	tumor necrosis factor-α
uPA	urokinase plasminogen activator
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau
VSMC	vascular smooth muscle cell
WAP	whey acidic protein
WT	wild-type
ZO-1	zonula occludens-1 (protein)

1 Introduction

1.1 Part I: Cardiovascular development

The general pattern of embryonic vascular system is highly conserved between vertebrates (Fig.1).

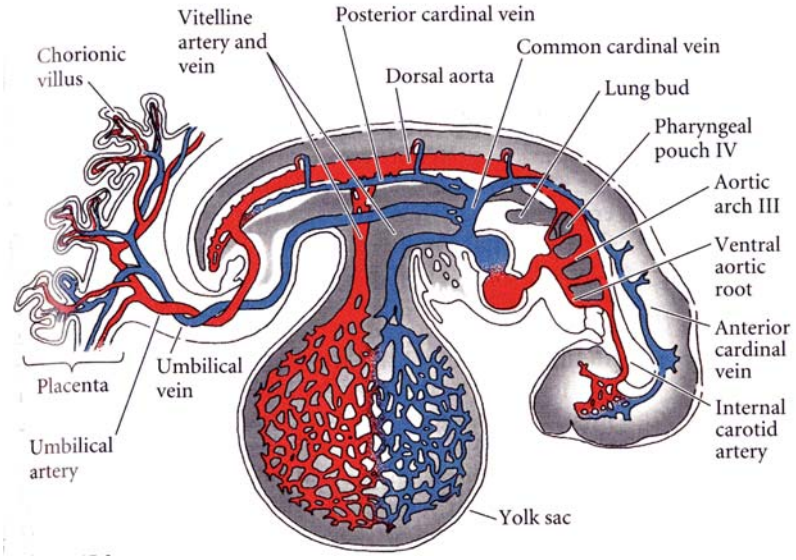


Figure 1: Circulatory system of a 4-week human embryo. Although at this stage all the major blood vessels are paired left and right, only the right vessels are shown. Arteries are shown in red, veins in blue. (After Carlson, 1981)

The morphogenesis of the embryonic vasculature begins with the appearance of angioblasts in mesodermal tissues. Angioblasts are defined as endothelial precursor cells which have not yet incorporated into the endothelial tissue of the vessels. After their specification, the angioblasts associate into vascular cords. The assembly of angioblasts to form a blood vessel is termed vasculogenesis. After the initial vasculature is established, it is extended throughout the embryo as a result of a process termed angiogenesis. Still later, the embryonic vascular system is extensively modified by endothelial remodeling, which involves the enlargement and splitting of existing vessels and extension of new vessels. Remodeling can also include the regression or complete disappearance of existing vessels. The final stage of vascular development is maturation, which involves a reduction in the proliferation of endothelial cells, their morphological change and the recruitment of vascular wall components (Fig.2).

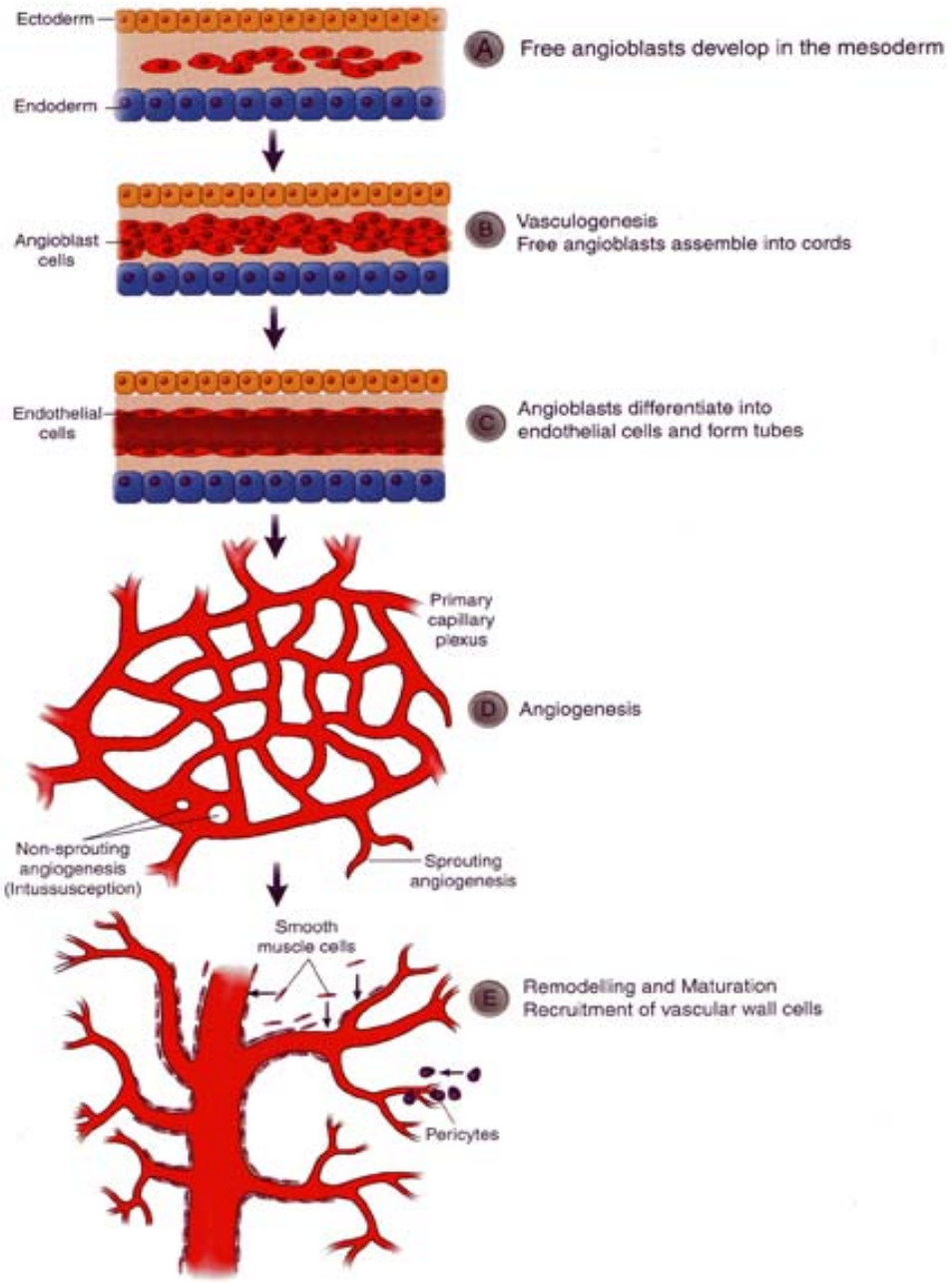


Figure 2: Schematic representation of the major processes involved in vascular development. Initially, (A) angioblasts differentiate from the mesoderm and then form cords either at the location where they emerge or at distant location, following migration (B). (C) The endothelial cells in the cords now differentiate and form tubes. (D) The primary vascular plexus is then extended and elaborated by angiogenesis. (E) Vascular remodeling occurs, resulting in the formation of large and small vessels. Finally, the endothelium matures and mesenchymal cells are recruited to become components of the vascular wall.

Despite rapid progress, the molecular mechanisms underlying many aspects of embryonic vascular development remain unclear. For example, very little is known concerning the precise origin of angioblast precursors in the embryo.

Early studies suggested that the embryonic vasculature might originate from invasion of the embryos by vessels from extraembryonic tissues. However subsequent studies have demonstrated that the intraembryonic vasculature develops *in situ* from intraembryonic precursors.

1.1.1 Molecules involved in early mesodermal differentiation of endothelial cells

1.1.1.1 Flk-1/VEGF

Angioblast differentiation in the mesoderm requires the activity of vascular endothelial growth factor (VEGF) and its receptor, Flk-1. They act in a paracrine way, VEGF expression being restricted to the endoderm and ectoderm and Flk-1 in the mesodermal endothelial cells. Heterozygous mice carrying a single copy of the VEGF die on E10.5 from severe perturbation of vessel development, including the disruption of dorsal aorta formation (Carmeliet et al., 1996). Differentiation of endothelial cells, growth of existing vessels, lumen formation and spatial organization of vessels are also significantly impaired. Homozygous mutant mice die at the same developmental stage but show more severe vascular abnormalities and tissue necrosis (Carmeliet et al., 1996) suggesting that the threshold levels of VEGF are critical for most steps of vascular development. At E8.5, mutant mice lack the dorsal aorta over its entire length. They also show reduced expression of endothelial markers (flk-1, flt-1, tie-2 and PECAM/CD31) suggesting that endothelial development is delayed but not completely eliminated.

Ablation of Flk-1 function in mice leads to a total absence of blood vessel formation (Shalaby et al., 1995). These embryos also lack the hemangioblastic cell lineage and do not develop blood. The difference between the VEGF and Flk-1 mutant phenotypes suggests that another Flk-1 ligand may be active during early mesoderm induction and could partially rescue the VEGF knockout (Breier et al., 1996). It seems that Flk-1 and VEGF are required for angioblast differentiation, but that the amounts of VEGF ligands determine angioblast survival (Risau, 1997).

1.1.1.2 Fibroblast growth factor

Members of the FGF family, especially bFGF, play a critical role in the induction of the mesodermal germ layer during the earliest stages of embryogenesis. In Xenopus laevis, they are potent inducers of ventral mesoderm, which will form the blood islands and some muscle tissue (Godsave et al., 1988; Isaacs et al., 1992; Slack et al., 1987; Tannahill et al., 1992). Experiments using in vitro avian epiblast cell culture have shown that FGF induces expression of the receptor tyrosine kinase gene flk-1(Flamme et al., 1995a) also known as VEGFR2, which is a marker of the endothelial cell lineage. It has also been shown that the vasculogenic mesoderm and endothelial cells fail to develop in Xenopus embryos lacking the FGF-receptor 1 activity (Flamme et al., 1995a). More recently, experiments using delivery of FGF-2 from beads have shown that FGF-2 could induce cells from the epithelial quail somite to differerentiate into angioblasts (Poole et al., 2001).

1.1.2 The blood islands

Blood islands have been observed in the mesodermal layer of the murine yolk sac. The blood island anlagen give rise to hemangioblastic focal aggregations, in which the peripheral cells differentiate into endothelial cells and the inner cells become blood cells (Ferkowicz and Yoder, 2005; Pardanaud et al., 1987; Wilt, 1974). Experiments in which the inner cells are removed show that blood formation is precluded without affecting the development of vascular structures. Later in development, after the blood islands have formed in the splanchnopleura, they anastomose to form a continuous primary vascular network (Haar and Ackerman, 1971; Houser et al., 1961).

1.1.3 The hemangioblast

The intimate temporal and spatial association of hematopoietic and endothelial cell development has led to the hypothesis that both lineages arise from a common precursor. This putative precursor cell has been called the hemangioblast. Experiments with embryoid bodies confirm the existence of such a hemangioblast with both endothelial and hematopoietic potential (Baron, 2003). But different studies suggest that the hemangioblast exists: Flk-1 is expressed in the extraembryonic yolk sac blood islands that contain both hematopoietic and endothelial lineages. However, this expression is only maintained in the endothelial precursors (Dumont et al., 1995). Studies showed that mice mutant in the flk-1 gene develop neither blood nor vascular tissue (Shalaby et al., 1995), suggesting that a single cell type may be affected early during development.

In addition to Flk-1, the hematopoietic and endothelial cell lineages express other genes in common during early embryogenesis. These include the Tie and Tek (Tie-2) receptor tyosine kinases (Dumont et al., 1992; Korhonen et al., 1994), the QH1 and MB1 antigens (Pardanaud et al., 1987), TGF-β1 (Akhurst et al., 1990), the transcription factor c-ets-1 (Pardanaud and Dieterlen-Lievre, 1993), the cell adhesion molecules PECAM-1 (Baldwin et al., 1994; Newman et al., 1990) and CD34 (Fina et al., 1990), the angiotensin-converting enzyme (ACE) (Caldwell et al., 1976), the von Willebrand factor (Hormia et al., 1984), the cell adhesion glycoproteins P-selectin and E-selectin (Gotsch et al., 1994), and the transcription factor SCL/TAL-1 (Kallianpur et al., 1994). In many cases, expression of these molecules is maintained in only one lineage.

1.1.4 Endothelial proliferation

Once endothelial cells differentiate in the embryo, they proliferate and migrate before assembling into blood vessels. They become quiescent only when the vascular network has matured in the adult, where their turnover is extremely slow. There are different factors regulating endothelial cell proliferation. Both FGF and VEGF are mitogens of capillary endothelial cells in culture (Folkman and Shing, 1992). But only VEGF is specific for endothelial cells (Ferrara et al., 1992). Ectopic VEGF in quail, chick and frog leads to dramatic alterations of vascular structures (Cleaver et al., 1997; Drake and Little, 1995; Flamme et al., 1995a; Wilting and Christ, 1996).

Platelet-derived growth factor (PDGF) is also implicated in endothelial proliferation. In vivo it acts as an inducer of angiogenesis and is chemotactic for endothelial cells (Battegay et al., 1994). The endothelial cells of capillaries express both PDGF-B and its receptor PDGF-β, suggesting an autocrine stimulatory system (Holmgren et al., 1991). In vitro experiments also indicate that PDGF influences the angiogenic proliferation of endothelial cells in an autocrine fashion (Battegay et al., 1994).
Certain factors inhibit the angiogenic proliferation of endothelial cells (Klagsbrun, 1991). These include thrombospondin (Good et al., 1990), platelet factor IV (Taylor and Folkman, 1982), γ-interferon (Friesel et al., 1987), protamine (Taylor and Folkman, 1982), angiostatin (O'Reilly et al., 1994), and TNF-α (Folkman and Shing, 1992). TGF- β inhibits both endothelial cell proliferation (Antonelli-Orlidge et al., 1989) and migration (Sato and Rifkin, 1989). Hyaluronic acid (HA) also downregulates endothelial cell proliferation.

1.1.5 Assembly of blood vessels

The formation of the mature vascular system is achieved by a coordination of vasculogenesis and angiogenesis (Pardanaud et al., 1989; Risau and Lemmon, 1988). Vasculogenesis is almost exclusively limited to the establishment of the primary vascular plexus in the embryo, whereas angiogenesis extends and remodels the primitive embryonic vasculature. Vasculogenesis and angiogenesis are two different cellular mechanisms and are regulated by different molecular mechanisms.

1.1.5.1 Vasculogenesis

As mentioned, the earliest step in the development of the vascular system is the specification of mesodermal cells to become endothelial cells. These cells soon organize into a primitive vascular plexus via vasculogenesis (Fig. 3a). Vasculogenesis is defined as the coalescence of free angioblasts into loose cords or the fusion of blood islands (Poole and Coffin, 1989). Some definitions state that this assembly of angioblasts must occur in situ in absence of significant cell migration, but this is not always the case, as will be explained later. Vasculogenesis is therefore

responsible for the formation of the primordia of the major blood vessels and of a homogenous capillary network.

Formation of the blood islands, the dorsal aortae, the endocardium, and the cardinal and vitelline veins is accomplished by vasculogenesis (Coffin and Poole, 1991; Kadokawa et al., 1990; Pardanaud et al., 1987; Pardanaud et al., 1989; Poole and Coffin, 1988; Poole and Coffin, 1989; Risau and Flamme, 1995). Establishment of the vasculature of most organs occurs by angiogenesis, but the vascular network of certain endodermal organs, including liver, lung, pancreas, stomach, intestine and spleen occurs by vasculogenesis (Pardanaud et al., 1989). Vasculogenesis involves a coordinated and sequential series of steps, including differentiation, migration, adhesion and maturation, that results in the coalescence of individual migratory angioblasts into a continuous tubular endothelium (Coffin and Poole, 1988).

The development of the endocardium by vasculogenesis has been described in the mouse embryo. An extensive vascular plexus, lying adjacent to the promyocardial layer, undergoes remodeling to form a single endothelial tube.

The fusion of blood islands into a capillary plexus via vasculogenesis seems to require additional vasculogenic factors present in the embryo. The formation of a capillary plexus will not occur in embryoid bodies derived from mouse embryonic stem cells unless they are implanted in the peritoneum of host mice, which suggests that factors are required for vasculogenesis which are not present in the embryoid bodies.

Based on experiments in quail, Pool and Coffin (1991) distinguished two types of vasculogenesis. In vasculogenesis type I, the angioblasts associate to form a mature vessel *in situ* at the location where they differentiate in the mesoderm. There is no significant migration of angioblasts. In vasculogenesis type II, angioblasts may migrate significant distance from their original location and then associate into a vessel at a distant location.

1.1.5.2 Angiogenesis

Once the primitive vascular plexus is formed, vascular structures are extended and propagated into avascular tissues via a process called sprouting angiogenesis. In addition, the structure of the primitive vascular plexus is modified by the splitting or

fusion of established vessels via a process called nonsprouting angiogenesis or intussusception (Folkman and Klagsbrun, 1987; Klagsbrun, 1991; Patan et al., 1996b). The mechanisms for these 2 types of angiogenesis are different.

1.1.5.2.1 Sprouting angiogenesis

Sprouting angiogenesis involves true sprouting of capillaries from preexisting blood vessels of the primary vascular plexus (Fig.3b). Proteolytic degradation of the extracellular matrix is coupled with mitotic proliferation of the sprouting endothelial cells. These endothelial cells exhibit extensive migratory ability. In angiogenic extensions in the brain, the endothelial cells at the tip exhibit filiform processes which may represent pathfinding mechanisms (Wilting and Christ, 1996). As the new vessel extends and takes shape, endothelial cells begin to differentiate and the basement membrane forms along the newly sprouting structure (Ausprunk and Folkman, 1977). This differentiation involves the formation of a lumen and functional maturation. Sprouting angiogenesis is found for vascularization of the yolk sac, embryonic kidney, thymus, brain, limb bud and choroid plexus (Ekblom et al., 1982; Jotereau and Le Douarin, 1978; Le Lievre and Le Douarin, 1975; Stewart and Wiley, 1981). Brain is a typical organ where sprouting angiogenesis occurs. Intersomitic veins and arteries are also formed by sprouting angiogenesis (Coffin and Poole, 1988). Sometimes, angiogenesis can occur simultaneously with vasculogenesis (Fig. 3c), for example during vascularization of the lung (Baldwin, 1996). Sprouting angiogenesis is the predominant mechanism later in development, during somatic growth, corpus luteum formation, placental formation and tissue regeneration (Augustin et al., 1995; Demir et al., 2006; Folkman and Klagsbrun, 1987; Kadokawa et al., 1990; Klagsbrun, 1991; Sariola et al., 1983). In adults, sprouting angiogenesis is linked to pathological processes such as tumor growth, inflammatory reaction, wound healing and diabetic retinopathies (Ferrara, 1995; Folkman, 1995; Folkman and Shing, 1992; Hanahan and Folkman, 1996; Sholley et al., 1984).

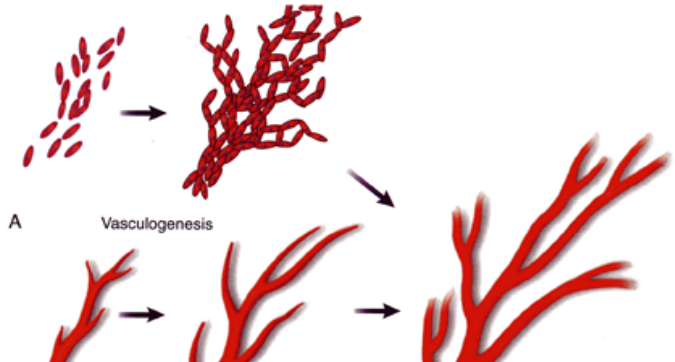
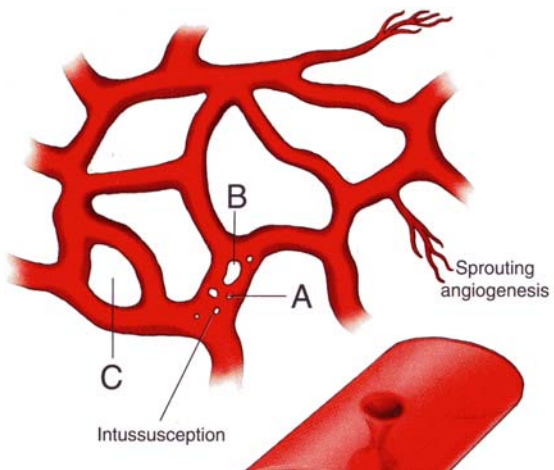


Figure 3: Schematic representation of the basic mechanisms of vascular development. (A) Vasculogenesis is the aggregation of angioblasts in the mesoderm to form blood vessels. Angioblasts either coalesce at the location where they emerge from the mesoderm or they migrate through tissues and form blood vessels at a distant site. (B) Angiogenesis involves the formation of new vessels from

1.1.5.2.2 Non-sprouting angiogenesis or intussusception

This second mechanism of angiogenesis involves the splitting of preexisting vessels (Burri and Tarek, 1990; Caduff et al., 1986; Patan et al., 1993; Patan et al., 1996a). Non-sprouting angiogenesis occurs by proliferation of endothelial cells within a vessel. It results in the formation of a large lumen. If the lumen is so large that there is disk-like zone of contact between opposite walls of a vessel, intercellular junctions are formed between the endothelial cells. This contact zone forms a column or pillar which then becomes perforated centrally, forming a canal within the pillar. This canal becomes invaded by pericytes and is eventually stabilized by the deposition of connective tissue fibers as collagen. The pillar then enlarges along the length of the vessel, fully splitting a vessel into 2 (Fig.4). In some organs, non-sprouting angiogenesis can occur together with sprouting angiogenesis. This is the case in the developing lung (Patan et al., 1993; Risau, 1997). Non sprouting angiogenesis also occurs in the avian yolk sac (Flamme and Risau, 1992).



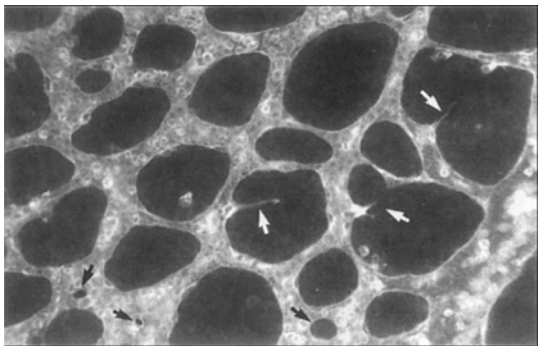


Figure 4: Left: In capillary networks, sprouting angiogenesis and nonsprouting angiogenesis, or intussusception, can occur simultaneously. Intussusception involves the formation of transcapillary pillars which split a capillary blood vessel into two. Initially, the pillar creates a small intervascular space (A), but the space subsequently enlarges (B and C) and forms a much larger intervascular region. After they have formed, the resulting intervascular spaces cannot be distinguished from those created by sprouting angiogenesis.
Right: Sprouting and non-sprouting angiogenesis in the 3-day-old quail yolk sac. Black arrows point to the intussusceptive pillars and white arrows indicate the long tips of sprouts. (From Risau, 1997)

1.1.5.3 Molecules involved in vasculogenesis and angiogenesis

A large number of molecules can modulate vasculogenic and angiogenic activity (Folkman and Klagsbrun, 1987). They can be soluble, associated with the cell membrane or with the extracellular matrix. Examples include angiogenin (Fett et al., 1985), angiotropin (Hockel et al., 1988), FGFs (Montesano et al., 1986), PDGFs (Holmgren et al., 1991) and TGF-α (Heimark et al., 1986). Some of their receptors are endothelial-specific markers, for example Flk-1, Flt-1, Tie-1 and Tie-2. Fibronectin has been implicated in endothelial cell proliferation and laminin in their subsequent maturation. Cell adhesion molecules such as certain integrins and cadherins also play an important role in vascular development.

1.1.5.3.1 Growth factors and their receptors

VEGF

VEGF is known to be mitogenic for endothelial cells and may be chemotactic for endothelial precursors (Breier et al., 1996).

In the embryo VEGF is expressed in regions undergoing both vasculogenesis and angiogenesis (Breier et al., 1992; Ferrara, 2001). In principle, VEGF is expressed in endodermal or ectoderamal tissues and flk-1 in the adjacent mesoderm (Cleaver et al., 1997; Dumont et al., 1995; Flamme et al., 1995a). Mice lacking a single allele of VEGF die around E10.5. They show abnormalities in vascular development, including defects in the in situ differentiation of endothelial cells, sprouting angiogenesis, lumen formation, formation of large vessels and spatial organization of the vasculature (Carmeliet et al., 1996; Ferrara et al., 1996). The heterozygous lethal phenotype implies that regulation of VEGF levels is essential for correct vascular morphogenesis. As said, VEGF also plays a role in angiogenesis. It is found in organs which are not juxtaposed to Flk-1 expressing endothelial cells and which are vascularized by angiogenesis, for example the kidney (Dumont et al., 1995). VEGF causes blood vessels to grow into the developing kidney from adjacent vascular structures. Also, VEGF spatio-temporal expression correlates with the ingrowth of blood vessels in the ventricular neurectodermal layer (Breier et al., 1992; Breier et al., 1996; Millauer et al., 1993). VEGF has also been shown to play a role in hypoxia-induced angiogenesis (Plate et al., 1993; Shweiki et al., 1992). In experiments using murine retina, hypoxia upregulates VEGF levels in migrating astrocytes (Stone et al., 1995). This upregulation is due to the increased transcription of VEGF by hypoxia-inducible factor-1 (Liu et al., 1995). But VEGF expression is also modulated by stabilization of VEGF mRNA (Ikeda et al., 1995).

PDGF

PDGF occurs as a homodimer or heterodimer of two isoforms, PDGF-A and PDGF-B (Beck and D'Amore, 1997). PDGF-B is involved in the autocrine stimulation of endothelial cells and angiogenesis. Transcripts for PDGF-B and its receptor PDGF-β are both present in the capillary endothelial cells of the human placenta, implying an autocrine signaling system (Holmgren et al., 1991). The endothelium of larger vessels maintains PDGF-B expression but does not express PDGF-β receptor,

suggesting a switch from autocrine to paracrine signaling when the endothelium recruits mesenchymal cells into the developing vascular wall. Capillary endothelial cells however are able to respond to PDGF, presumably due to the presence of PDGF-β receptor. Indeed, capillary density increases when PDGF-B is added to dermal wounds or to the chick chorioallantoic membrane (Pierce et al., 1992; Risau et al., 1992). PDGF-B was shown to be necessary for cord and tube formation of bovine aortic endothelial cells in vitro (Battegay et al., 1994). Expression of PDGF-β receptor was found only on the extending sprouts and forming endothelial tubes but not on the surrounding endothelial cells in the culture. Antibodies blocking PDGF-B activity reduced angiogenic activity, but antibodies against PDGF-A had no effect. Other studies suggest an indirect role of PDGF on endothelial cells. When myofibroblasts and endothelial cells are cultured together *in vitro*, PDGF stimulates the myofibroblasts to secrete a factor which causes vasculogenic aggregation of the endothelial cells into cords (Sato et al., 1993).
In contrast to the specific role of PDGF-B in angiogenesis, both PDGF-A and PDGF-B are implicated in vascular maturation and vascular wall development.

FGFs

Among the 23 FGFs, FGF-1 (aFGF) and FGF-2 (b-FGF) are both modifiers of angiogenesis (Fernig and Gallagher, 1994). aFGF promotes angiogenesis both *in vitro* and *in vivo* (Jouanneau et al., 1995). aFGF and bFGF show proliferative activity in vitro (D'Amore and Smith, 1993). FGF1 KO or FGF2 KO mice (single or double KO) do not show a defective vascular phenotype during development (Miller et al., 2000). This suggests functional redundancy or a non-essential role of FGFs in developmental vsculogenesis and angiogenesis. Mice knockout for FGFR1 and FGFR2 yield embryos that are arrested in their development before the onset of vascularization, because of the lack of mesoderm-inducing signals (Arman et al., 1998; Deng et al., 1994). Thus, these experiments of disruption of FGF/FGFR genes have not been very informative. Studies with explant or embryonic cultures have been more informative. Injection of a dominant negative FGFR into cultured day-9 mouse embryos induces incomplete branching of the yolk sac vasculature and intersomitic vessels, heart septation defects, and aangiogenesis defects in organs such as the brain (Lee et al., 2000). Endogenous FGF also induces vessel outgrowth

from embryonic heart explants (Tomanek et al., 2001). A proangiogenic phenotypes is observed in mice that overexpress FGF2 ubiquitously (Fulgham et al., 1999) or in the retina (Yamada et al., 2000). Overexpression of FGF2 (Sheikh et al., 2001) or FGF1 (Fernandez et al., 2000) in the heart leads to an increase in vessel density and arborescence. Mice that overexpress a dominant negative FGFR1 specifically in the retinal pigmented epithelium in the developing eye show branching defects in the choroid and and an avascular neonatal retina (Rousseau et al., 2003). This role for FGF (Branchless) in the branching process has also been observed in the Drosophila tracheal system (Affolter et al., 2003).

Flt-1 or VEGFR1

This receptor tyrosine kinase shows similarities to Flk-1 in overall structure and expression distribution (de Vries et al., 1992; Shibuya et al., 1990). It has a high affinity for VEGF and for placental growth factor (de Vries et al., 1992; Shibuya et al., 1990; Waltenberger et al., 1994). Its expression is associated with vascular development in mouse embryos and with neovascularization in wound healing (Peters et al., 1993). But Flt-1 is still expressed in the differentiated endothelium of adult vascular tissues, suggesting that it has a function in the quiescent endothelia of mature vessels. Targeted mutation of the flt-1 gene suggests that it is vasculogenesis rather than endothelial cell specification which is impaired. Indeed, such KO embryos develop endothelial cells in both intra- and extraembryonic tissues, but these endothelial cells do not properly assemble and organize into vessels (Fong et al., 1995). All vascular structures, including the major embryonic vessels, extraembryonic vessels, endocardium and capillary networks are disrupted. An increase in the endothelial cell number has been reported in the yolk sac and the endocardium which may result from a failure in contact inhibition. Thus, it has been suggested that Flt-1 signaling pathway may be involved in regulating the adhesion of endothelial cells to each other or to the extracellular matrix (Fong et al., 1995).

Flk-1 or VEGFR2

This receptor tyrosine kinase has a high-affinity for VEGF and is critical for both vasculogenesis and angiogenesis. As already mentioned, Flk-1 is initially present in precursors to both blood and endothelium, but it becomes restricted to endothelial

precursor cells. The expression of Flk-1 is particularly high during embryonic neovascularization and during tumor angiogenesis (Cleaver et al., 1997; Dumont et al., 1995; Flamme et al., 1995b; Fouquet et al., 1997; Liao et al., 1997; Millauer et al., 1993; Plate et al., 1993; Sumoy et al., 1997; Yamaguchi et al., 1993). Mice which lack the function of Flk-1 die between E8.5 and E9.5 due to defects in the development of both endothelial and hematopoietic cell lineages (Shalaby et al., 1995). Endothelial precursor cells do not coalesce into blood vessels by vasculogenic aggregation. In another experiment, glioblastoma cells having a dominant-negative construct of Flk-1 have been implanted into nude mice (Millauer et al., 1994). The angiogenic growth of vascular tissue in these tumors is significantly inhibited. These 2 experiments demonstrate the importance of Flk-1 in angiogenesis during development and during tumor growth.

Tie-2 (receptor for angiopoietin-1 and angiopoietin-2)

This tyrosine kinase is important for both vasculogenesis and angiogenesis. In the mouse, Tie-2 is expressed in endothelial precursors shortly after the onset of flk-1 expression (Dumont et al., 1995; Dumont et al., 1992). Mice lacking Tie-2 function die at E10.5, with defects in the integrity of the endothelium and defects in cardiac development (Dumont et al., 1994). They show vascular hemorrhage, possibly due to failure of endothelial proliferation or survival; indeed they show a decrease in the relative number of endothelial cells as development proceeds. They have distended yolk sac vessels and a ruptured and disorganized dorsal aorta. These experiments suggest that Tie-2 is not required for the differentiation of the endothelial cells, but is necessary for the expansion and maintenance of the lineage as vessels form by vasculogenesis.

Independent experiments have demonstrated that Tie-2 is also necessary for sprouting angiogenesis (Sato et al., 1995). Indeed its inactivation leads to an absence of capillary angiogenesis in the neurectoderm. The mutant mice have uniformly dilated vessels in the perineural plexus, abnormal and dilated vascular network in the yolk sac and a failure of branching of vessels in the myocardium. Because of these abnormalities in lumen diameter, it has been suggested that Tie-2 either modulates the activity of VEGF, which then regulates both sprouting and non-

sprouting angiogenesis, or is involved in recruitment of the vascular cell wall components which play a role in endothelial integrity.

Two ligands have been found for Tie-2 and are called angiopoietins (Davis et al., 1996). Angiopoietin-1 is expressed in proximity to developing blood vessels in the embryo, but it does not directly promote the proliferation of endothelial cells or tube formation *in vitro*. Targeted mutation of the angiopoietin-1 gene results in a phenotype similar to Tie-2 mutant mice: a vascular network lacking complexity of branching and heterogeneity of vessel size. These mice also show failure in the recruitment of vascular cell wall components, implying a role not only in initial vasculogenesis, but also in subsequent vessel maturation.

Angiopoietin-2 is a second ligand for Tie-2, but it does not activate it. Hence, it acts as an antagonist to Tie-2 function (Maisonpierre et al., 1997). It is expressed in the smooth muscle layer underlying the endothelium, in the dorsal aorta and the major aortic arches.

1.1.5.3.2 Extracellular matrix

ECM can modulate growth, differentiation and migration of endothelial cells *in vitro* (Risau and Lemmon, 1988). Extracellular matrix components such as fibronectin, laminin, vitronectin, collagens type I, II, IV and V comprise the environment in which angioblasts migrate and organize into cords which will form the primary vascular plexus. Some studies have analyzed the distribution of extracellular matrix molecules to determine the correlation with vascular development (Drake et al., 1990; Little et al., 1989; Risau and Lemmon, 1988). Other *in vitro* studies have directly assayed their ability to stimulate endothelial cell proliferation, migration, differentiation or vascular wall cell recruitment. They were done in two-dimensional assays, three-dimensional collagen gel assays and serum-free explant cultures of rat aorta (Bischoff, 1995; Grant et al., 1990).

Fibronectin

Vasculogenesis, the assembly of vessels from free angioblasts, takes place in a fibronectin-rich extracellular matrix (Mayer et al., 1981; Risau and Lemmon, 1988). In chick yolk sac, neighboring blood islands approach each other using fibronectin-rich

extensions (Mayer et al., 1981). As soon as the basic vascular network is established, fibronectin decreases and endothelial cells produce lamin and collagen IV. This dynamism was shown in avian blood vessel development in general (Risau and Lemmon, 1988), during the development of the endocardium (Drake et al., 1990) and the chick chorioallantoic membrane. Mice lacking a functional fibronectin gene have severe defects in blood vessel and heart development and in some cases a complete absence of the endocardium and the dorsal aorta (George et al., 1993). The extraembryonic vasculature does not develop and blood island development is disrupted. This shows the important role of fibronectin in the proliferative and migratory events of early vasculogenesis and angiogenesis.

Collagens

Different members of the collagen family possess different regulatory activities during vascular development. Endothelial tube formation *in vitro* is associated with the deposition of collagens type I and III-V (Iruela-Arispe et al., 1991). Endothelial cells cultured on interstitial collagens type I and III proliferate in all directions (Madri and Williams, 1983). However, endothelial cells cultured on basement membrane collagen type IV form highly organized tube-like structures. Endothelial cells grown in three-dimensional collagen type I matrix also organize into branching and anastomosing tubes (Montesano et al., 1983). Inhibition of collagen deposition or collagen cross-linking prevents angiogenesis (Ingber, 1991). Loss of collagen type I α-chain gene function results in the rupture of blood vessels in the developing embryonic vasculature (Lohler et al., 1984).

1.1.5.3.3 Cell adhesion molecules

Just as the dynamic changes in the composition of the extracellular matrix are important for endothelial behavior, so are the adhesive receptors that regulate the interactions of endothelial cells with their environment.

Vascular endothelial cadherin

VE-cadherin or cadherin-5 mediates calcium-dependent homophilic binding at adherens junctions between endothelial cells and is associated with catenins and the actin cytoskeleton (Breier et al., 1996). Agents that increase monolayer permeability (such as thrombin and elastase) cause a significant decrease in VE-cadherin at cell boundaries, suggesting a specific role in the control of endothelium permeability (Lampugnani et al., 1992). VE-cadherin is expressed from early on in blood islands and later in the vasculature of all organs, including the endocardium, the dorsal aorta, the intersomitic vessels and the brain capillaries (Breier et al., 1996). Cells transfected with the VE-cadherin gene in vitro are inhibited for proliferating (Caveda et al., 1996). Disruption of VE-cadherin in mouse ES-derived embryoid bodies by gene targeting experiments reveal that endothelial cells remain dispersed and fail to organize into vascular structures (Vittet et al., 1997).

Integrins ($\alpha_5\beta_1$ and $\alpha_v\beta_3$)

The role of integrins during vascular development is well characterized (Luscinskas and Lawler, 1994; Stromblad and Cheresh, 1996). Integrins generally mediate cell-ECM and occasionally cell-cell adhesion. They are heterodimers consisting of an α subunit and a noncovalently associated β subunit. Both are integral membrane proteins. Many different α and β subunits exist and many of these can associate to form different functional receptors (Baldwin, 1996). Endothelial cells from large vessels express $\alpha_2\beta_1$ $\alpha_3\beta_1$ $\alpha_5\beta_1$ and $\alpha_v\beta_3$. Endothelial cells from microvasculature express $\alpha_1\beta_1$ $\alpha_6\beta_1$ $\alpha_6\beta_4$ and $\alpha_v\beta_5$ (Luscinskas and Lawler, 1994). These vascular integrins serve as receptors for collagen, laminin, fibronectin and thrombospondin. Integrin $\alpha_5\beta_1$ is the receptor for fibronectin. The blocking of either subunit's function results in major defects in early vasculogenesis. Mouse embryos in which integrin α_5 function has been ablated are defective in blood vessel and blood island formation (Yang et al., 1993). The phenotype is similar to that of fibronectin loss of function experiments. Embryos then die on E10 or 11 due to numerous morphological defects. In quail embryos, the blocking of the binding of β_1 to its ligands with an anti-integrin antibody results in vasculogenic defects, including failure of lumen formation in the dorsal aorta (Drake et al., 1992). In summary, loss of $\alpha_5\beta_1$ integrin function causes vasculogenesis to be arrested after the stage when angioblasts form cords but before they have organized into tubes.

The β_3 family of integrins is essential for normal angiogenesis and vascular cell survival. For example integrin $\alpha_v\beta_{3,}$ which interacts with vitronectin, fibrin and fibronectin, is expressed at the tips of newly formed sprouting blood vessels in human wounds but is absent from normal skin (Brooks et al., 1994; Clark et al., 1996). As the vessels mature, its expression declines. During angiogenesis in the chick chorioallantoic membrane, $\alpha_v\beta_3$ expression increases. When antibodies are used to block its function, neovascularization is impaired, whereas preexisting vessels are unaffected (Brooks et al., 1994). As a control, antibodies against the related $\alpha_v\beta_5$ integrin had no effect. It was also shown that apoptosis of proliferative angiogenic endothelial cells occurs when the interaction of $\alpha_v\beta_3$ integrin with its substrates is disrupted (Brooks et al., 1994).

1.1.5.4 Endothelial cell migration

Endothelial cell migration is required during both vasculogenesis and angiogenesis (Christ et al., 1990; Noden, 1988; Noden, 1990; Poole and Coffin, 1989; Wilting et al., 1995). Experiments using quail-chick chimeras show that transplanted angioblasts are highly invasive and may migrate quickly over long distances (Noden, 1988; Noden, 1990). They invade the surrounding mesenchyme and contribute to the formation of veins, arteries and capillaries. Migratory distances of up to 400um have been observed (Klessinger and Christ, 1996). Despite the invasive character of angioblasts, they never cross the midline of the embryo (Wilting and Christ, 1996). Similar transplantation experiments showed that the notochord is the source of signals which create this barrier (Klessinger and Christ, 1996). The migration of angioblasts immediately precedes the formation of the endocardium, the ventral aortae and the cardinal and intersomitic veins in the avian embryo (Coffin and Poole, 1991). Blockage experiments which interrupt the path of migration show the importance of the migration for the formation of these structures.

1.1.5.5 Molecules involved in endothelial cell migration

1.1.5.5.1 VEGF/Flk-1

In frog embryos, the hypochord expresses diffusible VEGF and thus creates a signal gradient which may explain the directed migration of flk-1 expressing endothelial cells from the lateral mesoderm to the midline of the frog embryo. Moreover, exogenous VEGF can cause aberrant migration and proliferation of endothelial cells in the frog embryo (Cleaver et al., 1997). In homozygous flk-1 mutant mice, no mature endothelial or hematopoietic cells are present (Shalaby et al., 1995). However, the construct allows expression of β-galactosidase and it is detected at high levels in the region of the connecting stalk and in an aortic arch. It is thus possible that angioblasts cannot migrate from these sites to locations where the elements of the primary vasculature would normally differentiate.

1.1.5.5.2 Fibronectin

Fibronectin is involved in endothelial cell motility during vascular development. *In vitro* experiments have demonstrated that fibronectin can stimulate the migration of vascular endothelial cells. Moreover, the distribution of fibronectin in the chick embryo is associated with both migrating angioblasts prior to their coalescence into vessels, and with early steps of angiogenesis, when capillaries are extending and invading avascular tissue (Risau et al., 1988). Also, application of a pentapeptide which blocks the fibronectin receptor on endothelial cells results in the inhibition of endothelial cell migration both *in vitro* and *in-vivo* (Christ et al., 1990; Nicosia and Bonanno, 1991). For example, this blocking reagent impairs the migration of precardiac mesoderm (Linask and Lash, 1988).

1.1.5.5.3 Integrin $\alpha_v\beta_3$

In addition to its role in maintaining and stabilizing early vascular structure (discussed previously), integrin $\alpha_v\beta_3$ is also implicated in endothelial cell migration and in proteolytic modification of the extracellular matrix. Integrin $\alpha_v\beta_3$ colocalizes with active matrix metalloproteinase-2 in growing blood vessels and the two bind to each other *in*

vitro (Brooks et al., 1996). The degradation of the underlying basement membrane is a prerequisite for invasive angiogenic cells to extend new sprouts into adjacent tissues.

Vitronectin has binding sites for integrin $\alpha_v\beta_3$ and for the plasminogen activator inhibitor-1 (PAI-1). These two binding sites overlap and it has been suggested that plasminogen activator may bind to PAI-1, displacing it from vitronectin and thus inducing cell migration by allowing the receptor-ligand interaction. It has been shown that VEGF can upregulate the expression of integrin $\alpha_v\beta_3$ and PAI-1, and that both plasminogen activator and PAI-1 are upregulated in migrating endothelial cells (Pepper et al., 1991; Pepper and Montesano, 1990). These interactions provide a molecular basis for the coordination of cell migration and matrix degradation.

1.1.6 Vascular remodeling

Once the primary capillary plexus is established in the embryo, it is remodeled and matures into larger and smaller blood vessels. One of the processes by which this architecture is acquired has been called pruning, by analogy to trimming a tree (Risau, 1997). Pruning was first described in the embryonic retina and involves the removal of excess endothelial cells which form redundant channels (Ashton, 1966). In these excess capillaries blood flow ceases, the lumens are obliterated, and the endothelial cells retract towards adjacent capillaries. They don’t die by apoptosis (Augustin et al., 1995). They may dedifferentiate to become either muscular or supportive elements of the vascular cell wall (Ashton, 1966; Risau, 1997).

In addition to the trimming of excess endothelial cells, the embryonic vasculature undergoes dynamic changes in morphology, called remodeling (Beck and D'Amore, 1997; Risau, 1997). Remodeling involves the growth of new vessels and the regression of others as well as changes in the diameter of vessel lumens and vascular wall thickness. Blood flow as well as tissue demand are key regulators of vessel maintenance (Ashton, 1966; Risau, 1997). It seems that only a few numbers of embryonic blood vessels persist into adulthood (Risau and Flamme, 1995).

1.1.7 Remodeling, patterning and maturation

Dramatic changes occur after the circulation of blood cells has been established. Usually the larger vessels such as arteries or veins develop from the fusion of capillaries after the formation of the primary vascular plexus. Early vessels have thick endothelial cells with weak adherence and incomplete basement membrane formation, but it changes as blood flow increases and endothelial cells mature. Anastomoses disappear, capillaries may split by intussusception, the direction of blood flow may change many times and adherence between endothelial cells increases dramatically. With vessel maturation, a basement membrane forms, gradually thickens and becomes less heterogeneous (Wolff and Bar, 1972). Vessels become shaped by mechanical forces generated by the circulation (Resnick and Gimbrone, 1995). Hemodynamic forces can cause the changes in the expression of PDGF, FGF, TGF-β and tissue factor from endothelial cells. These factors can also modify endothelial cell adherence (Griendling and Alexander, 1996; Resnick and Gimbrone, 1995). But the determination and pattern of the vasculature does not only depend on blood pressure since growth and formation of blood vessels proceeds in the absence of a heart. The final maturation of the vasculature requires interaction of endothelial cells with each other, with the surrounding extracellular matrix and with adjacent mesenchymal support cells such as pericytes and smooth muscle cells.

The initial plexus becomes remodeled into larger veins and arteries and smaller venules, arterioles and capillaries. The endothelia lining these different vessels have different properties (Kumar et al., 1987). The endothelium of large vessels controls blood pressure through vasoconstriction and vasodilatation. The endothelium of small vessels plays a role in the exchange of gas and nutrients with the tissues (Risau and Flamme, 1995). The capillary endothelium is divided into 3 different subtypes: continuous, discontinuous and fenestrated (Bennett et al., 1959; Risau and Flamme, 1995). These morphological differences reflect different permeability of the vessels in different tissues. Continuous capillaries are found in the central nervous system, the lymph nodes and muscle. They are composed of endothelial cells perforated by the vessel lumen (intraendothelial canalization) and have been called seamless endothelia (Wolff and Bar, 1972). The lumen formation has been postulated to result from vacuolization and fusion of vacuoles. Discontinuous capillaries are found in the

liver, bone marrow and spleen. They have clustered pores of 80-200um diameter, located at each end of the endothelial cell. Fenestred capillaries are found in the kidney glomeruli, the choroid plexus, the endocrine glands and the gastrointestinal tract. They have large pores and are more permeable to to low-molecular-weight hydrophilic molecules. This is consistent with their presence in tissues involved in secretion, filtration and absorption (Levick and Smaje, 1987). VEGF is a permeability factor which has been shown increase permeability and fenestration (Roberts and Palade, 1995).

As the vascular endothelium begins to mature, endothelial cells synthesize multiple proteins of extracellular matrix which form a basement membrane. It is composed of fibronectin, laminin, entactin/nidogen, collagen and a heparin sulfate proteoglycan (Grant et al., 1990). This extracellular matrix maintains cell polarity and regulates proliferation, adhesion and differentiation of endothelial cells (Grant et al., 1990). The deposition of extracellular matrix helps to establish the patterning of the primary vascular plexus and is an early indication of blood vessel maturation.

After the morphological changes associated with pruning and remodeling of the vascular plexus, mesenchymal cells are recruited to give mechanical and physiological support to the endothelium. Pericytes are recruited to the small capillaries, and smooth muscle cells and adventitial fibroblasts are recruited to larger vessel to form their vascular wall (Le Lievre and Le Douarin, 1975; Schwartz and Liaw, 1993). Pericytes cover only a fraction of the surface of capillaries. They might regulate the permeability, proliferation and integrity of endothelial cells (Crocker et al., 1970; de Oliveira, 1966; Rhodin, 1968). Only pericytes and endothelial cells are included in the mature capillaries (Orlidge and D'Amore, 1987). *In vitro*, pericytes can inhibit capillary endothelial cell growth and this is mediated by TGF-β (Antonelli-Orlidge et al., 1989).

Larger vessels recruit a different type of vascular supportive cells, called the smooth muscle cells (SMC) which is essential for the physiological properties of these vessels. Early SMCs express α-actinin (Gabbiani et al., 1981; Owens and Thompson, 1986) and later express additional differentiation genes, such as SM22 and calponin (Duband et al., 1993).

These processes finally give a vast repertoire of specialized blood vessels. Three main layers have been identified in the major blood vessels. The tunica intima is the innermost layer. It is composed of the endothelium, the basement membrane and

internal elastic tissue. The tunica media surrounds the tunica intima. It is composed of SMCs with elastic tissue. The tunica adventitia surrounds the inner layers with fibrous connective tissue, elastic tissue and mesenchymal cells. Arteries are surrounded with a thick smooth muscle cell layer. Veins, which face a lower pressure, have less smooth muscle in their walls. They can stretch to become a temporary reservoir of blood.

1.1.7.1 Molecules involved in vessel maturation and patterning

1.1.7.1.1 PDGF

In addition to playing a role in angiogenesis, PDGF is important for the recruitment of vascular wall components (Beck and D'Amore, 1997). PDGF is expressed in the endothelial cells, whereas its receptor, PDGF-β, is found in adjacent mesenchyme (Holmgren et al., 1991). The model is that the endothelium secretes PDGF to recruit and stimulate the proliferation of mesenchymal cells in the vicinity. Experiments involving the targeted mutation of the PDGF-B and PDGF-β genes support a role for this signaling system in vascular wall cell recruitment (Leveen et al., 1994; Soriano, 1994). Mice mutant for either gene display a range of anatomical and histological abnormalities, including dilatation of the heart and blood vessels. Mutant mice die at about the time of birth from fatal hemorrhages, when embryonic blood pressure increases. The hemorrhages and vessel dilatation are attributed to a lack of pericytes throughout the capillary network. Other experiments suggest that PDGF-B secreted form endothelial cells recruit and stimulate proliferation of SMCs (Beck and D'Amore, 1997).

1.1.7.1.2 TGF-β

Contact between endothelial cells and SMCs or pericytes leads to the activation of TGF-β expression (Antonelli-Orlidge et al., 1989). TGF-β then leads to the inhibition of proliferation and migration of endothelial cells (Orlidge and D'Amore, 1987; Sato and Rifkin, 1989), the induction of SMC and pericyte differentiation, and the stimulation of extracellular matrix deposition (Basson et al., 1992). These effects lead

to the differentiation and maturation of the developing blood vessels. When the function of TGF-β is disrupted in mice, mutant mice show defects in both vasculogenesis and hematopoiesis (Dickson et al., 1995). Endothelial proliferation, however, is not affected, suggesting that the defects lie in the terminal differentiation. Similar defects are observed in mice lacking the TGF-β receptor type II (Oshima et al., 1996).

1.1.7.1.3 Tie-2, angiopoietin-1 and angiopoietin-2

Tie-2 is a receptor tyrosine kinase expressed in the vascular endothelium. In addition to being important in the early events of vasculogenesis and angiogenesis (Dumont et al., 1994), it is also required for vascular remodeling (Sato et al., 1995). Targeted mutation of the Tie-2 gene results in a disorganized vasculature and the absence of angiogenic sprouting. There is also little distinction between the large and the small blood vessels in the head and in the yolk sac (Sato et al., 1995). The ligands for Tie-2 are angiopoietin-1 (Davis et al., 1996) and angiopoietin-2 (Maisonpierre et al., 1997). Mice lacking functional angiopoietin-1 have defects similar to mice lacking functional Tie-2 receptor. Moreover, their endothelial cells are poorly associated with smooth muscle cells or pericytes, which are present in reduced numbers. Their endothelial cells are abnormally rounded, indicating that they have not acquired polarity. Angiopoietin-2 is an antagonist to angiopoietin-1 and Tie-2 (Maisonpierre et al., 1997). It is expressed only at sites of vascular remodeling, such as the dorsal aorta and the aortic branches. Overexpression of angiopoietin-2 results in defects similar to those seen in angiopoietin-1 or Tie-2 deficient embryos.

A model has been developed which proposes a role for a number of these molecules in the maturation of blood vessels (Armulik et al., 2005; Folkman and D'Amore, 1996). Mesenchymal cells produce angiopoietin-1, which activates the Tie-2 receptor on nearby endothelial cells. In response to the Tie-2 activation, the endothelial cells release a PDGF signal which acts to recruit nearby mesenchymal cells. In the case of pericytes, this signal is PDGF-B and in the case of SMCs, the signal is PDGF-A. Once the mesenchymal cells have contacted the endothelium, TGF-β is activated. The presence of TGF-β serves to reduce the proliferation of both endothelial and vascular wall cells, to induce their differentiation and to stimulate extracellular matrix deposition (Fig.5).

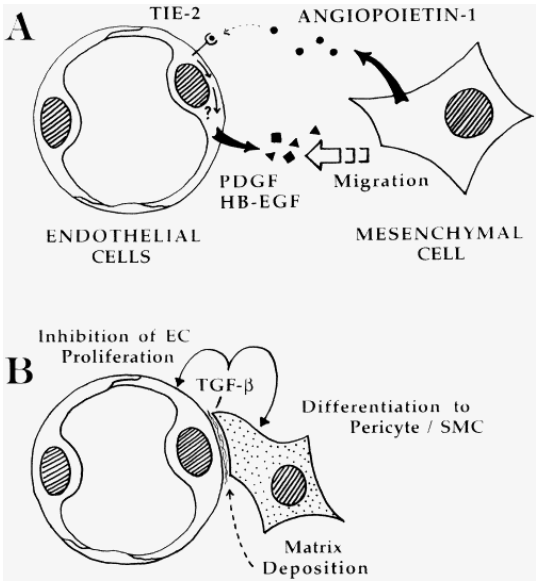


Figure 5: Model for the recruitment of the cellular vascular wall components. Angiopoietin-1 is secreted by mesenchymal cells and binds to the Tie-2 receptor located on the endothelial cells. This receptor activation triggers the release of factors from the endothelium which cause a chemotactic attraction of mesenchymal cells. These factors include PDGF-A or HB-EGF for the recruitment of smooth muscle cells to large vessels, or PDGF-B for the recruitment of pericytes to the capillaries. When these mesenchymal cells contact the endothelium, TGF-β is activated and causes vessel maturation.
From Folkman and D'Amore, 1996)

1.1.7.1.4 Tie-1

Expression of Tie-1 in the embryo is specific to endothelial cells. Mouse embryos homozygous for a disrupted Tie-1 gene die at about E13.0, when the mutant mice begin to die as a result of multiple vascular defects (Puri et al., 1995; Sato et al., 1995). Mutant embryos show edema and localized hemorrhaging and die due to the loss of integrity of the microvasculature. Thus Tie-1 is not necessary for the early steps of endothelial cell differentiation or vasculogenesis, but is required for later aspects of endothelial cell survival, maintenance, or proliferation.

1.1.7.1.5 Extracellular matrix molecules

A clear correlation between dynamic changes in extracellular matrix composition and endothelial cell maturation has been established. Fibronectin around endothelial cells is associated with their proliferation and migration. But as endothelial cells mature, levels of fibronectin gradually decrease whereas there is a corresponding increase in the levels of surrounding laminin and type IV collagen (Risau and Lemmon, 1988). Fibronectin thus appears to be associated with the early steps of endothelial development, whereas laminin may be an early marker for vascular maturation (Risau, 1991). Collagen IV might be even better than laminin to stabilize vessel walls

during vessel maturation, since cultured endothelial cells are more adhesive to a substrate composed of collagen IV than to one composed of laminin (Herbst et al., 1988).

A summary of the molecules involved in the assembly of blood vessels is shown in Fig. 6.

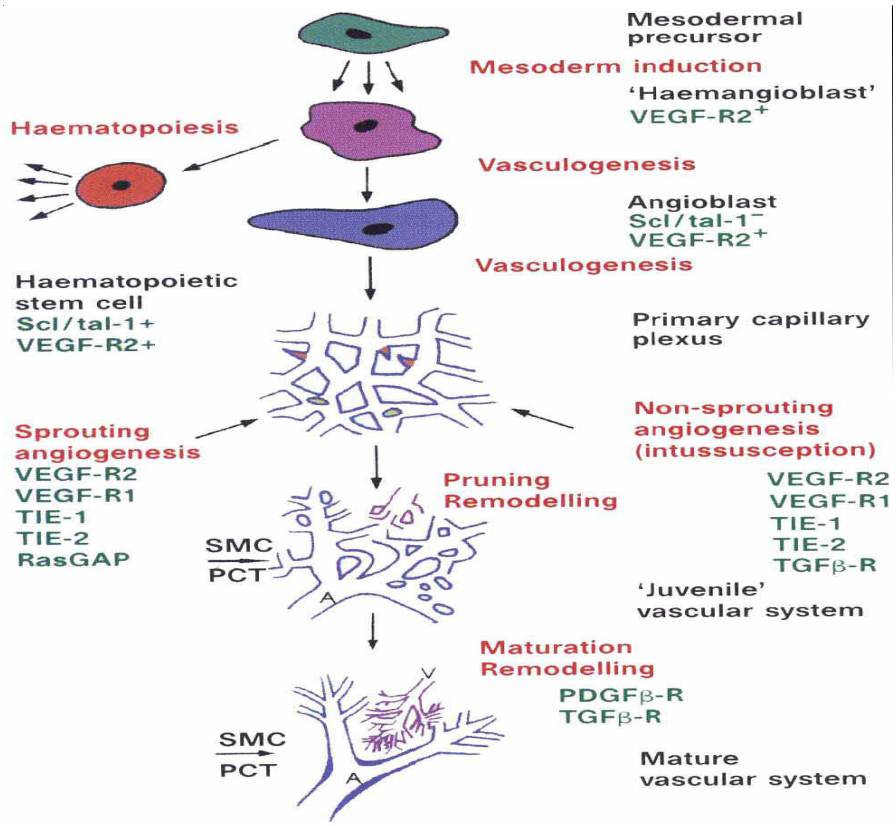


Figure 6: The processes (red labels), molecules (green labels) and appearances (black labels) involved in vascular development.

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1.2 Part II: Apoptosis and involution of the mammary gland

1.2.1 Generalities

1.2.1.1 History of apoptosis: p53, Bcl-2, apoptosis in C.elegans

In mammals, p53 represents an emergency molecule which enables tissues to eliminate aberrantly functioning or irreparably damaged cells. But apoptosis (in greek: falling leaves) can also happen through a variety of signaling channels that do not depend on p53, for example when apoptosis happens when a cell looses its anchorage to extracellular matrix. This special form of apoptosis is called anoikis and occurs without p53 activity.

The first indication of contributions of other proteins to the regulation of apoptosis came from studies on the function of Bcl-2 (B-cell lymphoma gene-2) oncogene. In genomes of human lymphatic tumors, the bcl-2 gene becomes an oncogene through chromosal translocation, which places it under the control of a promoter with high constitutive expression (Tsujimoto et al., 1984). When a construct harbouring this Bcl-2 oncogene was inserted into the germ-line of mice so that it was expressed in lymphocyte precursor cells, there was no effect on the long-term survival of these mice (Fig.1). But expression of an oncogenic myc transgene led to lymphomas and the death of a large number of mice. The concomitant expression of the two transgenes led to offsprings having a more rapid death rate.

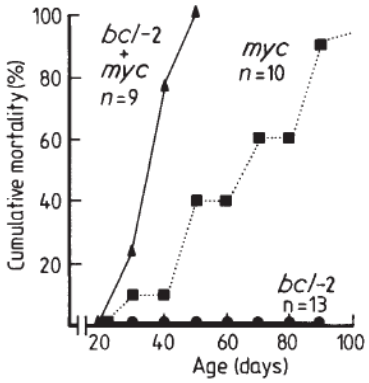

Fig.1: Mice bearing the IgG-bcl-2 transgene don't experience more mortality than WT mice. The Myc transgene leads to a greatly increased mortality of mice from lymphoma. However, when both Bcl-2 and Myc transgene are present in the germ line, the mortality is greatly increased.

The fact that Bcl-2 alone could not trigger tumor formation indicated that it does not act as a typical oncogene like myc or ras which alone emits growth-promoting signals. In fact careful study of the lymphocyte population indicated that Bcl-2 oncogene prolonged the life of lymphocytes, although they were not actively proliferating. Hence it promoted cellular survival (Vaux et al., 1988). This discovery introduced the concept that impaired apoptosis is central to tumour development (Hanahan and Weinberg, 2000). The Myc oncogene on its own acted as a potent mitogen. However, when myc and bcl-2 were acting together, the malignancy of the B-cell lymphocytes was more aggressive; this is because Myc drives rapid proliferation, and its death-inducing effects are neutralized by the life-prolonging actions of Bcl-2. .

In the late 1980s, apoptosis was widely studied in C.elegans, where 131 cells out of 1090 die to create the 959 nematode. This work led the 2002 Nobel Prize to Brenner, Holvitz and Sulston.

They saw that apoptosis needs CED-4 and CED-3 in order to happen. CED-9 on opposite has been shown to be anti-apoptotic, and to be negatively regulated by EGL-1 (Ellis et al., 1991; Hengartner et al., 1992). Convergence between the two fields revealed that CED-9 was the worm counterpart of the mammalian Bcl-2 (Hengartner and Horvitz, 1994) and CED-3 was shown to be the counterpart of caspase -1, the mammalian cysteine protease that cleaves interleukin-1β (Yuan et al., 1993). See Figure 2.

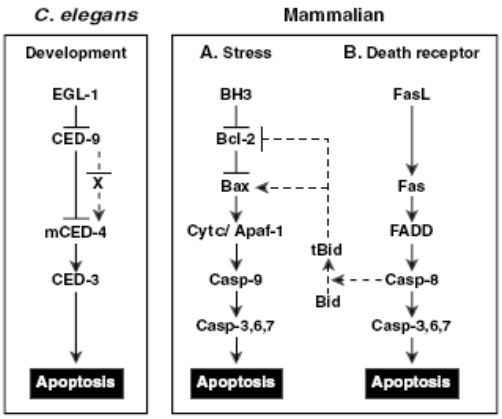

Figure 2: Pathways to apoptosis in C. elegans and mammalian cells. The pathway for programmed cell death in C. elegans (left panel) is compared with the two distinct pathways in mammals (right panel): the stress pathway (A), triggered by diverse cytotoxic conditions such as cytokine deprivation, DNA damage and anoikis, leads to activation of initiator caspase-9, whereas the death receptor pathway (B), triggered by aggregation on the plasma membrane of receptors of the tumour necrosis factor (TNF) family (here typified by Fas), leads to activation of initiator caspase-8. The stress and death receptor pathways are largely independent but may be linked via activation of the BH3-only protein Bid in certain cell types (see text). This model of the stress pathway now appears to be too simplistic (see text and Figure 4)

1.2.1.2 Apoptosis vs necrosis

Prior to the discovery of apoptosis, cells in metazoan tissues were thought to be eliminated solely by necrosis. But unlike necrosis, apoptosis is a genetically programmed cell death, and as indicated in Table 1, these two processes are quite different:

The stimulus provoking apoptosis can be a programmed tissue remodeling in the organism, for example during development. The reason can also be maintenance of cell pool size, genomic damage, metabolic rearrangement, hypoxia or imbalances in signaling pathways. On opposite, necrosis can be provoked by metabolic stresses, absence of nutrients, changes in pH, temperature, hypoxia or anoxia.

There is also a morphological difference between cells undergoing apoptosis or necrosis. In apoptosis, individual cells are affected, the cell volume is decreased, chromatin is condensed, lysosomes are unaffected and mitochondria are initially

morphologically normal. There is no inflammatory response and the apoptotic bodies are consumed by neighboring cells. In opposite necrosis affects groups of cells, the cell volume is increased, chromatin is fragmented, lysosomes are abnormal and mitochondria are morphologically aberrant. There is a marked inflammatory response and the cell is lysed.

The difference is also seen molecularly: apoptosis requires gene activity for the program to take place. The DNA is cleaved at specific sites. Intracellular calcium is increased and the ion pumps continue to function. In opposite, necrosis doesn't need any gene activity, the DNA is randomly cleaved, intracellular calcium is unaffected and ion pumps function is lost.

Table1: Apoptosis vs necrosis

	Apoptosis	**Necrosis**
Provoking stimuli		
	programmed tissue remodeling	metabolic stresses
	maintenance of cell pool size	absence of nutrients
	genomic damage	changes in pH, temperature
	metabolic derangement, hypoxia	hypoxia, anoxia
	imbalances in signaling pathways	
Morphological changes		
Affected cells	individual cells	group of cells
Cell volume	decreased	increased
Chromatin	condensed	fragmented
Lysosomes	unaffected	abnormal
Mitochondria	morphologically normal initially	morphologically aberrant
Inflammatory response	none	marked
Cell fate	apoptotic bodies consumed by neighboring cells	lysis
Molecular changes		
Gene activity	required program	not needed
Chromosomal DNA	cleaved at specific sites	random cleavage
Intracellular calcium	increased	unaffected
Ion pumps	continue to function	lost

1.2.1.3 Apoptosis is required for development and homeostasis

Cell death during embryonic development is essential for successful organogenesis and the crafting of complex multicellular tissues. The evolutionary advent of differentiated cell types may have necessitated controlling death as well as division in order to keep neighboring cells interdependent and insure the proper balance of each cell lineage. Apoptosis also operates in adult organisms to maintain normal cellular homeostasis. This is especially critical in long-lived mammals that must integrate multiple physiological as well as pathological death signals, which for example includes regulating the response to infectious agents. Gain- and loss-of-function models of genes in the core apoptotic pathway indicate that the violation of cellular homeostasis can be a primary pathogenic event that results in disease. In addition to its role in embryonic development, evidence indicates that insufficient apoptosis can manifest as cancer or autoimmunity, while accelerated cell death is evident in acute and chronic degenerative diseases, immunodeficiency, and infertility. Huntington disease or Alzheimer are examples of such degenerative diseases, where unproper folding of proteins activates the unproper folding response (UPR), which leads to apoptosis (Danial and Korsmeyer, 2004).

1.2.1.4 Different forms of apoptosis

It is now clear that there are variations in the morphological events associated with cell death and these probably reflect distinct molecular mechanisms. At least 10 genetically programmed cell death pathways have been defined which occur in different situations and in response to diverse stimuli (Melino et al., 2005).

1.2.2 Intrinsic apoptosis pathway: role of the mitochondrion

Bcl-2 was then found to operate at the outer membrane of the mitochondrion. This was at first surprising since mitochondria were thought to be specialized only for the generation of ATP. Soon the role of mitochondria in the apoptotic program was clarified. Cytochrome c, key player in the apoptosis, normally resides between the

inner and outer mitochondrial membranes, where it transfers electrons as part of oxidative phosphorylation. But upon triggering of apoptosis by certain signals, the outer membrane becomes depolarized and cytochrome c spills out of the mitochondrion into the cytosol. Once present in the cytosol, cytochrome c associates with Apaf proteins to form the apoptosome and trigger a cascade of events that yield apoptotic death. Therefore, evolution chose the mitochondrion as a site of energy production and release of a messenger, cytochrome c, which is responsible for cell death.

1.2.2.1 The Bcl-2 family and its 3 subfamilies

In fact the Bcl-2 protein is a member of a large family of proteins: the Bcl-2 protein family, which is complex and involves at least 24 Bcl-2 related proteins. Some like Bcl-2 are anti-apoptotic, but majority are pro-apoptotic. We distinguish 3 sub-families (Fig. 3):

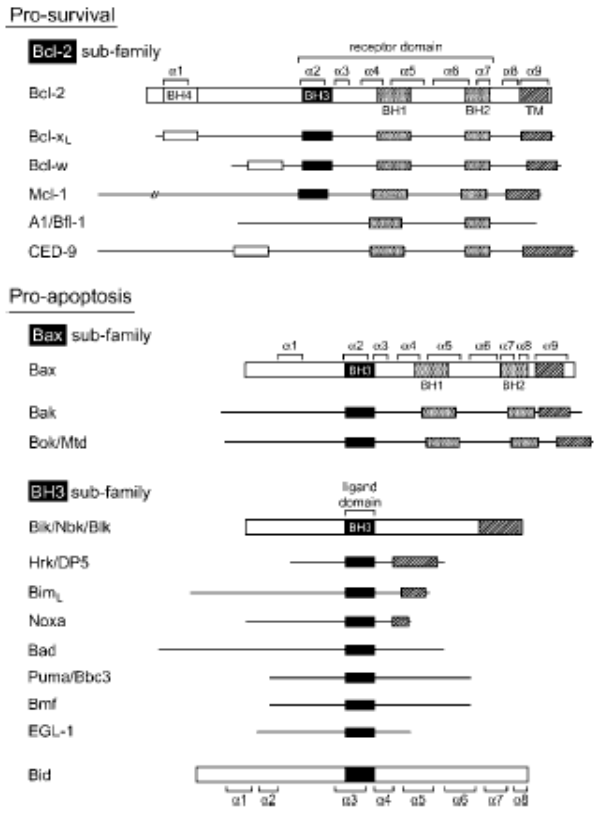

Fig.3: Structure of the Bcl-2 family and its 3 subfamily members: the Bcl-2, Bax and BH3-only subfamilies.

The Bax subfamily includes Bax, Bak and Bok. They normally reside inactive in the outer mitochondrial membrane or in the cytosol. If activated, these molecules act at

the surface of the outer mitochondrial membrane. Inactivation of either Bax or Bak alone has little consequence in mice, but elimination of both genes dramatically impairs developmental apoptosis in many tissues, resulting in perinatal death (Lindsten et al., 2000). This suggests that Bax and Bak have functional redundancy. Simultaneous lack of Bax and Bak, like loss of Bim (Bouillet et al., 1999; Bouillet et al., 2002), also perturbs thymic selection and lymphoid homeostasis (Rathmell et al., 2002).

In response to cytotoxic signals, Bax and Bak undergo conformational change and form oligomers associated to the membrane (Nechushtan et al., 2001). All these events can be blocked by Bcl-2 overexpression, since it acts upstream of Bax/Bak. It is not clear how the oligomer forms. Perhaps some Bax/Bak molecules assume a BH3 donor-like conformation while others retain their BH3 groove and behave as 'receptors'.

In healthy but not apoptotic cells, a small proportion of Bak is found in association with VDAC2 in the mitochondrial outer membrane (Cheng et al., 2003). It is thus not clear yet if Bax and Bak form themselves pores or if they act on voltage-dependant activated channels, but the result is damage to the outer membrane of mitochondria, which causes the release of apoptotic mediators into the cytosol:

-cytochrome c, which activates Apaf-1 to form the so-called apoptosome or wheel of death (Liu et al., 1996). The apoptosome cleaves procaspase 9 into caspase 9. Caspase stands for **c**ysteine **asp**artyl-specific prote**ase**. Caspase 9 then cleaves procaspase 3. Then a serie of cleavages ensues in which one protease activates the next one by cleaving it.

-Smac/Diablo and Omi, which antagonize the function of the anti-apoptotic IAPs (Inhibitors of Apoptosis Proteins) (Suzuki et al., 2001). Theses IAPs normally block caspase action.

-endonuclease G, which helps CAD (caspase-activated DNAse) in DNA fragmentation (Parrish et al., 2001).

The Bcl-2 subfamily includes Bcl-2 itself, its close relatives Bcl-X_L, Bcl-w, and the more divergent Mcl-1 and A1. These molecules are anti-apoptotic or pro-survival and when they are active they bind to Bax or Bak and thus inhibit the release of cytochrom c from the mitochondrion to the cytosol. All antiapoptotic Bcl-2 family members have oncogenic potential. However in the totality of malignancies, mutations that directly affect antiapoptotic Bcl-2 family members appear to be surprisingly rare. However, certain oncogenic mutations probably act indirectly to increase their expression levels. In any case high levels of expression in tumors must be interpreted with caution, because a tumor is often less differentiated than the surrounding normal tissue and expression levels of Bcl-2 family members often change markedly during differentiation.

The BH3-only subfamily includes Bim, Bik, Bad, Bmf, Hrk, Noxa and Puma. The members of this family are pro-apoptotic. In their inactive state, they are limited to the cytosol, but upon activation by pro-apoptotic signals, they are translocated to the mitochondria (Puthalakath and Strasser, 2002). The multiplicity of mammalian BH3-only proteins allows sophisticated control over the initiation of cell death (Figure 4). Individual BH3-only proteins are expressed only in certain cell types, and some appear to monitor particular subcellular compartments for stress or damage, and to respond to specific cytotoxic signals. For example, Bim is required for deletion of autoreactive lymphocytes *in vivo* (Bouillet et al., 2002) and for apoptosis of T cells *in vitro* following cytokine deprivation, calcium flux or treatment with Taxol (paclitaxel) but not markedly for apoptosis induced by γ-irradiation (Bouillet et al., 1999). Bad is required for the death that follows deprivation of glucose (Danial et al., 2003) or of epidermal growth factor (Ranger et al., 2003). Bmf is required for anoikis, the apoptosis that epithelial cells undergo following their detachment from the extracellular matrix (Puthalakath et al., 2001); interestingly, anoikis is thought to limit metastasis. In another link to tumorigenesis, Noxa (Oda et al., 2000) and Puma (Nakano and Vousden, 2001) are both induced by the tumour suppressor p53. Importantly, they have now been shown to be critical for apoptosis following genotoxic damage and Puma also for the death induced by several drugs (Villunger et al., 2003).

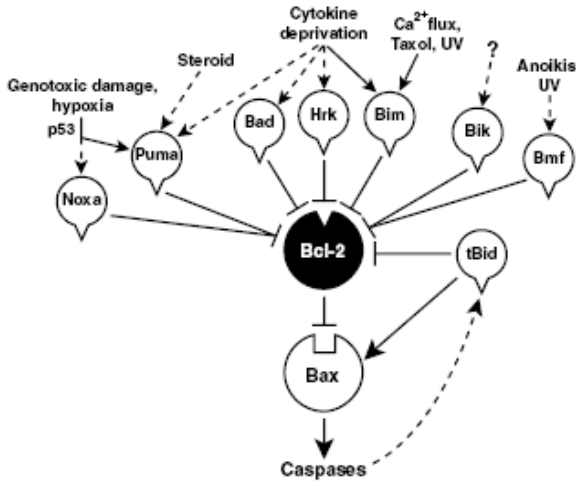

Fig.4: Various stresses seem to operate through different BH3-only pro-apoptotic proteins

Besides the fact that the individual pro-apoptotic BH3-only members can be activated by different signals, the way how individual BH3-only proteins are recruited to induce apoptosis is also different (Puthalakath and Strasser, 2002). Hrk/DP5 are regulated primarily at the transcriptional level, as well as Noxa and Puma (Harris and Johnson, 2001; Imaizumi et al., 1999). The BH3-only proteins that are produced constitutively are maintained in a latent form until activation by diverse mechanisms. For example, Bad is sequestered by 14-3-3 scaffold proteins after phosphorylation by kinases such as Akt/PKB and protein kinase A (Zha et al., 1996), and its activation requires dephosphorylation, for example by calcineurin (Wang et al., 1999). Conversely, Bik/Nbk activation requires phosphorylation, possibly by casein kinase II (Verma et al., 2001). Bid instead undergoes cleavage by caspases or granzyme B, perhaps regulated by phosphorylation (Desagher et al., 2001).

Bim and Bmf seem to be sentinels that check the cytoskeleton state (Puthalakath et al., 1999; Puthalakath et al., 2001). In healthy cells, both predominant forms of Bim (the splice variants Bim_{EL} and Bim_{L}) are sequestered to the dynein motor complex on microtubules via the dynein light-chain DLC1 (also known as LC8) (Puthalakath et al., 1999). Sucrose gradient experiments show that upon stress such as cytokine deprivation or UV treatment, Bim relocates from high sedimentation coefficient fractions (containing the microtubules) to low sedimentation coefficient fractions. More detailed experiments showed that Bim relocalized to fractions corresponding to mitochondria, where apoptosis procedes. In an analogous way, Bmf is bound to the myosin V motor complex through interaction with DLC2 (Puthalakath et al., 2001). Intriguingly, Taxol, which affects microtubules, promotes release of Bim but not Bmf,

whereas anoikis frees Bmf but not Bim. In contrast, UV irradiation of cells releases both Bim and Bmf (Lei and Davis, 2003).

Bim can also be trancriptionally regulated. In the case of cytokine-deprived cells, upregulation of Bim is seen. In hematopoietic cells, this happens through the transcription factor FKHR-L1 (Dijkers et al., 2002); in neuronal cells, it happens through JNK activation (Harris and Johnson, 2001). Certain Bim transcripts, generated by alternative splicing, encode smaller proteins, for example Bim_S that lack the restraining DCL1-binding motif and are therefore very potent death inducers (Marani et al., 2002), but their low abundance makes their physiological relevance unclear.

For several BH3-only proteins, optimal docking on their prosurvival relatives probably requires not only their BH3 domain but also a membrane targeting function. With Bim, for example, that domain is required both for mitochondrial targeting and proapoptotic activity (Yamaguchi and Wang, 2002).

At first it has been thought that all the BH3-only proteins bind to all Bcl-2 subfamiliy members, but quantitative analysis could reveal if there are some significant preferences. There might be more specialization among the pro-survival relatives that presently envisioned (Nijhawan et al., 2003).

Bid seems to be exceptional among the BH3-only proteins. Although it can bind to both Bcl-2- and Bax-like proteins *in vitro,* mutagenesis studies have suggested that the latter are the functionally relevant targets (Wang et al., 1996). Incubation of activated (cleaved) Bid with mitochondria was shown to promote oligomerization of membrane-bound Bak and rapid cytochrome *c* release but Bid was not detectable within cross-linked Bak oligomers, leading to the suggestion that Bid activates Bax and Bak by a 'hit-and-run' mechanism (Korsmeyer et al., 2000).

Mouse genetic studies (Ranger et al., 2001) suggest that the survival of every cell type requires protection by at least one Bcl-2 homolog. Despite overlapping expression patterns, inactivation of individual genes leads to diverse phenotypes, presumably because the different proteins are more abundant in particular tissues. Bcl-2 is essential for the survival of kidney and melanocyte stem cells, as well as

mature lymphoid cells (Kamada et al., 1995; Nakayama et al., 1993; Veis et al., 1993); Bcl-x_L for neuronal and erythroid precursor cells (Motoyama et al., 1995; Wagner et al., 2000); Bcl-w for sperm cell progenitors in adults (but not juveniles) (Meehan et al., 2001; Print et al., 1998); Al for neutrophils (Hamasaki et al., 1998); and Mcl-1 for successful implantation of the zygote (Rinkenberger et al., 2000).

Other genetic studies clearly indicate that homeostasis requires an appropriate balance between the level of prosurvival proteins and that of their BH3-only antagonists. Overexpression of the former provokes an abnormal accumulation of cells within the haematopoietic compartment (Ogilvy et al., 1999) and neuronal lineage (Farlie et al., 1995), presumably by impairing physiologically important death signals delivered through upregulation of BH3-only proteins. Conversely, the consequences of inadequate levels of prosurvival proteins can be suppressed by also reducing the level of BH3-only killers: loss of just a single allele of Bim is sufficient to prevent the kidney failure of Bcl-2-null mice (Bouillet et al., 2001).

1.2.2.2 Model for the mode of action between Bcl-2 family members

All members of the Bcl-2 family share in common at least one BH3 domain. This domain corresponds to an α-helix. It constitutes the way how different members of the family bind to each others to interact and eventually inhibit opposite family members to determine if the caspase proteolytic cascade should be activated or not. The α-helix of a BH3 domain of pro-apoptotic BH3-only members binds into the hydrophobic groove formed by the BH1 and BH3 domain of the anti-apoptotic Bcl-2 subfamily members (Hinds et al., 2003). See figure 5. Some of the family members have a hydrophobic sequence which has the feature of a transmembrane domain. It is important for their targeting to intracellular membranes.

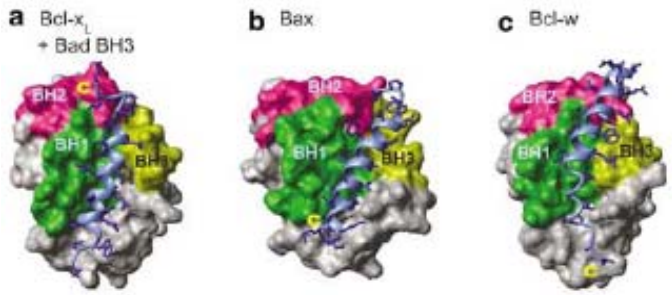

Fig.5: Binding of an α-helix from a BH3 domain into the groove formed by the domains of Bcl-2 subfamily members

1.2.3 Extrinsic or receptor-activated apoptotic pathway

If signals triggering apoptosis don't originate from within the cell, we speak about extrinsic apoptotic pathway. In this case the pro-apoptotic signal is initiated outside the cell and activates pro-apoptotic cell surface receptors. They are transmembranic and are called death receptors. Once they bind their ligands in the extracellular space, the death receptors activate a cytoplasmic caspase cascade which converges on the intrinsic apoptotic pathway.

The ligands of the death receptors are members of the tumor necrosis factor (TNF) family, which includes TNF-α, TRAIL, Fas Ligand (FasL), APO3L. Originally, TNF-α was found to cause the death of cancer cells. But later these ligands were also found to cause the death of normal cell types that display appropriate receptor on their surface. There are numerous cognate receptors. The mostly known are Fas, TNFR1, DR3, DR4 and DR5. They all share in common a cytoplasmic death domain. When activated by ligand binding, the death domains of the receptors bind and activate the protein FADD (Fas-associated death domain protein) in the cytoplasm. The resulting complex is termed DISC (death-inducing signaling complex). The DISC then triggers self-cleavage of procaspases 8 and 10, which then activate the executioner caspases 3, 6 and 7. This converges to the signaling of the intrinsic pathway. In addition, caspase 3 can cleave the BH3-only protein Bid, which act on Bak and Bax to leave the mitochondrial channels open.

Some cell types in the body rely only on an intrinsic or extrinsic apoptotic pathway to trigger their own cell death, while others can use both. Suicide of a cell by the extrinsic pathway can happen if it secretes a ligand for one of the death receptors that is displayed on its cell surface. This would be an autocrine fashion to initiate apoptosis. The choice between intrinsic and extrinsic pathway has some

consequence regarding the anti-apoptotic proteins of the Bcl-2 subfamily. In cells which can activate the extrinsic program, the overexpression of Bcl-2 is not of great help, since the death receptors can circumvent the mitochondrion-based program by acting directly on the caspase cascade. Figure 6 depicts a summary of the intrinsic and extrinsic apoptotic pathway and the communication from extrinsic to intrinsic through Bid.

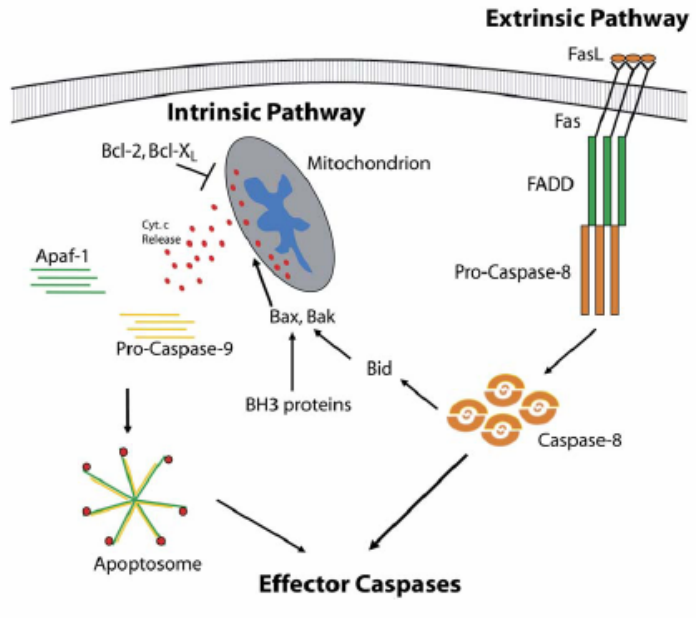

Fig.6: A summary of the intrinsic and extrinsic pathway and the communication from extrinsic to intrinsic through Bid.

1.2.4 Bcl-2 family and the cell cycle

Intriguingly, in addition to its central role in regulating apoptosis, the Bcl-2 family influences the transition between quiescence and proliferation. High levels of Bcl-2 do not affect the proliferation rate of continuously cycling cells (O'Reilly et al., 1996). Significantly, however, G0 cells overexpressing Bcl-2 (or Bcl-x_L, Bcl-w) are slow to enter the S phase when stimulated with growth factors (O'Reilly et al., 1996). Consistent with these *in vitro* studies, B and T cells in Bcl-2 transgenic mice turn over more slowly. Conversely, overexpression of Bax (O'Reilly et al., 1996) and Bad (Chattopadhyay et al., 2001) neutralizes the cell-cycle barrier imposed by Bcl-2, and T cells from bax transgenic mice enter into cycle more rapidly in response to IL-2 stimulation than normal T cells (Brady et al., 1996).

Importantly, the antiapoptotic and cell-cycle aspects of Bcl-2 function are separable, since mutation of a conserved tyrosine abrogates the cell-cycle constraint but not the survival benefit (Huang et al., 1997).

1.2.5 Role of BH3-only subfamily members in oncogenesis

The oncogenic potential of antiapoptotic Bcl-2 subfamily members suggests that proapoptotic relatives could be tumour suppressors. But since there is redundancy within both the BH3-only group and the Bax-like family, their tumour suppressor function may only arise in specific cell types or in specific situations. Indeed, although loss of Bim alone elevates tumour incidence in mice within the first 12 months of life, loss of even a single allele of Bim dramatically accelerates leukemogenesis in mice expressing a *myc* transgene during B lymphopoiesis (Egle et al., 2004). Thus, Bim is indeed a potent tumour suppressor, at least in B cells. Although inactivation of Bid does not perturb homeostasis during development (Yin et al., 1999), 50% of Bid-deficient mice develop chronic myelomonocytic leukaemia by two years of age (Zinkel et al., 2003). Noxa and Puma are particularly attractive candidate tumour suppressors, because both are transcriptionally induced by the tumour suppressor p53 (Oda et al., 2000).

Since Bax and Bak have largely redundant function (Lindsten et al., 2000), it is certainly necessary to inactivate both genes (and perhaps also Bok in tissues expressing this Bax-like protein) in order to get tumour promotion. Indeed Bax-null mice acquire few spontaneous tumours (Knudson et al., 2001). Nonetheless, loss of Bax has enhanced transformation by potent oncogenes in several cell types, for example by myc in B-lymphoid cells (Eischen et al., 2001). Furthermore, some human colorectal and haematopoietic tumours exhibit mutated Bax or Bak (Kondo et al., 2000; Meijerink et al., 1998; Rampino et al., 1997). Finally, loss of Bax in the HCT116 colorectal cell line abolishes the (p53-independent) apoptotic response to nonsteroidal anti-inflammatory drugs but only partially reduces the p53-dependent response to the chemotherapeutic agent 5-fluorouracil (Zhang et al., 2000). These observations suggest that certain cytotoxic signals are Bax-specific in their action and/or that Bak expression in certain cell types is insufficient to mediate apoptosis in the absence of Bax.

1.2.6 Therapeutic modulators of apoptosis

Since impaired apoptosis plays a central role in the pathogenesis of many diseases, research is trying to find novel agents that engage the cell death machinery (Reed, 2002; Reed, 2003). The goals are either to preserve cell viability after acute injury (e.g. to limit tissue damage from myocardial infarction) or to delete malignant or autoreactive cells. Promising attempts to minimize cell death are focused on caspase inhibitors (Nicholson, 2000). Strategies to enhance cell death include targeting either Bcl-2 or the IAPs, or engaging the death receptor pathway (Ashkenazi, 2002).

The rational for the use of Bcl-2 as a target is the following: conventional therapies that damage DNA or perturb microtubules work by indirectly activating the cell death program (Brown and Wouters, 1999). Hence, direct activation of the cell suicide machinery should be advantageous (Reed, 2003). First evidence is that overexpression of Bcl-2 contributes to oncogenesis in a number of mouse tumour models. Second, Bcl-2 overexpression renders many cultured cell lines refractory to chemotherapeutic drugs and radiation (Huang et al., 1997). Finally, Bcl-2 is known to function downstream of the p53 tumour suppressor (Strasser et al., 1994), the function of which is lost in most tumours (Sherr, 2001). Hence, the diminished apoptosis in the tumour cell due to that loss might be overcome by directly targeting Bcl-2. Diverse strategies are being developed: antisense oligonucleotides (Banerjee, 2001), RNA interference (Hannon, 2002) and delivery of BH3-like peptides which target the deep groove of Bcl-x_L, Bcl-2 and Bcl-w (Vieira et al., 2002).

1.2.7 Involution in the mammary gland: a 2-phase process

Apoptosis can be easily studied in the mouse mammary gland at the physiological and genetic level. Within 6 days of weaning in the mouse, most of the secretory epithelium is removed and the gland is then remodeled to a pre-pregnant state. During involution a coordinated process of alveolar programmed cell death (PCD) and lobular-alveolar remodeling restructures the mammary gland. Simple removal of

the suckling stimulus triggers this process. With loss of suckling, milk accumulates within alveolar lumens, and levels of systemic lactogenic hormones fall. Apoptosis is easily studied in the mouse mammary gland by using a forced weaning protocol in which the pups are removed at the peak of lactation (10 days) before they naturally wean. This brings the mammary gland to a synchronous involution and allows the study of morphological events and their associated molecular mechanisms.

Analysis of the involution and glucocorticoid administration has revealed two distinct phases of postlactational involution. The first stage is reversible and involves programmed cell death of the alveolar cells, which are shed into the lumen. In Balb/c mice, the peak of apoptotic cells is seen at day 2 of involution. Apoptotic cells are seen up to 8 days of involution (Lund et al., 1996). There is no rearrangement of the lobulo-alveolar structure. The second stage is irreversible and involves the activity of proteinases which degrade the extracellular matrix and basement membrane on which the epithelial cells are lying. Hence, there is remodeling of the lobulo-alveolar structure.

The two stages exhibit characteristic changes in gene expression or activity. First-stage changes include up-regulated expression of sulfated glycoprotein-2 (SGP-2), tissue inhibitor of metalloproteinases-1 (TIMP-1), interleukin-1β converting enzyme, cell cycle control proteins (c-Jun, JunB, JunD, c-Fos, and c-Myc), and decreased expression levels of milk protein genes (Lund et al., 1996). Second-stage changes include increased expression levels of matrix metalloproteinases gelatinase A and stromelysin-1 and serine protease urokinase-type plasminogen activator (Lund et al., 1996). The level of proteinases increases particularly at 4 days of involution. Expression of TIMP-1 was downregulated.

In-situ hybridization revealed that the proteinases are secreted by stromal cells, and not by myoepithelial cells, neither by apoptotic cells nor by invading macrophages. The immunohistochemistry on the other hand reveals that the proteins are located close to the myoepithelail cells. This difference between in-situ and immunohistochemistry apparently indicates that fibroblasts synthesize and secrete the MMPs, which are then bound to ECM close to the myoepithelial cells. This role

played by stroma cells indicates that involution requires the collaboration between epithelial and stromal mesenchymal cells.

Teat-sealing experiments or the use of transplanted mammary gland which is not connected to the tit, as well as the use of oxytocin KO mice demonstrated that the first phase, illustrated by programmed cell death, is regulated by local factors within the individual gland and not by circulating hormones. Indeed the other mammary glands were left intact in the two first models and the pups could suck the milk, but this was not sufficient to prevent programmed cell death (Li et al., 1997).

In opposite, the second phase is dependent on the decrease of circulating hormones and can be delayed by the administration of glucocorticoid. In the teat-sealing experiment, the second phase of involution did not occur. Moreover, pellet implantation in a single tit followed by involution revealed that the cells closer from the pellet had a lactation-like morphology, whereas the cells distant from the pellet were normally undergoing involution (Feng et al., 1995).

The use of genetically modified mice has revealed a number of factors that either promote, or delay, involution and apoptosis. These include members of the Bcl-2 family. Deletion of the anti-apoptotic Bcl-X gene does not affect lactation or the first day of involution, but it accelerates apoptosis at the second day of involution (Hennighausen and Robinson, 2001). Bax KO or the gain-of function of Bcl-2 obtained by transgenic overexpression delay the first phase of involution when examined after 48 hours (Schorr et al., 1999). As demonstrated by the number of apoptotic cells, Bcl-2 gain-of-function seems to be a more potent inhibitor of apoptosis than Bax KO. Examination of later stage indicated that gain-of-function of Bcl-2 delayed the second phase of involution, but this was not the case for Bax KO, as measured by the percentage of surface covered by epithelial cells. This difference indicates that also there is redundancy between the different Bcl-2 family members, *in vivo* each member has specific nonredundant role in fine-tuning the propensity of a cell to undergo apoptosis.

1.2.7.1 The first phase of involution

1.2.7.1.1 LIF-Stat3-cEBP-IGFBP-5 axis

However, because of redundancy or because they are not essential components of the primary regulatory pathways, many of the Bcl-2 regulatory factors make only minor contribution to the involution process. One of the primary pathways is the Jak/Stat pathway, which is activated by cytokines and growth factors, resulting in phosphorylation and specific dimerisation, translocation into the nucleus and activation of transcription of target genes. While Stat5 is important for lobuloalveolar development (Teglund et al., 1998), Stat3 is necessary for the initiation of apoptosis and involution (Chapman et al., 1999; Humphreys et al., 2002). The activation of apoptosis by Stats has been investigated in a number of *in vitro* systems. In myeloid leukemia cells, Stat3 seems to induce apoptosis since its overexpression accelerated interleukin-6 (IL-6) or leukemia inhibitory factor (LIF)-induced apoptosis and a dominant negative blocked apoptosis induced by these cytokines (Minami et al., 1996). Conversely, in a pro-B cell line Stat3 seems to suppress apoptosis after induction of gp-130 receptor (Fukada et al., 1996). In T cells as well, Stat3 is required for the survival in response to IL-6 (Takeda et al., 1998). Hence, the role of Stat3 in apoptosis is cell-type specific. In the mammary gland, Stat 5 is activated during pregnancy and lactation but is rapidly down regulated in involution, whereas Stat 3 is specifically activated at the start of involution (Liu et al., 1997; Philp et al., 1996). The reciprocal activation of Stat 5 and 3 at the onset of apoptosis suggests opposing roles for these Stats in the regulation of apoptosis in the mammary gland. It should be mentioned that Stat5a-deficient mice do not undergo precocious involution, suggesting that Stat3 can induce apoptosis through alternative mechanisms than inactivating Stat5a (Liu et al., 1997). Conditional KO of Stat 3 by deletion of the exon containing its important tyrosine does not impair lactation. However, it results in delayed involution in the mammary gland when examined at I2, I3 and I6 (Chapman et al., 1999). At I2 and I3, although there were some shed apoptotic cells, the morphological structure of the gland remained intact. At I6, there was some remodeling but it looked like at I3 in the control. In Stat3 CKO glands, Stat5a level remains high longer. Insulin growth factor binding protein-5 (IGFBP-5), a target of Stat3, was also downregulated in Stat3 CKO mammary glands. Similar but more extensive mutation of Stat3 but with a more extensive exon deletion including removal of exons 15-22 including the DNA binding and SH2 domain reveals a delay in the in the initiation of the irreversible phase of involution. This was also seen by zymography, where MMP9 (gelatinase B) but not MMP2 (gelatinase A) activity was

delayed in the CKO mammary glands (Humphreys et al., 2002). So apparently both phases are delayed in the Stat3 CKO.

The cytokine leukemia inhibitory factor (LIF) is the principal activator of Stat3 in vivo. The cytokine IL-6, which also acts through gp130, is not the principal activator of Stat3 in vivo, since in KO of IL-6, Stat3 phosphorylation status does not change (Humphreys et al., 2002). The KO of LIF results in diminished apoptosis, delayed involution, lack of P-Stat3, reduction in cleaved caspase-3 and in C/ebpδ levels at involution (Kritikou et al., 2003). These mammary glands did not present any difference in the level of Bax or Bcl-X. They had a decreased level of P-Erk, suggesting that LIF, in addition to increasing P-Stat3 dependant apoptotic stimuli, also decreases P-Erk dependant survival stimuli. Experiments on cell culture argue for this: cells treated with LIF or with U0126 (the inhibitor of MEK) showed a modest increase in apoptosis, whereas cells treated with both simultaneously showed a dramatic potentiation of apoptosis.

IGF-BP-5 overexpression at a level similar to the one measured at apoptosis induces premature cell death at the beginning of lactation (L2), but the effect was transient and not present any more later in lactation (L10). These mammary glands showed elevated levels of cleaved caspase-3 and plasmin, as well as decreased level of the anti-apoptotic Bcl-2 and Bcl-X_L.

1.2.7.1.2 C/EBPδ

CCAAT/enhancer binding protein delta (C/EBPδ) is a crucial mediator of pro-apoptotic gene expression events in mammary epithelial cells. In mammary glands knock-out for C/EBPδ, involution is delayed as seen morphologically, the pro-apoptotic genes encoding p53, BAK, IGFBP5 and SGP2/clusterin are not activated, while the anti-apoptotic genes coding for BFL1 and Cyclin D1 are not repressed (Thangaraju et al., 2005). Consequently, p53 targets such as survivin, BRCA1, BRCA2 and BAX are not regulated appropriately and protease activation is delayed. Furthermore, expression of MMP3 during the second phase of involution is perturbed in the absence of C/EBPδ.

1.2.7.1.3 Akt

In mammary glands from wild-type animals, the level of Akt decreases at involution day 2 and remains lower later in involution. The phosphorylation of a known substrate of Akt, GSK-3β, follows the same pattern. This suggests a potential role for Akt, which is known as a survival factor, in involution of the mammary gland. Transgenic mice having a constitutively active form of Akt (myristoylated) under activation of the MMTV promoter show delayed involution (Schwertfeger et al., 2001) as seen morphologically. In the mammary gland from these mice, the number of apoptotic cells at involution is decreased, and peaks at I6 or I8 instead of I4 in the wild-type controls (FVB background). In these mice, the decrease in β-casein and WAP expression normally seen during the five first days of involution occurs only after 5 days of involution. Interestingly in these glands, the levels of P-Stat3 did not decrease as compared to control glands. Although MMP-3 level increased, TIMP-1 levels increased as well and remained high until I10, probably accounting for the delay in apoptosis. Confirmation that Akt is critical for regulation of mammary gland involution still requires the analysis of KO or CKO animals.

Recently, it has been shown that Stat3 induces the expression of negative regulatory subunits of PI(3) kinase, resulting in diminished levels of P-Akt (Abell et al., 2005). During involution, level of P-Akt is decreased; this is not due to PTEN, since it is also decreased, but rather to PI3kinase, which looses its activity. PI3 kinase has regulatory and catalytic subunit. Interestingly, a change in the regulatory subunit composition is seen at involution: regulatory p55α and p50α subunit are up regulated, whereas p85 α and β are downregulated. Regulatory p55 γ as well as catalytic subunits level do not change during involution. These changes were seen at the protein and RNA level, indicating that they are transcriptionally regulated. In Stat3 KO glands, the levels of p55α and p50α subunit are reduced compared to WT at involution. There was no change in the level of p85 in Stat3 KO compared to WT glands. Thus Stat3 selectively upregulates p55α and p50α during involution. This represents a new mechanism by which the LIF-Stat3 axis may act to downregulate the survival factor PKB during involution.

1.2.7.1.4 Death receptor pathway

Fas (Apo-1/CD95) is a 45-kDa cell-surface receptor of the TNF/nerve growth factor receptor family whose signal transduction pathway mediates apoptosis of Fas-bearing cells after binding with FasL. Although the Fas/FasL system was originally described in the context of lymphocyte-mediated apoptosis, new data have shown that Fas and FasL are widely expressed and function in many tissues outside the immune system.

It has been shown that the protein levels of Fas and FasL increase during involution. However, this pattern of expression does not reflect the mRNA level, which is present as the same level through the pregnancy, lactation and involution (illegitimate transcription). Immunohistochemistry revealed that they were expressed by glandular epithelial cells. Mice deficient for Fas had reduced apoptosis as seen with TUNEL staining (Song et al., 2000).

Microarray data from a study focused at the transition lactation/involution indicate that there is a difference in the expression profile of genes belonging to the extrinsic and intrinsic pathway of apoptosis (Clarkson et al., 2004). Four death receptor ligands, Tnf (TNFα), Tnfsf6 (Fas ligand), Tnfsf10 (TRAIL) and Tnfsf 12 (TWEAK) had a pic of expression after 1 day of involution, and then returned back to normal levels. These receptors bind to the death receptors Fas, TNFR-1, TNFR-2, DR3 and DR4. In contrast to this transient expression of death receptor ligands, components of the mitochondrial (intrinsic) pathway exhibited delayed but sustained induction, often starting after 2 days of involution. This was the case for some caspases, IGFBP-5, Apaf, Bax.

So apparently during involution the death receptor pathway would be activated before the intrinsic mitochondrial pathway.

1.2.7.1.5 TGF-β3

It is known that TGF-β superfamily members are involved in apoptosis. Mullerian inhibiting substance causes regression of the Mullerian ducts during the male sexual development. BMP signals to induce apoptosis in the rhombomeres 3 and 5 and in the interdigit field of the chick limb. In the mammary gland, TGF-β3 is upregulated at very beginning of involution although there is no change in the transcript of TGF-β1

and 2 (Nguyen and Pollard, 2000). In-situ hybridization shows that it is expressed by lobuloalveolar cells. Immunohistochemistry shows that it is expressed in lobuloalveolar cells, as well as in the extracellular matrix, indicating that it can be secreted. Injection of physiological levels of pituitary hormones prolactin and oxytocin at weaning do not suppress TGF-β3 expression, indicating that TGF-β3 is an actor of the first phase. Sealing one mammary gland also shows increased TGF-β3 expression, although the rest of the glands are accessible to the pups and the level of circulating hormones are thus normal. TGF-β3 can be the cause of cell death. Indeed, transgenic mice expressing TGF-β3 upon betalactoglobulin gene promoter show nuclear localization of SMAD 4 and apoptotic cells at 1-3 days lactation. Immunohistochemistry revealed that P-Stat3 was found in the nucleus of the mice having a sealed tit and also in mice expressing TGF-β3. So P-Stat3 might be a downstream target of TGF-β3. Transplantation of mammary glands from TGF-β3 WT or KO animals show less apoptotic cells in the TGFb3 KO transplanted mammary glands compared to WT transplanted mammary glands. For a reminder, the transplanted mammary glands are not connected to tits, and so will undergo apoptosis due to the accumulation of local factors. It is possible that TGF-β3 act in the mammary gland trough disrupting the matrix attachment of the lobuloalveolar epithelial cells, causing anoikis. This is consistent with the role of TGF-b in matrix remodeling in other organs.

A summary of the various pathways which converge to apoptosis in the mammary gland is shown in fig.7.

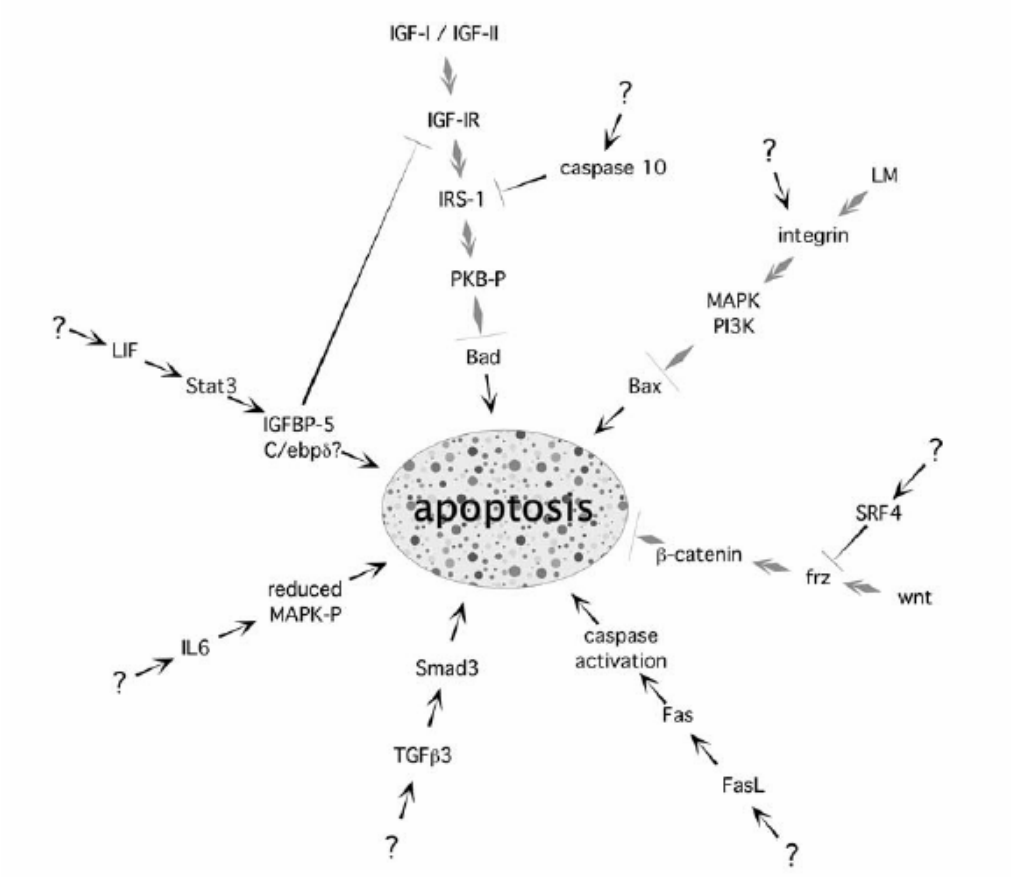

Fig. 7: Summary of the various apoptotic pathways leading to apoptosis in the first phase of mammary gland involution. Several pathways are activated and required for apoptosis during involution, but the mechanisms for triggering them are not always known (question marks). Some are pro-apoptotic pathways (black arrows), while others inhibit survival pathways that would otherwise be operational during lactation (grey arrows).

1.2.7.2 The second phase of involution

1.2.7.2.1 A process dependant on proteases

48 hours after the start of involution, the transition to the second phase occurs: alveoli start to collapse and adipocytes begin to re-fill. Two main families of matrix degrading proteases are activated during mammary gland remodeling, the matrix metalloproteases (MMPs) and serine proteases uPA and tPA involved in the activation of plasminogen to plasmin. These families share similar matrix substrates. Plasmin directly degrades matrix proteins such as fibrin and lamin, and also activates MMP precursors like pro-MMP3, MMP-9 and MMP-13. uPA is the plasminogen

activator involved in tissue remodeling events, whereas tPA acts primarily on the circulatory system.

MMP3 is expessed in the mammary stroma. Mice expressing a mammary targeted autoactivating MMP3 display accelerated involution through basement membrane degradation and epithelial apoptosis late in pregnancy (Sympson et al., 1994). However removal of MMP3 from the mammary gland has no effect on mammary apoptosis but rather has an effect on adipocyte maturation (Alexander et al., 2001).

MMP11 is expressed by fibroblasts surrounding degenerating ducts. MMP7 is detected in the mammary gland during lactation and involution by RT-PCR. MMP 14 is expressed in the stroma and its mRNA levels do not change through lactation and involution. MMP2 levels increase during involution. *In situ* hybridization show that it is expressed by the stroma (Wiseman et al., 2003). Immunohistochemistry show that it is secreted to the mammary epithelial and myoepithelial cells (Dickson and Warburton, 1992). MMP9 was not detected by *in situ* hybridization but is upregulated at involution as seen by zymography (Lund et al., 2000). It is produced by macrophages in the mammary gland.

Plasminogen activation pathway is also involved in mammary gland involution. This happens through upregulation of the mRNA of uPA (Lund et al., 1996). Fibroblasts and macrophages express uPA during involution. Mice deficent for plasminogen display impaired involution (Lund et al., 2000). The requirement for plasmin during involution is likely due to its direct action on the ECM rather than its MMP activating function, since MMP2 and MMP9 are still activated in its absence.

The cysteine proteases cathepsins B and L are lysosomal enzymes that can be secreted and active in the extracellular environment. At least cathepsin L plays a role in involution, since its mRNA level is increased and a cathepsin-L specific inhibitor delays involution and a cathepsin-L specific inhibitor delays involution.

The activity of MMPs is prevented trough expression of their inhibitors, the TIMPs (tissue inhibitor of metalloproteinases). Implantation of a TIMP-1 release pellet delays alveolar regression through involution (Talhouk et al., 1992). TIMP-2 and 3 levels decrease during lactation, but there is no change at the transition to involution.

However, TIMP-3 deficient mice show accelerated involution with premature epithelial apoptosis and MMP2 activation compared to the wild type glands (Fata et al., 2001). In addition, the reversibility after pup removal was lost in these TIMP3 KO mice, as evidenced by the loss of pup weight, suggesting a role for TIMP3 in the re-initiation of lactation after pups removal. This gives unique functions to TIMP-3, since other TIMP family members were not able to compensate. It has been shown that nidogen, an ECM molecule which binds integrin, is cleaved by plasmin and MMP3 during mammary gland involution (Alexander et al., 1996). Moreover, the transmembrane protein E-cadherin, which is involved in cell-cell adhesion, is also cleaved during involution (Vallorosi et al., 2000). Therefore proteases can also disrupt cell-ECM and cell-cell contact during involution. MMP3 and MMP7 also have the potential to cleave Fas ligand from the cell surface to generate apoptosis (Vargo-Gogola et al., 2002). Indeed, Fas ligands have a MMP-7 cleavage site in their sequence. Table 1 summarizes the proteases involved during involution of the mammary gland.

Protease	Alternative name	Expressed in mammary gland by:
MMP2	**Gelatinase A**	**stroma**
MMP3	**Sromelysin-1**	**stroma**
MMP7	**Matrilysin**	**epithelium**
MMP9	**Gelatinase B**	**Stroma (macrophages)**
MMP11	**Stromelysin-3**	**Stroma (fibroblasts)**
MMP14	**MT1-MMP**	**stroma**
Plasmin(ogen)	**PLG**	**Produced in liver as plasminogen**
uPA	**PLAU**	**stroma**
tPA	**PLAT**	
Cathepsin B	**CTSB**	**Secretory epithelium**
Cathepsin L	**CTSL**	**Secretory epithelium**

Table 1: ECM-degrading proteases in the mammary gland involution

1.2.7.2.2 Adipocyte differentiation

A third phase during mammary gland involution can be distinguished, which is the re-differentiation of adipocytes. MMPs don't only play a role in ECM protein degradation, they are also important for adipocyte differentiation. Both plasmin and MMP3 play a role in this process (Selvarajan et al., 2001). MMP3 KO mice have the same level of

apoptosis than their wild.type control mice. However, they display accelerated adipocyte differentiation during involution (Alexander et al., 2001).

1.2.7.2.3 Phagocytosis

Phagocytosis of a large number of cells and debris is an important constituent of the remodeling process. Inflammatory mediators like Il-6 and Lif, which are activated earlier in involution, probably attract macrophages, since they can be seen in big number at day 4 involution (Stein et al., 2004).

1.2.8 References

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2 Aim of this thesis

So far Memo was known to be expressed in breast cancer cell lines and to be involved in *in-vitro* cell migration. The goal of this thesis is to analyze the role of Memo in *in vivo* processes. For this, we wanted to:

- study the expression pattern of Memo in adult and in embryonic development. See if it is expressed more in epithelium than mesenchymal cells or if it is expressed more in migrating cells.

- generate a knockout mouse and analyze its phenotype: identify if any organ presents any defect, and if yes which one.

- generate a conditional knockout mouse in case the knockout mouse was lethal (this was the case) and cross it with a transgenic mouse expressing Cre in the organ of choice (we chose the WAPiCre mouse, since the mammary gland is an organ well studied in the lab and since it allows deletion of the gene of interest in the luminal epithelial cells of the mammary gland in late gestation and lactation).

- analyze if Memo plays a role in the organ of choice (since we chose the mammary gland, analyze if the major events occurring during lactation and involution (morphology, proliferation, differentiation or apoptosis) are affected upon Memo deletion.

3 Results

3.1 Part I: Memo is required for vascular integrity during mouse embryonic development as shown by knockout study

Patrick Kaeser, Régis Masson, Francisca Maurer, Jean-François Spetz, Bernard Kuchemann, Patrick Kopp and Nancy Hynes

3.1.1 Abstract

Studies from our lab recently led to the discovery of Memo (mediator of ErbB2-driven cell motility), a novel 297 amino acid protein shown to be required for ErbB2- and other receptor tyrosine kinase-driven cell motility. Inhibition of Memo expression had consequences on the microtubule network which could not grow towards the periphery of the cells upon heregulin stimulation. It also had consequences on the actin cytoskeleton, since more actin stress fibers were seen. Here we show the physiological function of Memo by disruption of the Memo gene in mice. Memo was expressed ubiquitously in adult organs and during embryogenesis. Memo knockout embryos showed hemorrhages and died at about embryonic day 13.5. Memo knockout embryos had a malformed neural tube. By morphological study and by using various markers, we show that deficiency in Memo had no significant effect on vasculogenesis, but had consequences on vascular integrity.

3.1.2Introduction

Memo (mediator of ErbB2-driven cell motility) is a novel molecule identified in our lab as a protein binding to the phospho-Tyr 1227 of the ErbB2 receptor (Marone et al., 2004). A single Memo protein is encoded in the human and mouse genome, but Memo homologs are found in all branches of life including in C.elegans (Gerlai et al., 2000).
With the help of transwell chamber assay, it has been shown that in vitro Memo is required for efficient heregulin-driven motility of breast carcinoma cells (Marone et al., 2004), hence its name Memo. It has also been shown that in vitro Memo is required for efficient FGF2- and EGF- induced cell migration, indicative of a widespread role for Memo in receptor tyrosine kinase-induced cell motility.
Inhibition of Memo expression markedly reduced the network of microtubules growing to the periphery. It has also been shown that the actin stress fibres appeared to be increased and it has been hypothesized that this effect is a consequence of an improper microtubule network.

To explore the biological function of Memo, and in order to check if Memo also plays a role in *in vivo* cell migration events, we generated a mouse knockout for Memo. This was achieved by targeting the exon 2 of Memo. We found that Memo is expressed ubiquitously in adult organs as well as in organs of the developing embryo. Unexpectedly, we did not see any defect in migration *in vivo*, despite the presence of a lot of migrating events during development. Instead, we found that Memo is essential for embryonic development, and specifically for vascular integrity.

3.1.3 Results

Mouse Memo gene characterization

A blast of the human nucleotide sequence of Memo AF132961 was performed using NCBI. Among the sequences producing significant alignments, we took the ref. NM_133771 as corresponding to Memo transcript in mouse.
We searched for mouse Memo genomic localization and organization through the Ensembl Mouse Genome Browser (http://www.ensembl.org/) (Hubbard et al., 2002).

It revealed that in mouse, Memo transcript is on genomic location 72760268-72817650 bp on chromosome 17. Real comparison with Ensembl data and NCBI transcript revealed that the first exon could not be localized in the genome, probably because it is surrounded by a region difficult to sequence.
Since exon 1 could not be located, exon 2 is recognized and named as exon 1 in Ensembl.
It should be mentioned that the exon/intron structure given by Ensembl was not correct for exon 2 (exon 1 in Ensembl): indeed Ensembl gave a G as the first nucleotide of this exon. In fact this G is still belonging to the acceptor site of the intron-exon boundary. Indeed, an intron starts with a GT donor site and an AG acceptor site, giving the following sequence: exon-GTintronAG-exon.

The blast of transcript NM_133771 with Ensembl revealed that mouse Memo contains 9 exons.

Theoretical considerations for the targeting vector

Both Cre and Flp are site-specific recombinases that cleave DNA at a distinct target sequence and then ligate it to the cleaved DNA of a second identical site to generate a contiguous strand. This recombination reaction is carried out with absolute fidelity, such that no single nucleotide is gained or lost overall. The Cre-lox system comes from the bacteriophage P1 and the Flp-FRT system from the budding yeast Saccharomyces cerevisiae.
The strategy to knock-out Memo gene consisted in replacing an exon of the gene of interest by a sequence of this exon flanked by loxP sites (Figure 1). The presence of loxP sites allows the removal of the exon upon the Cre enzyme activation. This replacement was achieved by electroporating the targeting vector in ES cells from the 129/Ola strain. The targeting vector also contains a neo cassette for resistance upon G418 treatment. This allowed selection of ES cell clones where recombination of the targeting vector occurred.
Based on the fact that exon 1 (which contains the start codon ATG) could not be localized in the mouse genome we decided to choose exon 2 as the targeted exon for our construct. The number of nucleotides contained in exon 2 is not a multiple of 3 and so, any potential transcript which would go from exon 1 directly to exon 3 would

generate a potential protein which would share only the first 20 amino acids with wild-type Memo (figure 2). According to bioinformatical predictions, any change in the protein structure of Memo would lead to a complete change in the conformation of the protein. This comforted us in choosing exon 2 for the targeting, although the ATG start codon is located in exon 1.

PCR screening for ES clones that underwent homologous recombination in 5' and 3' of targeted Memo locus

In order to select for ES cell clones that underwent not only recombination but as well homologous recombination of the targeting vector, PCR was performed using a forward primer in 5' external to the vector and a reverse internal primer specific to the construction. PCR was as well performed using a forward primer specific to the construction and a reverse 3' external primer. Figure 3A shows that ES clones # 116, 159, 194 and 109 underwent correct homologous recombination. Sequence analysis of the PCR products was also performed in order to verify that the loxP and exon 2 sequence was correct (not shown). Southern blot analysis was also performed on these clones in order to verify that they recombined only 1 copy of the vector. Results indicate that they indeed recombined only 1 copy (figure 3B). ES clone 109 was used for mice generation.

Generation of MEMO KO mice

To obtain mice harboring one Memo knockout allele, ES clone 109, containing one recombined Memo allele, was transiently transfected with pCMV-Cre plasmid (gift from Prof. Patrick Matthias) in order to induce Cre-mediated removal of the 3-lox sites. The 240 resulting clones were screened by PCR. The figure 4 shows that clones 185 and 202 contained the KO allele of 1.5 kb in addition to the WT allele of 1.8 kb. ES clone 202 was used for aggregation experiment and to generate chimeras. The chimeras giving germ line transmission (GLT) were used to generate heterozygous knockout mice (Figure 5). Mice heterozygous knockout appeared normal and did not display any overt anatomical or behavioral abnormalities. Memo heterozygous knockout mice were interbred to generate full knockout mice. No mice

homozygous for the mutation were detected among >150 weaned progeny from heterozygous intercrosses (Table 1). Therefore, the mutation is recessive lethal.

Analysis of Memo RNA and protein localization

So far we knew that Memo is expressed in breast cancer cell lines (data not shown).
To get an idea in which organ or at which time point during development Memo could play an important role, we studied its pattern of expression in adult organs. This was first done by Northern blotting, since no antibody for Memo was available at that time. RNA from a total of 32 organs were harvested and analyzed. Northern blotting revealed only one band, suggesting that only one isoform is transcribed. It also revealed that Memo transcript (1555 bp) is ubiquitously expressed, and is particularly abundant in brown adipose tissue, thymus and testis (Figure 6A, lanes 5, 14 and 24).
Once we got the polyclonal antibody for the protein, we checked at the protein level through Western blotting. It revealed that Memo protein is also ubiquitously expressed in all 23 adult tissues examined, particularly in the skin (lane 1) and testis (lane 4) (Figure 6B).
In order to check if Memo was more expressed at a particular stage of embryonic development, Western blotting was performed in embryos from E10.5 to E18.5 using the polyclonal antibody. It revealed a constant expression of Memo in the examined stages (Figure 6C).
The results obtained by Northern and Western blotting on adult organs suggest that Memo is expressed in every organ, or that it is strongly expressed in a type of cells which is present in every organ, for example in the blood vessels.
To know if Memo was expressed in every cell type of the organs, we performed *in-situ* hybridization using an antisense probe and a sense probe as control. It revealed that in the embryo, Memo RNA is ubiquitously expressed (Figure 7). Expression of Memo was seen in epithelial but as well in mesenchymal cells. High magnification reveals abundant staining in the endocardiac cells of the heart and endothelial cells of blood vessels (Figure 7 G and I).
Immunohistochemistry using the polyclonal antibody did not show any difference in staining between wild-type and knock-out embryo, indicating that the polyclonal antibody is not suitable for immunohistochemistry (data not shown). We therefore developed a monoclonal antibody. Specificity of the staining for Memo using the

monoclonal antibody was assessed by comparing the staining in a wild-type embryo with the staining of a KO embryo (Figure 8A and B). Evident difference was seen in the liver (Figure 8C and D) and in the dorsal root ganglia (Figure 8E and F). Some background staining is seen in the KO embryo in the nerve fibers and in the conjunctive tissue.

Immunohistochemistry using this monoclonal antibody was established and was performed on embryos from E10.5 to E16.5. It revealed that Memo protein is ubiquitously expressed (Figure 9). However at E10.5, it is detected more abundantly in the somites. At E12.5-E13.0, it is mostly detected in dorsal ganglia, liver and cranial ganglia. There is strong staining in all the blood vessels, but this might be non specific since vessels are known to give some background. Later on, the staining is homogenous and ubiquitous. The only tissue which does not seem to express Memo is bone (E14.5-E16.5). Despite the fact that the monoclonal antibody also stains some organs in the knockout embryos, the monoclonal antibody can be used to distinguish wild-type and knockout embryos on sections. Results from the *in-situ* and immunohistochemistry taken together suggest that Memo is expressed in a ubiquitous manner.

Mice homozygous for the mutant allele die around E12.5 and E13.5 and show hemorrhages, edema as well as malformed neural tube

To determine when the homozygous die, embryos were examined from E10.5 to E14.5 (see Table2). Between E10.5 and E12.5 postcoitum, wild-type, heterozygous and homozygous mutant embryos were represented in a normal Mendelian ratio of 1:2:1 (Table 2). Memo null embryos at E10.5 and E11.5 were indistinguishable from wild-type and heterozygous littermates. Therefore, development to day 11.5 of gestation is largely independent of Memo.

Figure 10A shows a PCR analysis of gDNA from wild-type, heterozygous and knockout embryos. Figure 10B shows a Western blot analysis using the polyclonal antibody from protein extracts coming from the same embros. Both techniques are able to distinguish the genotypes of the embryos.

By E12.5, defects began to appear in Memo-null embryos. Although they were still present in the expected proportions (Table 2) and all were alive as demonstrated by beating hearts, many showed hemorrhages in the heart, liver, brain and cardinal vein

(Figure 11B and H). Some Memo-null embryos also exhibited malformation of the neural tube, which appeared bent instead of being straight (Figure 11B).
Memo knockout embryos had a pale yolk sac when compared to wild-type embryos (Figure 12). However, whole-mount immunohistochemistry with anti-CD31/PECAM antibody of yolk sacs of embryos revealed that the vasculature was still present as late as in E14.5 knockout embryos (Figure 11G and H).

By E13.5, the number of living Memo-null embryos is not found in the normal Mendelian ratio any more. Indeed the majority of knockout embryos did not show any beating of the heart. The phenotype of these knockout embryos is the same as the knockout embryos of E12.5. Hemorrhages were seen around the heart, liver, brain and cardinal vein but as well in the intersomitic blood vessels (Figure 11H). Some Memo-null embryos also exhibited pericardial edema at this stage. The edema was also evident in the space between the embryo and the yolk sac (Figure 12E and F). The neural tube was still malformed (Figure 10F). Yolk sac from knockout embryos of E13.0 and E13.5 showed a less complex vascular network than the one from wild-type embryos (Figure 12C, D, E, F).
In order to check if the vasculature in the placenta of KO embryos cannot be seen because of absence of vessels or because the vessels don't contain any blood, CD-31/PECAM whole-mount staining was performed on placenta from dead KO E14.5 embryos. It revealed that there were still vessels present in the placenta KO embryo (figure 12G and H). So the vasculature of the placenta was only apparently absent when examined macroscopically, probably due to lack of blood flow.
Later on at E14.5 and E15.5, no living knockout embryos were detected. Instead, they were deteriorating.
Therefore, homozygosity for Memo-null mutation leads to death between E12.5 and E13.5. This change in time of death might be due to incomplete phenotype penetrance or to the mixed background of the mice.

No morphological defect was detected in other organs

Histological examination confirmed the results obtained from macroscopic examination. H&E staining of sagittal cross-sections showed abnormal blood presence in the heart, aorta, cardinal vein and liver (Figure 13). No overt

abnormalities were observed in the skin, skeletal muscle, lung, kidney, pancreas, liver and heart of Memo knockout embryos until E13.5. Particular attention was given to the heart due to the phenotype of ErbB2 KO embryos (Lee et al., 1995); see discussion. But the endocardial cushions, trabeculae of the ventricles and valves were normal in the KO embryo, indicating that the hemorrhages were probably not due to an improper morphology of the heart.

Analysis of the vasculature in Memo knockout embryos

To analyze potential effects of the Memo knockout on overall vascularization, blood vessels of Memo wild-type and Memo knock-out embryos at stages E10.5 and E11.5 were visualized by whole mount staining with anti CD-31/PECAM (platelet endothelial cell adhesion molecule) antibody. CD-31 is a surface membrane glycoprotein of 130000 daltons found on platelets, endothelial cells, and certain white blood cell subtypes. It is thus mainly found in the vasculature (Newman and Albelda, 1992).
Only embryos of the same litter were compared. As shown in figure 13, Memo knockout embryos showed a well-developed peripheral vascular system at E10.5 with no obvious differences compared to wild-type embryos. At E10.5, side view and dorsal view did not present any difference between control and KO embryos (Figure 14A-F). Higher magnification in the cranial region showed a well developed network of the primary head veins and a normal vessel branch from anterior carotid arteries (Figure 14G and H). Higher magnification of the dorsal region also showed a well-developed vasculature with good ramifications at the surface of the skin (Figure 14I and J).
Closer view at the region surrounding the heart revealed that the transitional branchial system was correctly established in the KO embryos. Ventricle and atrium also looked similar to the control embryos (Figure 14 K and L).
Detailed view at the intersomitic vessels revealed a perfect sprouting of the intersomitic vessels from the dorsal aorta (Figure 14 M and N).
At E11.5, the general vasculature of the KO embryo was well established. Higher magnification of the skin showed a well ramified vascular network (data not shown).
In order to check for the integrity of the vasculature in later stage and to examine larger blood vessels in deeper layers of embryonic tissue, CD-31 immunostaining was established and performed on paraffin sagittal sections.

Comparison between wild-type and KO embryos did not show any major difference (Figure 15). Closer examination showed a normal aorta, cranial vessels and intersomitic vessels. Taken together, the presence of main blood vessels like aorta and smaller ones like the intersomitic blood vessels clearly demonstrated that the Memo gene is neither required for the formation of the dorsal aortae and primary plexus (vasculogenesis) nor for vessel sprouting (angiogenesis). However some embryos showed broken vessels (Figure 15D).

After the initial formation of the vascular plexus, vessels mature by the stabilization of the endothelial vascular network through a recruitment and differentiation process that ultimately results in the investment of vessel walls with mural cells (Carmeliet, 2000).Vascular smooth muscle cells (VSMCs) first appear on the ventral side of the aorta in E10.5 embryos, followed by migration to the dorsum (Takahashi et al., 1996). By E11.5, the aorta is completely enveloped by VSMCs. The knockout of Edg-1, a GPCR for sphingosine-1 phosphate, which is involved in cell migration, leads to defect in pericyte recruitment due to their loss of migration (Liu et al., 2000). To assess this process of vessel development in the Memo knockout embryos, VSMCs were identified using an antibody against SMαA. The vascular smooth muscles were properly stained in wild-type and knockout embryos (Figure 16A and B). In sagittal sections from E13.0 embryos, the dorsal aortae were completely surrounded by VSMCs in both wild-type and knockout embryos (Figure 16C and D). Correct pattern of VSMCs was also observed in smaller vessels like the intersomitic vessels (Figure 16 E and F).

Vessels of the lymphatic system are highly permeable and specialized for the uptake of fluid and macromolecules from the interstitium and their return to venous circulation. Embryonic development of the lymphatic vessels starts when a subset of endothelial cells in the cardinal vein commits to the lymphatic lineage and sprouts to form the primary lymph sacs (Alitalo et al., 2005; Oliver, 2004). Recent studies have identified specific transcription factors and growth factors required to regulate the development of lymphatic vessels (Taniguchi et al., 2007). In mice, the lymphatic vasculature starts to develop at embryonic day 10.5 (E10.5), when the cardiovascular system is already functioning.

Due to the presence of edema in the Memo knockout embryos, we checked for a marker of lymphatic vessels. LYVE-1 (lymphatic vessel endothelial hyaluronan receptor) is a receptor for the glycosaminoglycan hyaluronan (HA) which is

predominantly expressed in lymphatic vessels. To assess if the edema observed in Memo knockout embryos could be due to a problem in lymphatic vessels, we performed immunohistochemistry of LYVE-1 on frontal paraffin sections (Figure 17). Comparison between wild-type and KO embryos did not reveal any difference between the control and KO embryo.

In order to have a more detailed analysis of blood vessel morphology, immunofluorescence on frontal cross-sections of E13.0 was performed using CD-31 and SMA antibody. Figure 18A and B shows that the heart, liver, intestine, are properly vascularized. A higher magnification of the subcutaneous vessels close to the neural tube reveals that the vessels in the KO embryo are sparser and less regularly aligned (Figure 18D) than in the wild-type embryo (Figure 18C). A higher magnification of the dorsal aorta and surrounding vessels shows that the aorta has a normal morphology and is properly surrounded by the smooth muscle cells in the KO embryos. It also reveals that the smaller vessels in the KO embryos are more dilated than in their control counterpart, indicating a problem in the small vessels of the Memo KO embryos (Figure 18E and F).

Because of the hemorrhages seen and the dilated appearance of smaller blood vessels, endothelial cell proliferation and differentiation signaling factors, including angiopoietin-1 and -2, Flt-1, Flk-1, Tie-1 and-2 were analyzed by RT-PCR (Figure 19). No difference could be observed between control and KO embryos. RT-PCR of EPO, HIF-1α, mouse secretory leucocyte, Edg-1 and VE-cadherin was performed as well. No difference could be observed between control and KO embryos.

Thus the expression of genes required for early differentiation and assembly of endothelial cells into the vascular network was not impaired in the Memo KO embryos. Our data indicate that in Memo KO embryos, vasculogenesis and the phase of angiogenesis that entails vessel sprouting had properly occurred.

3.1.4 Discussion

Memo is a new protein that has recently been discovered in our lab. It has been shown to interact with ErbB2 through the phospho-tyr 1227 of ErbB2. In-vitro, it has been shown via the use of siRNA that Memo is playing a role in the migration of breast cancer cells upon stimulation by heregulin, a ligand which leads to activation of ErbB3/ErbB2 and ErbB4/ErbB2 heterodimers. This role of Memo in cell migration

has been attributed to a function of Memo in the microtubule outgrowth towards the periphery of the cell.

To get an idea of the *in-vivo* role of Memo, its pattern of expression was extensively analyzed in adult organs as well as in embryos. It revealed ubiquitous expression, and Memo was not seen stronger in the migrating cells than in other cells.

We also generated a conventional KO mouse to see which *in-vivo* functions could be perturbed when Memo is absent. Since Memo has been shown *in-vitro* to be involved in cellular migration and a lot of migration events are going on during embryonic development, we hypothesized that Memo KO could be embryonic lethal due to impaired migration.

The gastrulation is a period of development characterized by extensive migration and remodeling of the embryo germ layers. Due to the migration, some cells from the ectodermal layer become in contact with cells from the endodermal layer, and are induced to take new fate. In the mouse, the gastrulation occurs between E6 and E7.5. It is followed at E9 by a turning of the embryo, which then adopts a fetal position. Memo knockout embryos die between E13.0 and E13.5, a time when the complex events of gastrulation have already taken place. This indicates that Memo is not necessary for the migration events to occurr at gastrulation.

The neural crest is also prone to migration. Neural crest cells arise along the lateral margins of the neural folds at the boundary between the surface and neural ectoderm. During the process of neurulation, these cells detach from the periphery of the neural plate and migrate throughout the body, where they differentiate into a particular wide range of cell types present in many tissues and structures. Interestingly these include non-neural elements as well, such as glial cells, angioblasts and cardiac mesenchyme. Components of the aortico-pulmonary spiral septum of the outflow tract of the heart seen at E10 also originate from neural crest derivatives. Memo knockout embryos presented some defect in the closure of the neural tube. However Memo knockout embryos contained dorsal root ganglia as well as cranial ganglia, indicating that these derivatives of the neural crest could properly migrate. The blood vessels were formed in Memo knockout embryos, and no defect could be observed in the heart and its associated vessels, indicating that Memo is dispensable for the migration of neural crests components to the heart and vessels.

At about E7.5 and following gastrulation, the mesoderm on either side of the embryo segregates from the rest of the mesoderm in a position lateral to the neural tube. This

mesoderm then forms so-called somitomeres in the head and somites in the body, which develop in a cranio-caudal sequence. This process continues until about E14. In the head the somitomeres give rise to the musculature of the head and branchial arches. In the body, the somites give rise to the vertebrae, ribs and associated parts of the skeleton. They also give rise to the musculature. In the mid-cervical region, some somite-derived myoblast cells migrate into the pleuroperitoneal membranes which then differentiate into the musculature of the diaphragm. Memo knockout embryos don't show any defect in the branchial arch system, and skeleton as well as musculature don't show any change as compared to control littermates. Hence, Memo is not required for somite migration during embryogenesis.

Since Memo has been shown to interact with ErbB2, and to play a role in heregulin-induced cell migration, it is of interest to compare the phenotype of Memo knockout with ErbB2-, ErbB3-, ErbB4- and neuregulin- knockout embryos.

ErbB2 knockout embryos die by E10.75. They show a cardiac and a neuronal phenotype: they show absence of trabeculae in the heart ventricles and reduced size of endocardial cushions. They also have reduced Schwann cell number, abnormal cranial ganglia, hypoplasia of primary sympathetic ganglion chain (Lee et al., 1995).

ErbB3 knockout embryos die by E10.75 and also show cardiac and neuronal phenotype. They show thin endocardial cushions, hypoplastic heart valves and poor circulation. They also show abnormal neuroepithelial layer differentiation at the fourth ventricle and absence of fingerlike projections at the fourth ventricle choroid plexus. They have abnormal pancreas and stomach epithelium morphology (Erickson et al., 1997).

ErbB4 knockout embryos die by E10.5 and also show a cardiac and neuronal phenotype. They show a failure of development of cardiac myocytes as well as patterning defects in the cranial nerves (Gassmann et al., 1995).

Neuregulin knockout embryos die by E11.5. They show failure of endocardial cushion closure, poorly developed ventricular trabeculae, enlarged heart, enlarged pericardium and irregular heart beat (Meyer and Birchmeier, 1995).

Memo knockout embryos die later, between E12.5 and E13.5. It is not clear whether this time range is due to incomplete penetrance of the phenotype or to the mixed background of the mice analyzed. Memo knockout embryos don't show any defect in cardiac morphology. The endocardial cushions are well formed, the trabeculae (the finger-like projections of cardiomyocytes) and valves show correct morphology. The

cranial ganglia were also present in Memo knockout. Instead, Memo knockout embryos show hemorrhages and despite the fact that blood vessels are correctly formed, the small blood vessels seem less organized and more dilated than in control littermates.

A number of genes are important for vasculogenesis and vessel formation (Argraves and Drake, 2005). Their importance has been revealed through targeting deletion. The majority are associated with the VEGF pathway: Neuropilin 1 (Kawasaki et al., 1999), Neuropilin 1&2 (Takashima et al., 2002), VEGFR1 (Fong et al., 1995; Fong et al., 1999), VEGFR2 (Shalaby et al., 1995), VEGFR3 (Dumont et al., 1998), VEGFA (Carmeliet et al., 1996; Ferrara et al., 1996), some are related to angiopoietins and their receptors: angiopoietin 1 (Suri et al., 1996), Tie-2 (Dumont et al., 1994; Sato et al., 1995), others are related to cell-cell adhesion: VE-cadherin (Carmeliet et al., 1999; Crosby et al., 2005), connexin 45 (Kruger et al., 2000), Ephrin B2 (Wang et al., 1998), beta-catenin (Cattelino et al., 2003) or cell-ECM adhesion: alpha5 integrin (Francis et al., 2002; Yang et al., 1993), fibronectin (George et al., 1993), alpha v integrin (Bader et al., 1998), alpha 4 integrin (Yang et al., 1995).

We could not analyze all the genes mentioned here, but it is possible that the expression or localization of some of them is different in knockout compared to wild-type embryos.

Components of the hypoxia response also play a role in vascular development: HIF-1alpha (Kotch et al., 1999), VHL (Gnarra et al., 1997). Whether or not Memo plays a role in hypoxia response still needs to be analyzed.

Interestingly, Wave 2, a protein involved in cell migration, is necessary for proper angiogenesis *in vivo* (Yamazaki et al., 2003). Memo also plays a role in migration and in the cytoskeleton integrity. However, wave 2 is expressed predominantly in the vascular endothelial cells during embryogenesis, whereas Memo is ubiquitously expressed. The mechanisms how Memo influences the integrity of smaller blood vessels still need to be investigated.

Memo has been shown to interact with Shc (Marone et al., 2004). Interestingly, the knockout of ShcA shows enlarged pericardium and abnormal cardiac contractions. By E11.5, they have pale yolk sacs and their heart and cardiac outflow tracts are congested with blood (Lai and Pawson, 2000). Memo knockout embryos die later though. If Memo phenotype is dependant on Shc remains to be elucidated.

Here we have shown that Memo is essential for proper vascular maintenance in the embryo. However, we do not know yet the physiological function of Memo in other tissues or organs. Production of a conditional knockout of Memo will be helpful in solving this issue.

3.1.5 Materials and methods

Northern blotting and RT-PCR analysis

RNA was prepared by the Trizol method (GiBCO). For synthesis of the Memo probe, full length Memo cDNA was synthesized by using the forward primer 5'-CCCATCTTCCGGCGGCCGGCGGAG-3' and the reverse primer 5'-GAGTTGTGTAGCCCTTTATTAGC -3'. The PCR fragment was extracted and cloned in pGEM-T easy Vector System 1 (Promega, A1360). After digestion of the fragment, synthesis of the radiolabeled ^{32}P probe was performed using the random prime labeling kit from Roche

For RT-PCR analyses, purified RNA was reverse transcribed and PCR amplified by standard procedures.

Lysate preparation and Western blot analysis of Memo

To prepare lysates from organs and embryos, the frozen tissue was ground to a powder in liquid nitrogen and homogenized in lysis NP40 buffer containing 1% Nonidet P-40, 50mM Tris (pH7.5), 120mM NaCl, 5mM EDTA, 1mM EGTA, 2mM Na-vanadate, 20mM ß-glycerophosphate, 10µM/ml aprotinin, 10µM/ml leupeptin, 0.5mM PMSF, 50mM NaF and 1mM DTT. Cell lysates were subjected to SDS-PAGE, transferred to PVDF membranes, which were blocked in 5% nonfat milk for 30 minutes and incubated overnight at 4°C with the Memo polyclonal antibody. Membranes were then incubated with the specific secondary antibody (Amersham) coupled to horseradish peroxidase. Signals were detected by enhanced chemiluminescence (ECL; Amersham) and recorded by Kodak LS-OMAT film.

Targeted disruption of Memo

PCR synthesis of homology arms and loxP-exon2 were performed on 15ng/µl gDNA extracted from ES cells (129/OLA), 1.5mM MgCl2, 0.2mM dNTP mixture, 0.03 U/µl Red Hot Dna polymerase (Abgene, ref. AB-0406/A9), 0.005 U/µl Pwo polymerase (Roche diagnostics, ref. 1 644 947), respective primer pair (200nM each) in the following cycling conditions:

Pre-denaturation step 2 min. at 94°C, 14 cycles [15 s at 94°C – 3 min. at 70°C (-0.5°C per cycle)], 20 cycles [15 s at 94°C – 3 min. (+ 10 s per cycle) at 63°C], post elongation step 7 min. at 63°C, storage at 4°C.

Primer sequences were:

For synthesizing the 5' Homology arm (5'HA) of 3535 bp,

forward primer

5'- ACATTCAT**GGCCCTCGAGGCC**CATCTTAGAGCAGTCTTTGCATAGG-3'and

reverse primer

5'-AAGTATGA**GGCCATCCCGGCC**GGAGTCAGCAAAGCAACATATTACA-3'

were used.

For 3' Homology arm (3'HA) of 3458 bp,

forward primer

5'-AATTCCTAGCG**GGCCAGCTAGGCC**GCCTCTGGTTCCAGTTCCAGGGGAT-3'

Reverse primer

TGAAGATT**GGCCACTGAGGCC**TCAGCTTGCTTAAGTCTCACTTTGC

Briefly, each PCR product was purified, SfiI digested and ligated in a vector containing a pgk-neomycine cassette flanked with 2 loxP sites and 2 Frt sites. Sequencing of the exonic regions, loxP and FRT sites was performed to avoid any risk of PCR-induced mutations.

The targeting construct linearized by SalI restriction and overhanging ends were filled by the Klenow fragment. Purification was carried out by two successive rounds of phenol/chloroform extractions followed by an ethanol precipitation and two washes with 70% ethanol. Pure DNA was air dried and resuspended in sterile deionized water. ES cells were electroporated with the targeting construct and selected for resistance for G418. ES clones that underwent recombination and were hence resistant were initially screened for homologous recombination at Memo locus by PCR using 5' external forward primer 5'-

ATGGTGTGGCTGTTTTGCCTGGATGTGTGC-3' combined with a neo cassette specific reverse primer 5'-CTAAAGCGCATGCTCCAGACTGCCT-3'as well as with a neo cassette forward primer 5'- TCAGCAGCCTCTGTTCCACATACACTTC-3' combined with a 3' external reverse primer 5'-CCTTTACTTCCCCTCCTCAGCCTGACCTTC-3' using Expand Long Template PCR system (Roche # 11 681 834 001), according to manufacturer's instructions. Single integration was confirmed by Southern blotting using a neo specific radiolabeled probe. Two ES clones showed specific homologous recombination at Memo locus (3 lox allele) and were used for aggregation experiment and chimera production. Only clone 109 gave chimera with germline transmission.

Generation of mice and tissue preparation for analysis

ES clone 109, containing one recombined Memo allele, was transiently transfected with pCMV-Cre plasmid (gift from Prof. Patrick Matthias) in order to induce Cre-mediated removal of the 3-lox Memo allele. 240 clones were screened by PCR using forward primer 5'-GGCTCAGGGAATTCCTGCTCAGG-3' and reverse primer 5'-GGATCGAGAAACTTTCATACTACAGC-3'. Clone 202 was selected for aggregation experiment and generation of chimera with germline transmission. Memo heterozygous mice were inbred for generation of full knockout.

For histology, embryos were dissected. For immunohistochemistry on paraffin sections, they were fixed 24 hours at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4 then embedded in paraffin for preparing 5µm sections. For immunofluorescence, they were fixed overnight at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS) and frozen in optimal cutting temperature compound (OCT, Tissue Tek) for preparing 10µm sections.

In situ hybridization

RT-PCR using Fw primer 5'-CTTCCCATCATGTGCCCCTGT-3' and Rw primer 5'-GAGTTGTGTAGCCCTTTATTAGC-3'was performed using RNA from WT testis.

The PCR fragment was extracted and cloned in pGEM-T easy Vector System 1 (Promega, A1360). Sense and antisense probes were in-vitro transcribed using the DIG RNA Labeling Kit (SP6/T7) (Roche 1 175 025) after linearization of the vector at a SalI restriction site which is not present in the construct.

In situ hybridization on paraffin sections was performed with the Ventana Discovery XT system. Sections were pretreated 4 minutes with HCl and 36 minutes with citrate buffer pH 6. 30ug of (anti-) sense probe was applied for 6 hours. Anti-DIG antibody (1:2000) was applied for 32 minutes. After an amplification step using the avidin/biotin system, nitro blue tetrazolium chloride (NBT) and bromo- chloro- indolyl phosphate (BCIP) were used to detect the signal.

Immunohistochemistry

Immunohistochemistry on paraffin sections was performed with the Ventana Discovery XT system. Memo monoclonal antibody was used 1/50 from the purified fractions of 350ug/ml and sections were pretreated 64 minutes in Tris-EDTA pH 8. LYVE-1 antibody (R&D Systems Cat no AF2125) was used 1/200 and sections were pretreated 36 minutes in Tris-EDTA pH 8. α-smooth muscle actin antibody (Sigma, product no A 5228) was used 1/400 without pretreatment. CD-31 antibody (BD Pharmingen cat 550274) was used 1/50 and sections were pretreated 20 minutes with protease. Revelation was made using the DAB kit (Ventana), except for CD-31 which additionally required the TSA amplification kit (Ventana).

Memo monoclonal antibody preparation

2 Memo-peptides were selected for production of monoclonal antibodies: peptide 1470 (sequence Cys His Ala Tyr Lys Gln Val Asp Pro Ser Ile Thr Arg Arg) and peptide 1471 (sequence Cys Arg Asn Trp Gln Asp Ser Ser Val Ser Tyr Ala Ala Gly Ala Leu Thr Val His). The peptides were independently conjugated to keyhole limpet hemocyanin (KLH) and BSA using the “Imject Maleimide-activated Imunogen Conjugation Kit with mcKLH and BSA” (Pierce, 77607).

2 female Balb/c mice (age 6-8 weeks) were immunized with 25 ul of KLH-conjugated petide-mix (containing 12 ug KLH-peptide 1470 and 8 ug KLH-peptide 1471) which was mixed with 25ul of the adjuvant ImmunEasy (Qiagen). The mice were injected 4 times with adjuvant (s.c, neck) and once without adjuvant (final booster injection, i.p). All injections were given with a 2-week interval. Two days after the final injection the mice were killed and the spleen was removed. Splenic lymphocytes were fused with the myeloma cell line P3xAg8.653 (ATCC) and cultured according to standard protocols. Growing hybrids were tested for their ability to secrete antibodies by ELISA. ELISA plates were coated with a 1:1 mix of the BSA conjugates of peptide

1470 and 1471. The hybridoma tissue culture supernatants were used as 1st antibody. Hybrids showing the best ELISA signals were further analized by Western blot using cell culture extracts of myc-Memo overexpressing cells. Selected hybridomas were then subcloned twice and rescreened by ELISA.

Clone NH3-65J25 (specific for peptide 1471) was identified as most usefull and monoclonal antibody secreted by this clone was used in the immunofluorescence experiment.

NH3-65J25 cells were grown in IMDM with 15% FCS to high cell density without medium change to allow monoclonal antibody accumulation in the tissue culture supernatant. Hybridoma supernatant was then collected and antibodies were purified using the HiTrap Protein G HP column (GE Healthcare) according to manufacturer's instructions. An aliquot of the collected fractions was run on a gel and only fractions which showed high enrichment after Coomassie staining were pooled and used for immunofluorescence.

Immunofluorescence

Cryosections of 10 µm were prepared from overnight 4% Pfa fixed cryosections. Immunofluorescence was performed on cryosections postfixed 10 minutes at -20°C in acetone-methanol (1:1). Antibodies were used as followed: CD31 1/200 (Pharmingen), SMA 1/400 (Sigma). Prior to antibody incubation, sections were blocked for 15 minutes with 10% goat serum.

Secondary antibodies were AlexaFluor (from Molecular Probes). They were diluted 1/400.

3.1.6 Tables and Figures

Mating	MEMO+/+	MEMO+/-	MEMO-/-	Total pups
TOTAL	66	113	0	179
%	36.9	63.1	0	

Average of liter size = 5.77 pups

Table 1: Analysis of newborns genotypes resulting from Memo heterozygous knockout matings.
No living or dead Memo knockout (-/-) newborn was obtained.

	Number +/+	% +/+	Number +/-	% +/-	Number -/-	% -/-
10.5 dpc	9	**45**	7	**35**	4	**20**
11.5 dpc	24	**31.6**	33	**43.42**	19	**25**
12.5 dpc	8	**23.5**	17	**50**	9	**26.5**
13.5 dpc	14	**37.8**	18	**48.65**	5	**13.5**
14.5 dpc	2	**28.6**	5	**71.43**	0	**0**

Table 2: Analysis of genotypes of living embryos resulting from Memo heterozygous knockout matings

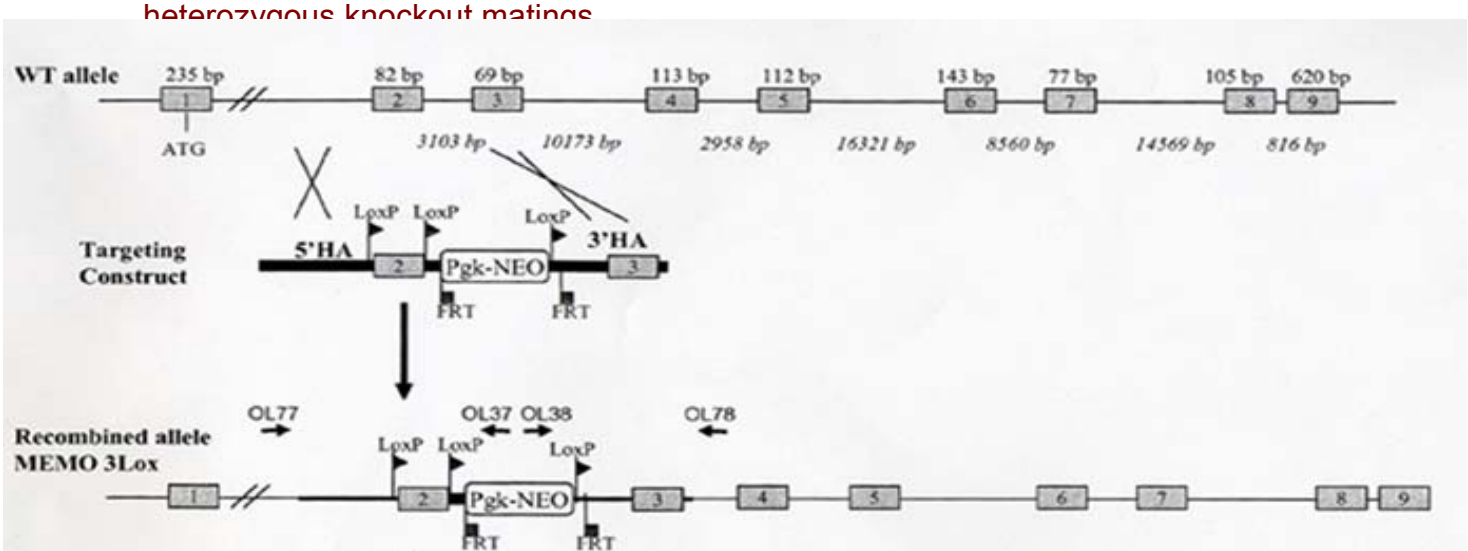


Figure 1: Targeting of Memo locus

The exon-intron structure of the mouse Memo gene shows that Memo contains 9 exons. The targeting vector harboring the 5'homology arm, the floxed exon 2, the Neo cassette and the 3' homology arm was electroporated into ES cells. This gives rise to the depicted Memo floxed allele.

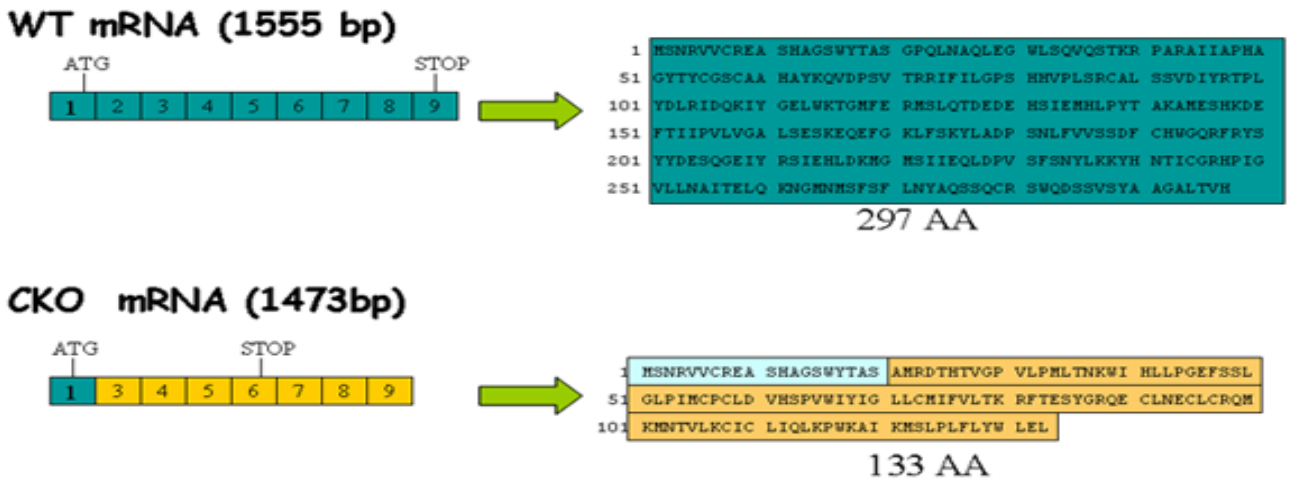
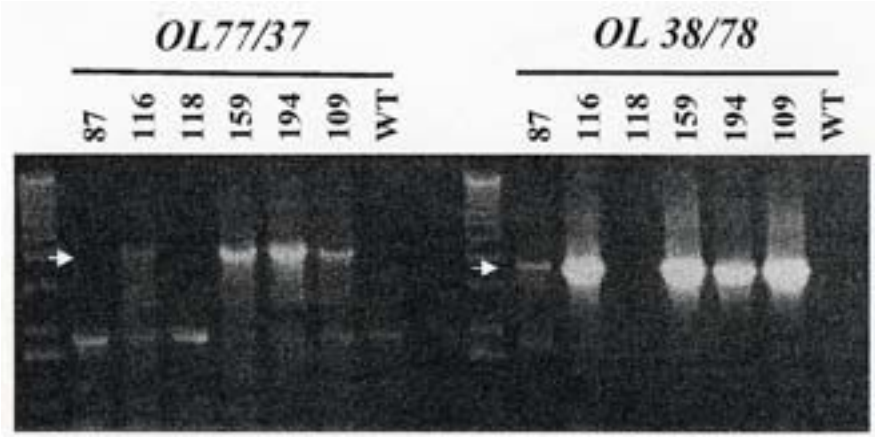


Figure 2: Protein sequence of mouse Memo and of putative protein after exon 2 deletion.
Analysis of protein sequence reveals that if any protein is translated from the mRNA after removal of exon 2, this protein would share only the first 20 amino acids with the wild-type Memo protein.

A



B

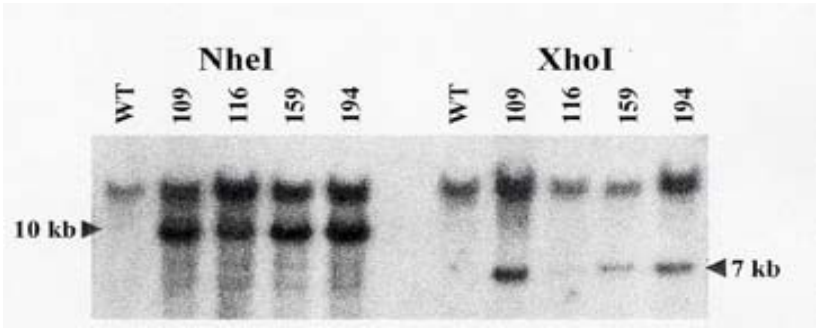


Figure 3: Screening for ES cells that underwent one homologous recombination

PCR screening for ES clones that underwent homologous recombination 5' and 3' of targeted Memo locus shows that ES clones #116, 159, 194 and 109 have done correct homologous recombination (Figure 3A).
Southern blotting of gDNA from ES cells having the recombined Memo locus show that clones #116, 159, 194 and 109 have the correct pattern after NheI and XhoI restriction (Figure 3B). Note that the 7kb fragment of clone 116 after XhoI restriction is very faint. The upper bands sharing the same size than the WT band is a background band corresponding to contamination by G418 resistant fibroblasts used as feeder cells.
Clone 109 was used for further experiments.

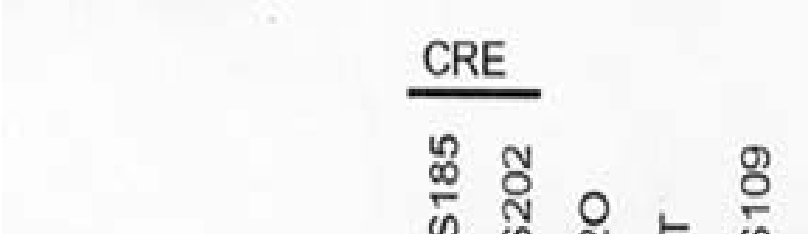


Figure 4: Screening for ES cells that have one Memo KO allele

PCR characterization of clones that underwent recombination and generation of KO allele after electroporation of the Cre recombinase in ES clone 109

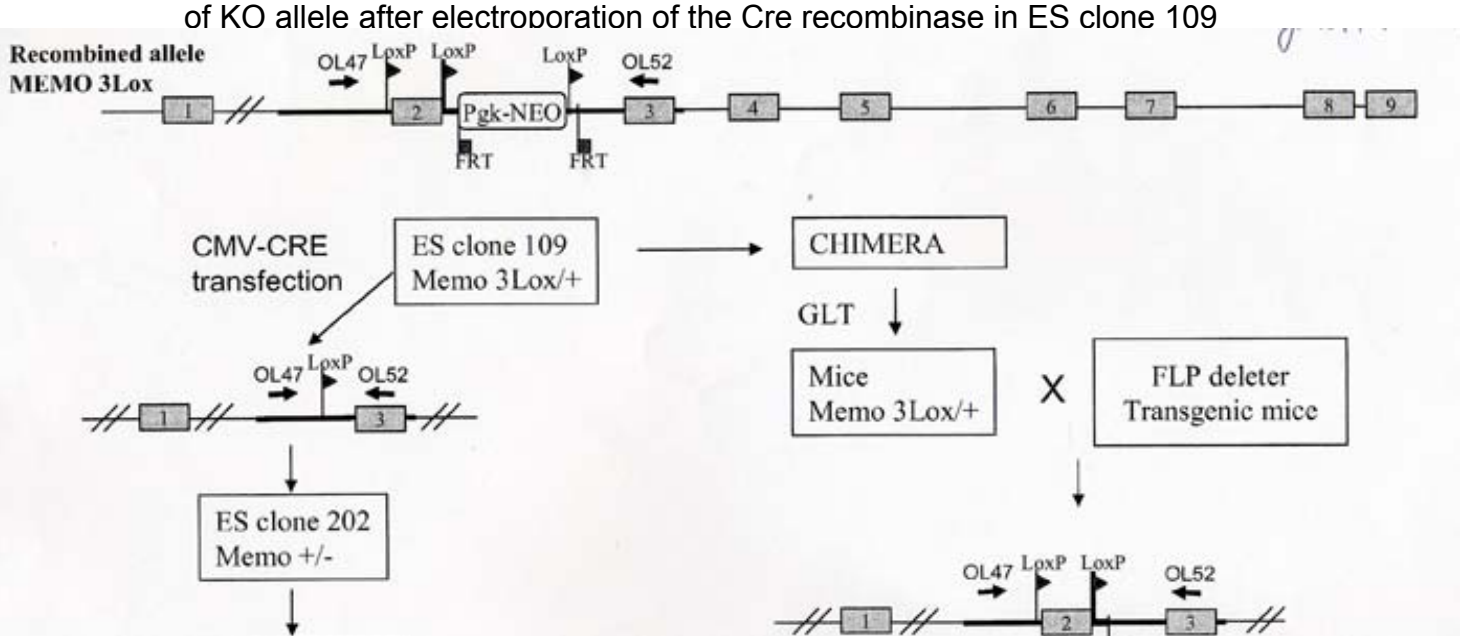


Figure 5: Generration of Memo KO mice
After electroporation of the targeting vector, clone ES 109 showed correct homologous recombination and was used for further experiments. After Cre transfection in clone 109, clone 202 showed a Memo knockout allele and was used to generate chimeras. The chimeras which gave germline transmission (GLT) were further used to generate Memo heterozygous animals.

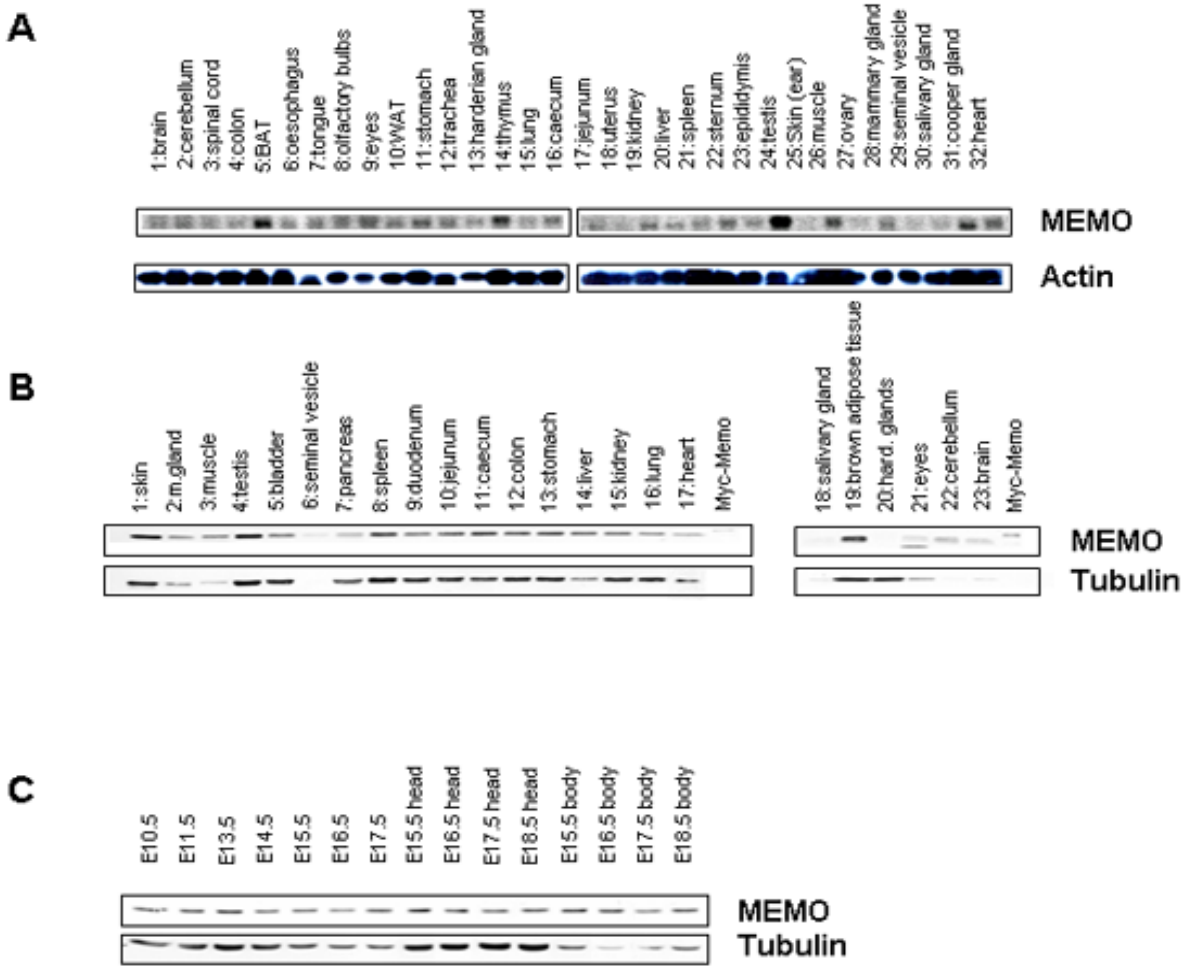


Figure 6: Analysis of Memo's expression pattern in adult organs and in embryos by Northern and Western blotting

A: Northern blotting on mouse adult tissues reveals an ubiquitous expression of Memo. Memo transcript is particularly expressed in the brown adipose tissue(lane 5), thymus (lane 14) and testis (lane 24).

B: Western blotting on mouse adult tissues reveals that the protein is ubiquitously expressed. It is particulary abundant in the skin (lane 1), testis (lane 4) and in the brown adipose tissue (lane 20). The Memo polyclonal antibody was used.

C: Western blotting on embryos from E10.5 to E18.5. For better clarity, separated analysis of head and body protein content was also performed for stages E15.5-E18.5. The same amount of protein was observed in all the examined stages during mid to late embryogenesis. The Memo polyclonal antibody was used.

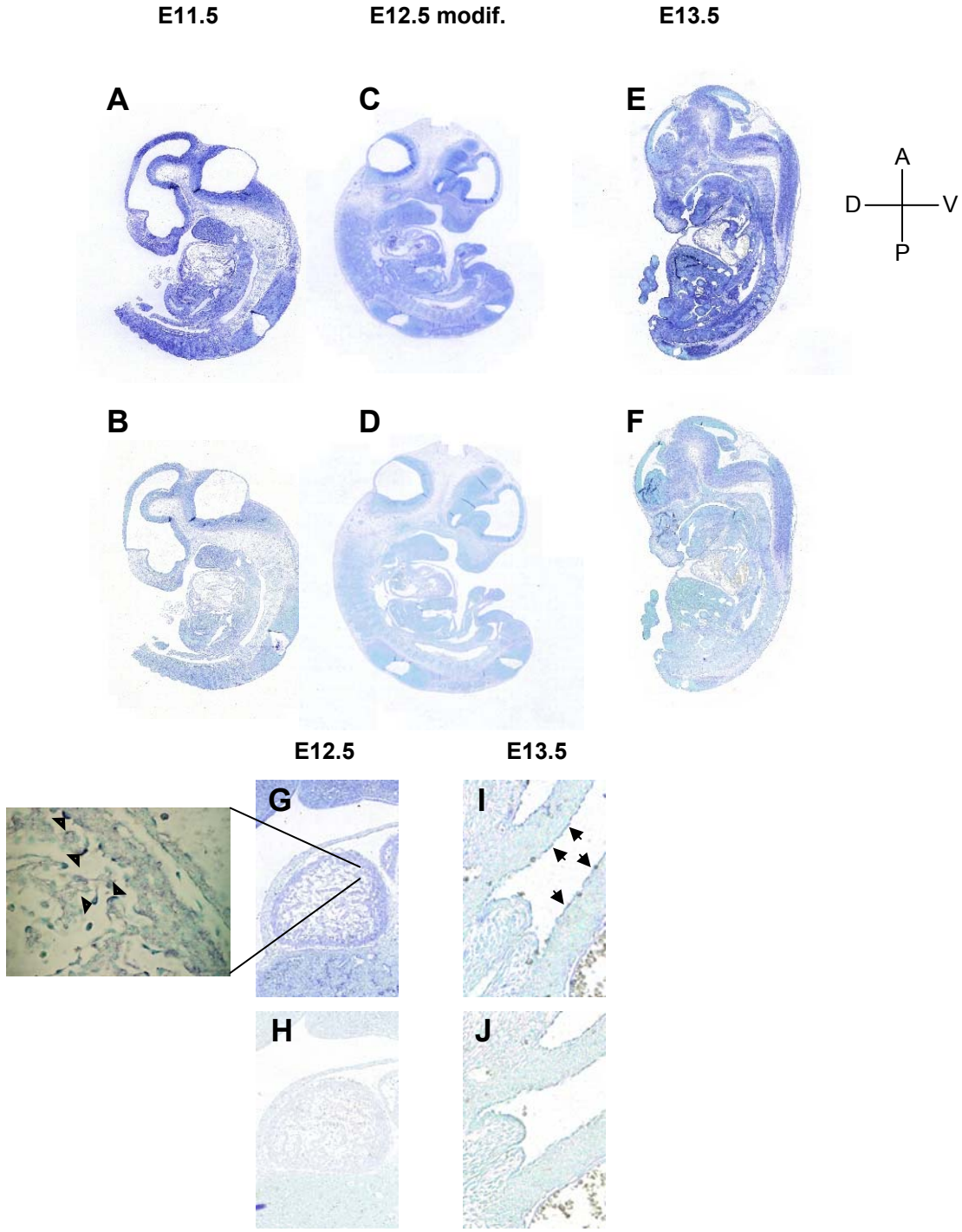


Figure 7: Ubiquitous expression pattern of Memo in embryos E11.5-E13.5
In-situ hybridization on paraffin sagittal cross-sections of WT embryos using an antisense (A, C, E, G, and I) and a sense (B, D, F, H and J) probe for Memo. (G and H) represent a higher magnification of the heart. The zoom shows a particularly strong staining in the endocardiac cells (arrowheads). (I and J) represent a higher magnification of the aorta and its valve. Note the endothelial cells bordering the aorta (arrows).

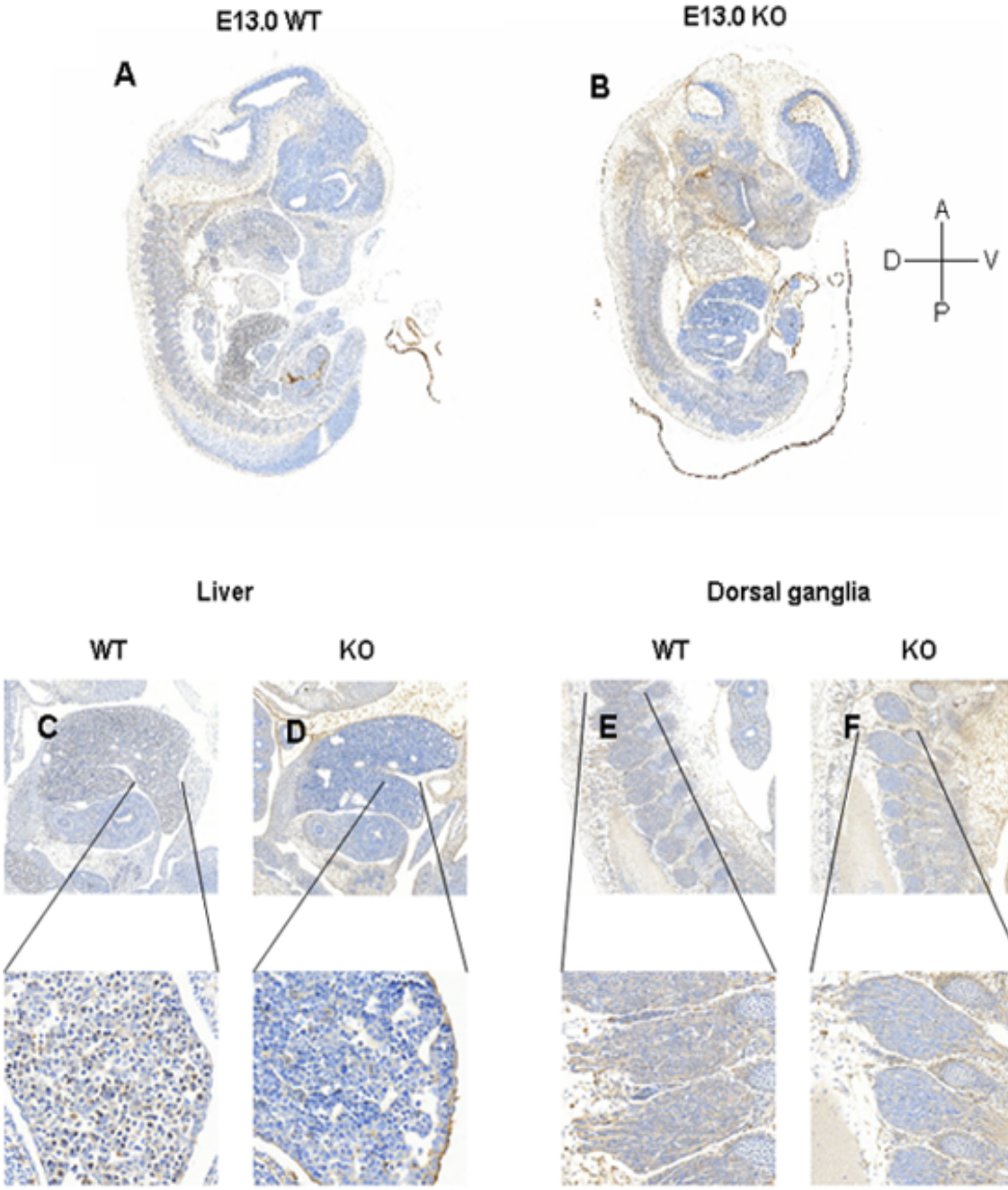


Figure 8: Use of Memo monoclonal antibody enables us to distinguish wild-type from knockout embryos

Comparison of Memo's expression pattern between WT and KO embryos by immunohistochemistry with the monoclonal antibody.

At E13.0, immunohistochemistry reveals a ubiquitous expression pattern in the wild-type embryo (A). Although some background staining can be seen in the conjunctive tissue in the KO embryo (B), difference in the staining between wild-type and knockout embryos can be observed in the liver (C and D) and in the dorsal ganglia (E and F).

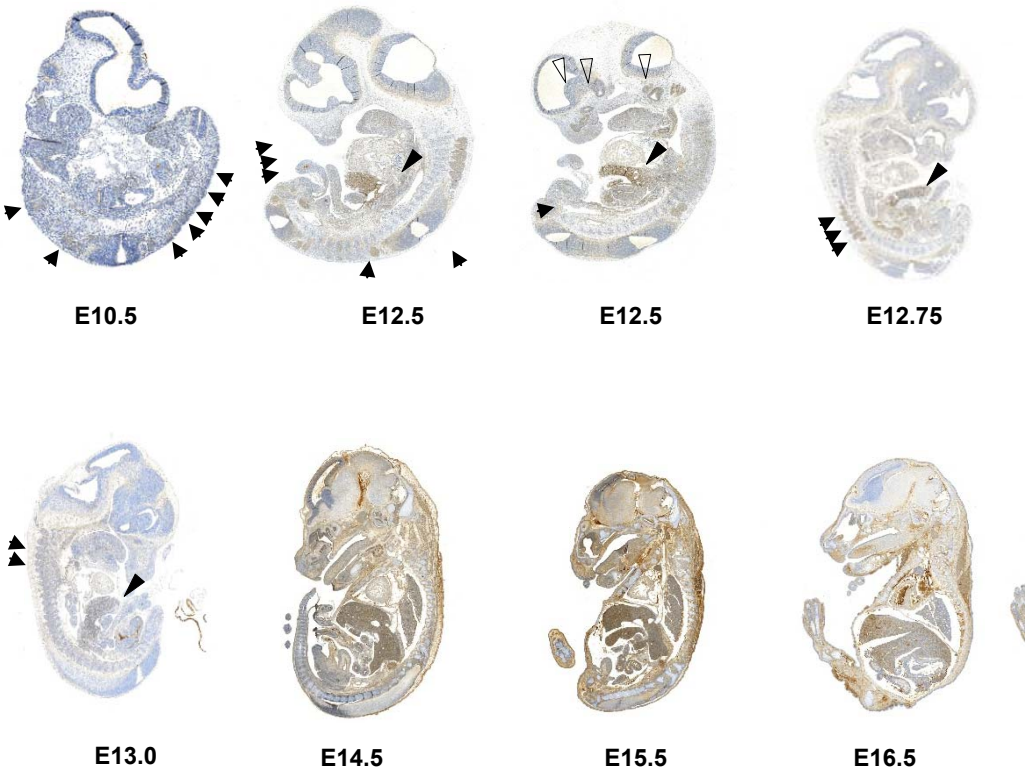


Figure 9: Analysis of Memo's expression pattern in embryos by immunohistochemistry

A: Immunohistochemistry with the monoclonal antibody was performed on embryos from stages E10.5-E16.5. At E10.5, the protein is mostly detected in the somites (black arrows) but its expression is ubiquitous. At E12.5-E13.0, it is detected in the dorsal ganglia (black arrows), liver (black arrowhead) and cranial ganglia (white arrowhead). Later on, the protein is ubiquitously expressed throughout the embryo.

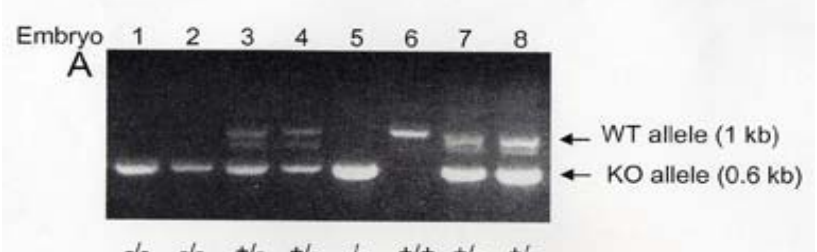


Figure 10: No protein is detected in the Memo knockout embryos by the plyclonal antibody.

A: PCR genotyping of KO E12.5 embryos obtained from matings of heterozygous knockout parents shows that pups 1, 2 and 6 are knockout. 3, 4, 7 and 8 are heterozygous and 6 is wild-type.
B: Western blot analysis of protein extracts coming from the same embyros as in (A) confirms the PCR result: No Memo protein is detected in the knockout embryos 1, 2 and 6.

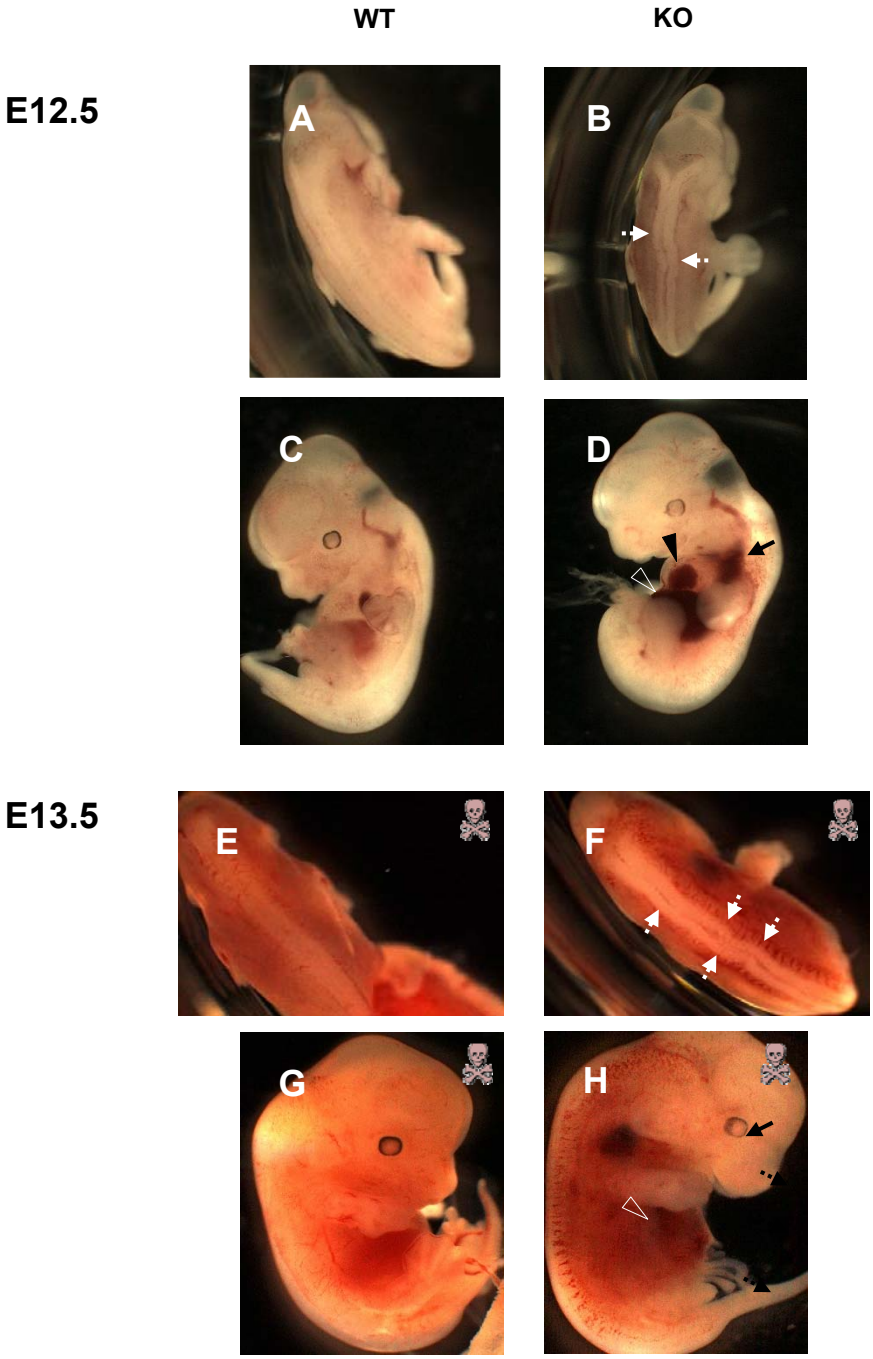


Figure 11: The Memo knockout embryos have malformed neural tubes and show hemorrhages.

Macroscopic examination of wild-type and K-O E12.5 and E13.5.

The dorsal view of the embryo shows that the neural tube is not correctly developed but is bent in the K-O embryos (dashed white arrows in B and F) compared to wild-type embryos (A and E).

On a left side view of E12.5 embryos, hemorrhages are seen in the heart (black arrowhead), cardinal vein (black arrow) and liver (white arrowhead) of the K-O (D). A side view of E13.5 shows intersomitic hemorrhages (dashed black arrows), as well as abnormal blood presence in the cardinal vein (black arrow) and liver (white arrowhead).

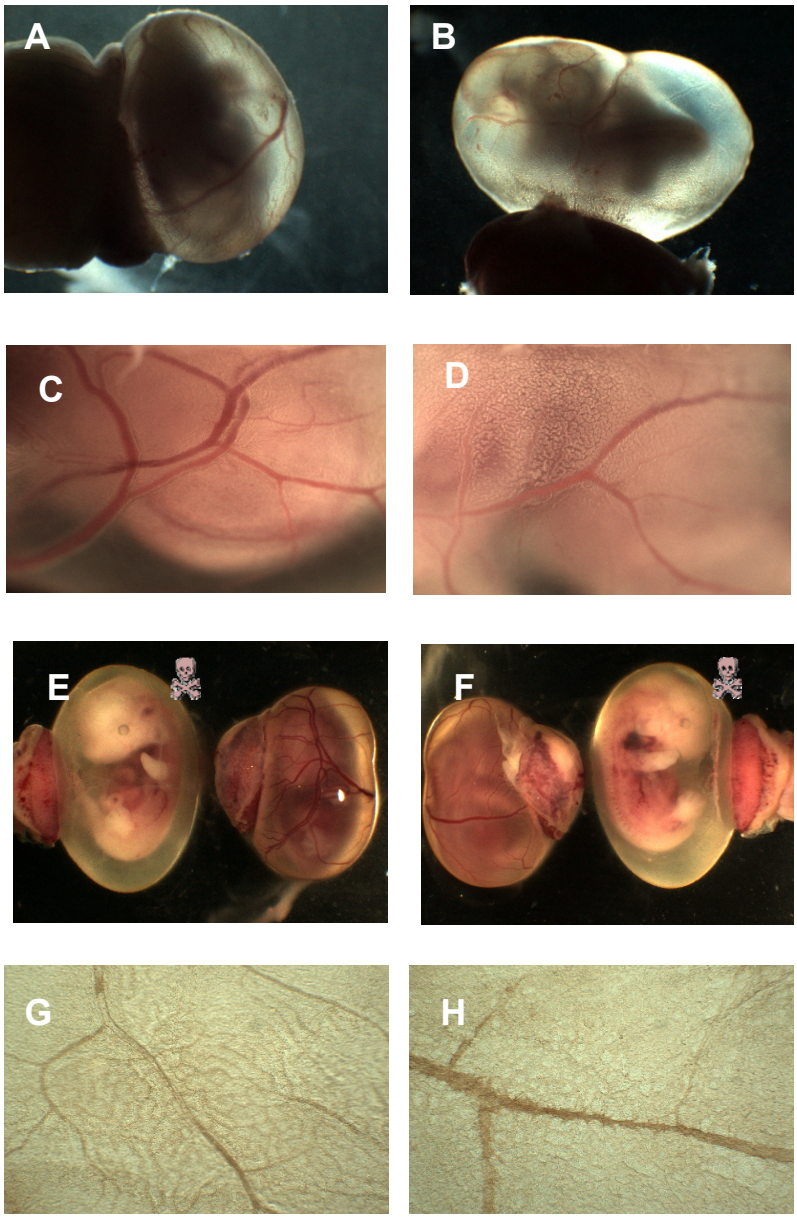


Figure 12: Abnormal placental vascularization in Memo knockout embryos
Examination of placenta of wild-type (A,C,E,G) and knockout (B,D,F,H) embryos.
Macroscopical view of embryos at E12.5 reveals a paler yolk sac in knockout embryos (B) compared to wild-type littermates (A).
Higher magnification of vessels of the placenta at E13.0 reveals that they slowly disappear in the knockout embryos (F). Wild-type littermate placenta is shown in (E).
At E13.5, a time when the knockout embryos were dead as indicated by non beating heart, there is no more trace of placental vascularization in the knockout embryos (left in C and right in D). Moreover, the space between the embryo and the placenta has increased, a sign of edema.
Whole-mount CD31 staining of placenta of wild-type (G) and knockout (H) embryos reveals that the endothelial cells are still present in the knockout embryos, despite the gradual absence of blood seen in (B), (D) and (F).

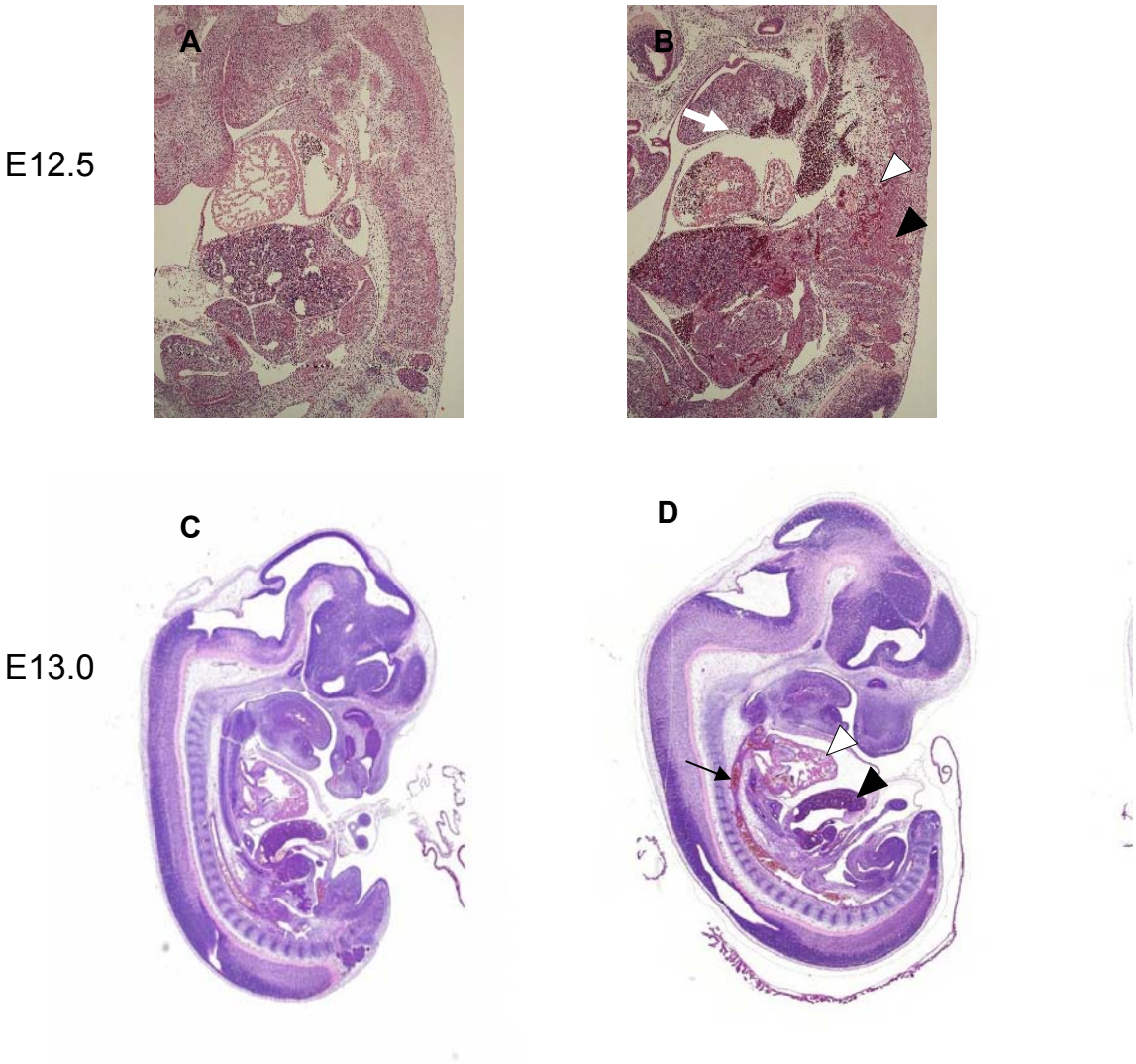


Figure 13: Histological sections confirm the presence of hemorrhages and edema, but shows that other organs are intact

H&E staining on paraffin cross-sections reveals the abnormal presence of blood in heart (white arrowhead), aorta (black arrow), cardinal vein (white arrow) and liver (black arrowhead) in knockout embryos (B and D) compared to wild-type embryos (A and C). No other defect could be seen in the morphology of heart, lung, pancreas for example.

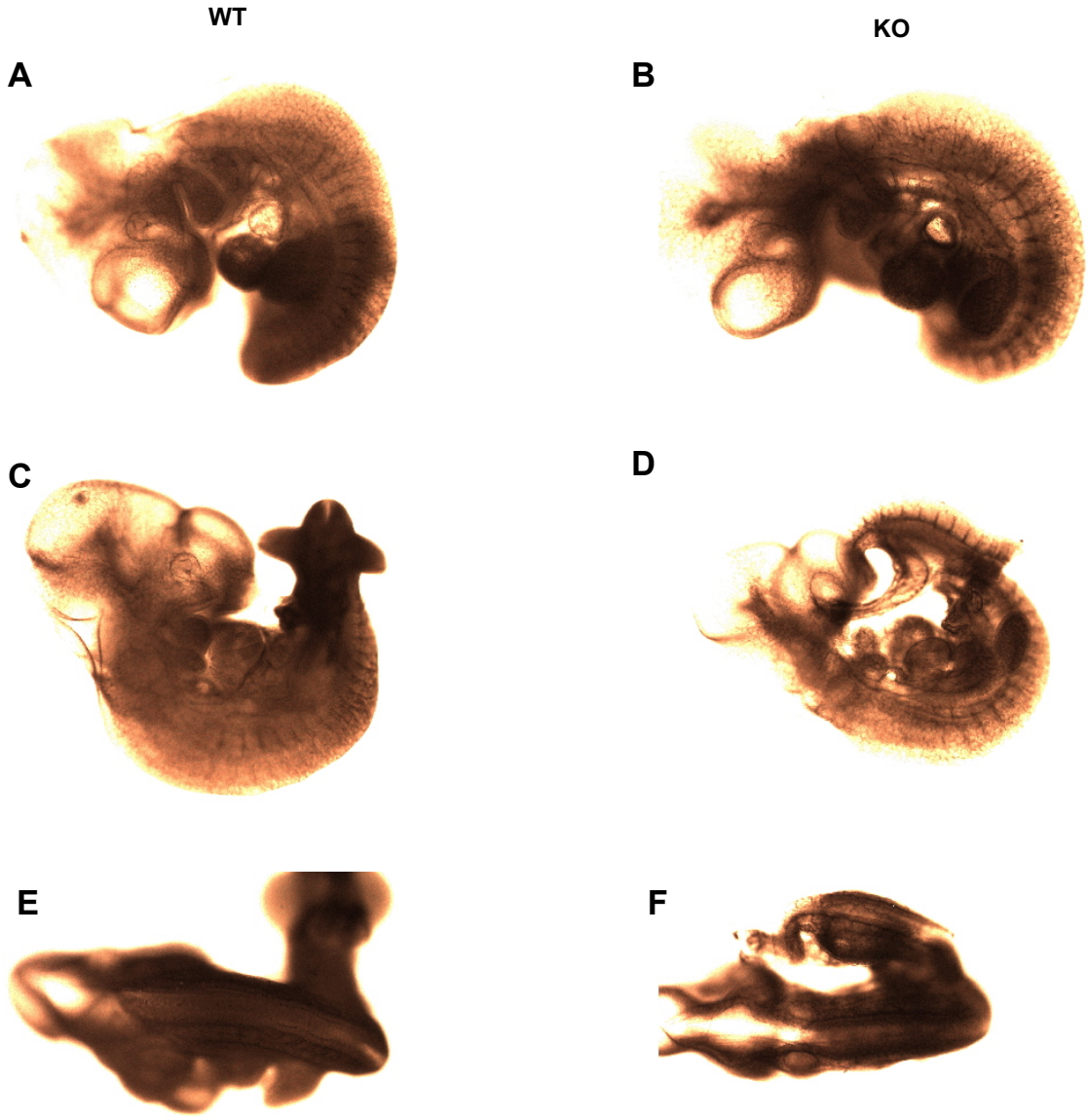


Figure 14: Memo knockout embryos have a normal network of blood vessels
CD-31 whole mount staining of E10.5.
Left (B) and right (D) side view shows that the gross vasculature morphology is intact in the KO embryos compared to wild-type embryos (A resp.C).
Dorsal view shows the normal presence of intersomitic vessels in KO (F) and wild-type (E) embryos.

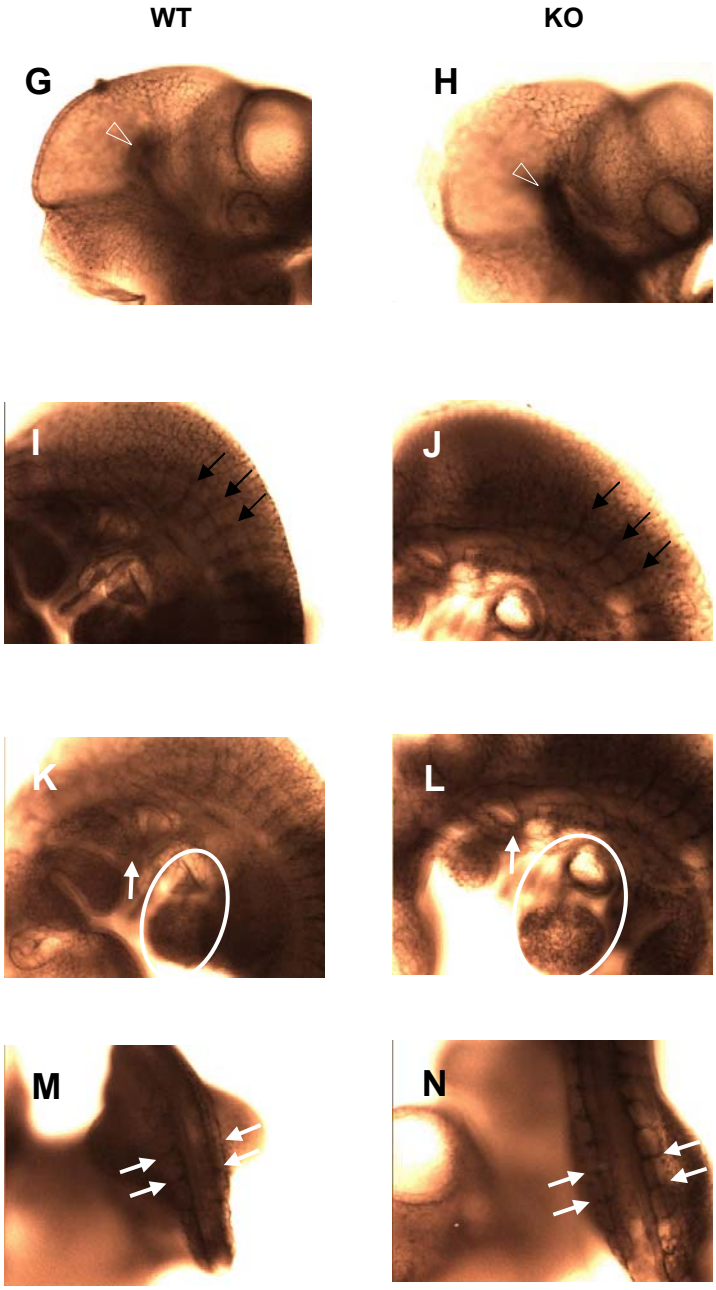


Figure 14 (continued): Memo knockout embryos have a normal network of blood vessels

Higher magnification shows a good network of primary head veins (G and H). The vessel branch from anterior carotid arteries is also correctly formed (white arrowhead in G and H). Vascular network in the back does not show any difference between WT (black arrows in I) and KO (black arrows in J) embryos. The transitional branchial arches have the correct morphology in the knockout embryos (white arrow in L) compared to wild-type embryos (white arrow in K). The atrium and ventricle are also well vascularized (white circle in K and L). Higher magnification of intersomitic vessels shows that their network is not affected in the knockout embryos (white arrows in N) compared to wild-type embryos (white arrows in M).

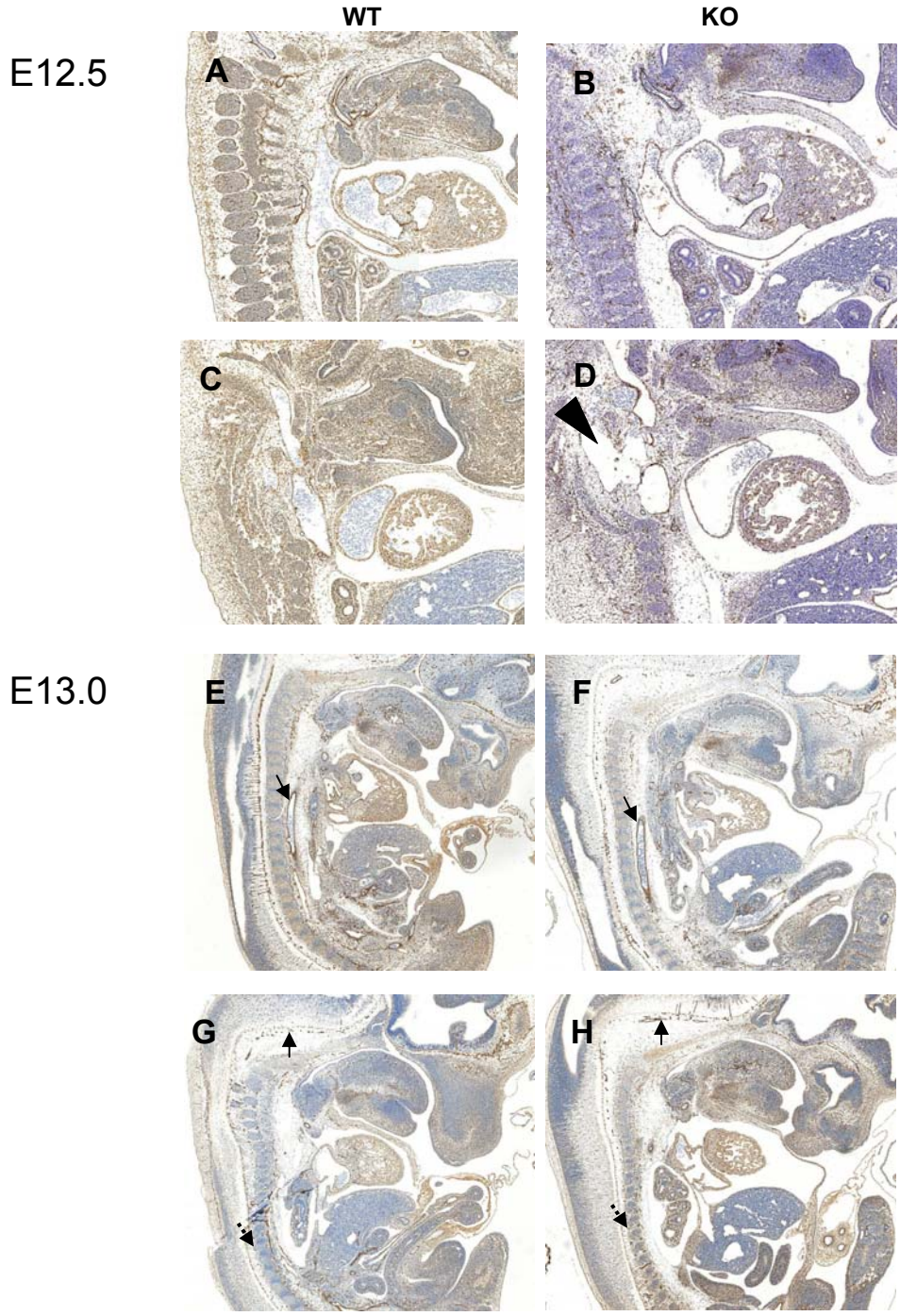


Figure 15: The deeper network of vessels is intact in Memo knockout embryos
CD-31 staining of sagittal cross sections of wild-type and KO E12.5 and E13.0 embryos. The atrium and ventricle morphology of the heart is intact in the KO embryos. Cranial vessels (black arrow in G and H), aorta (black arrow in E and F) and intersomitic vessels (dashed black arrow in G and H) show no abnormality. The arrowhead in (D) points to a dilated blood vessel.

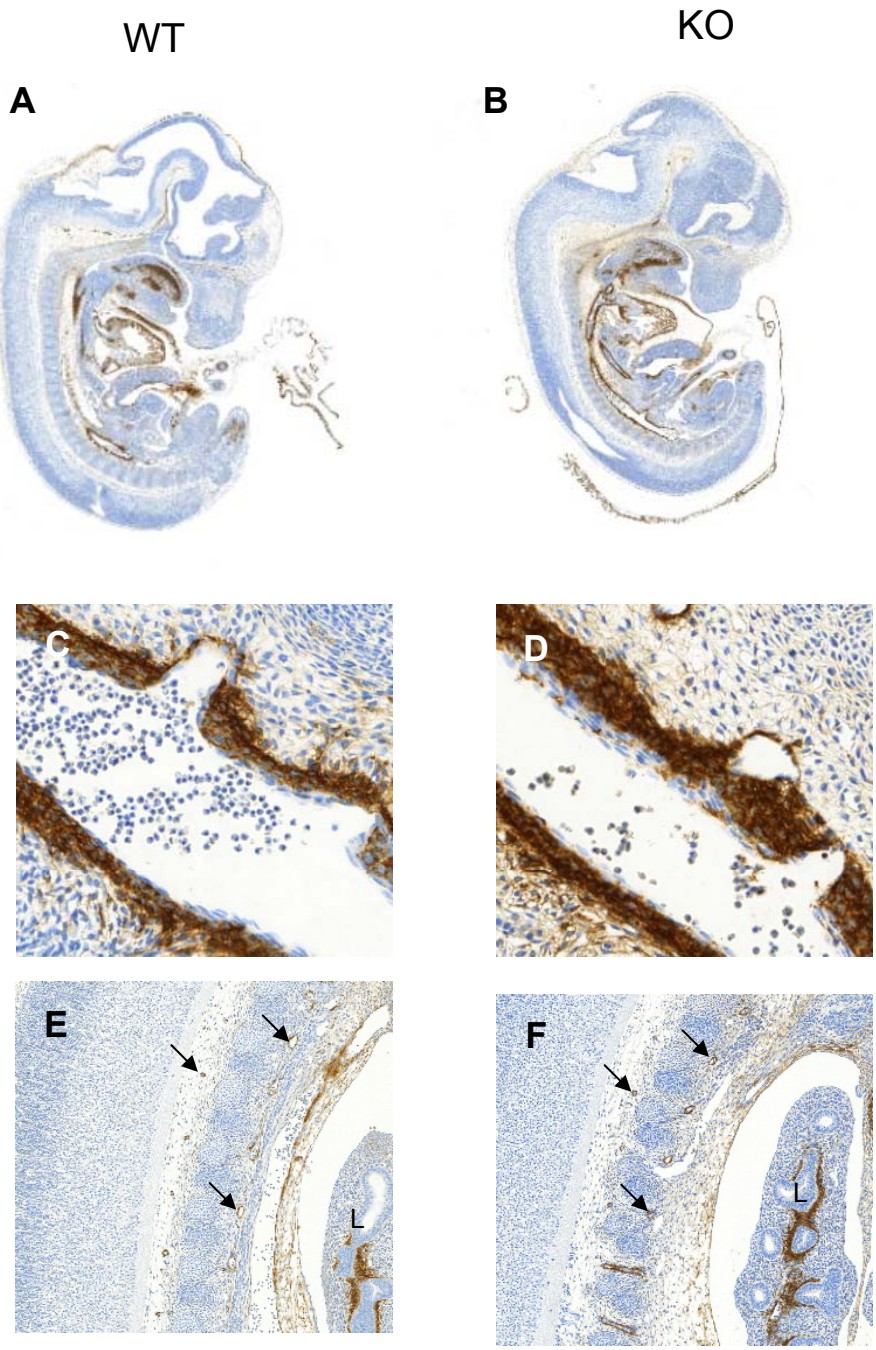


Figure 16: The smooth muscle cells are properly recruited to stabilize blood vessels of the Memo knockout embryos.

Smooth muscle actin staining of sagittal cross sections of E13.0 WT (A,C,E) and KO (B,D,F) embryos.

The dorsal aorta, heart and cranial vessels are normally stained in the KO embryo (B) compared to wild-type embryo (A).

Higher magnification shows that the dorsal portion of the dorsal aorta is stained as well (B and D).

Higher magnification shows that even smaller vessels as the intersomitic vessels are properly surrounded by smooth muscle cells (black arrows in E and F; L stands for lung).

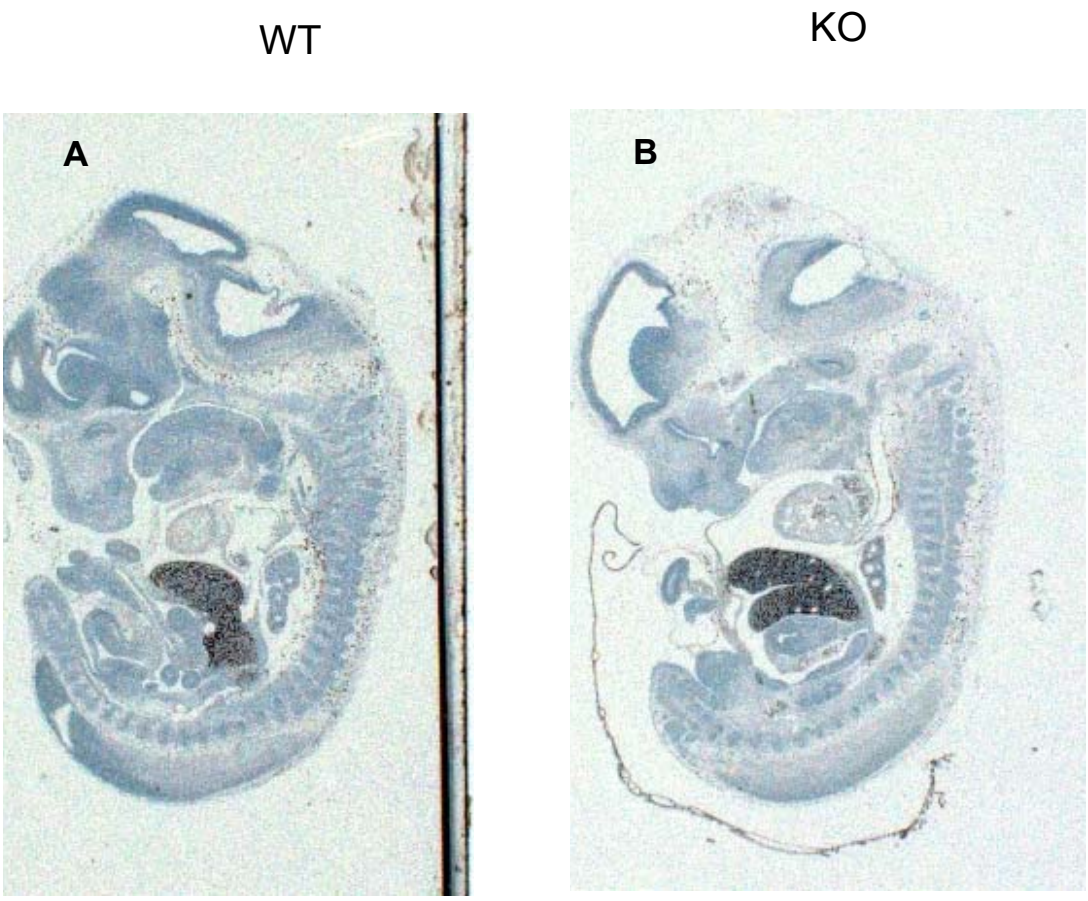


Figure 17: The lymphatic vessels are intact in the Memo knockout embryos.

LYVE-1 staining of sagittal cross sections of E13.0 WT (A) and KO (B) embryos.
The liver, heart and cranial region are normally stained in both WT and KO embryos.

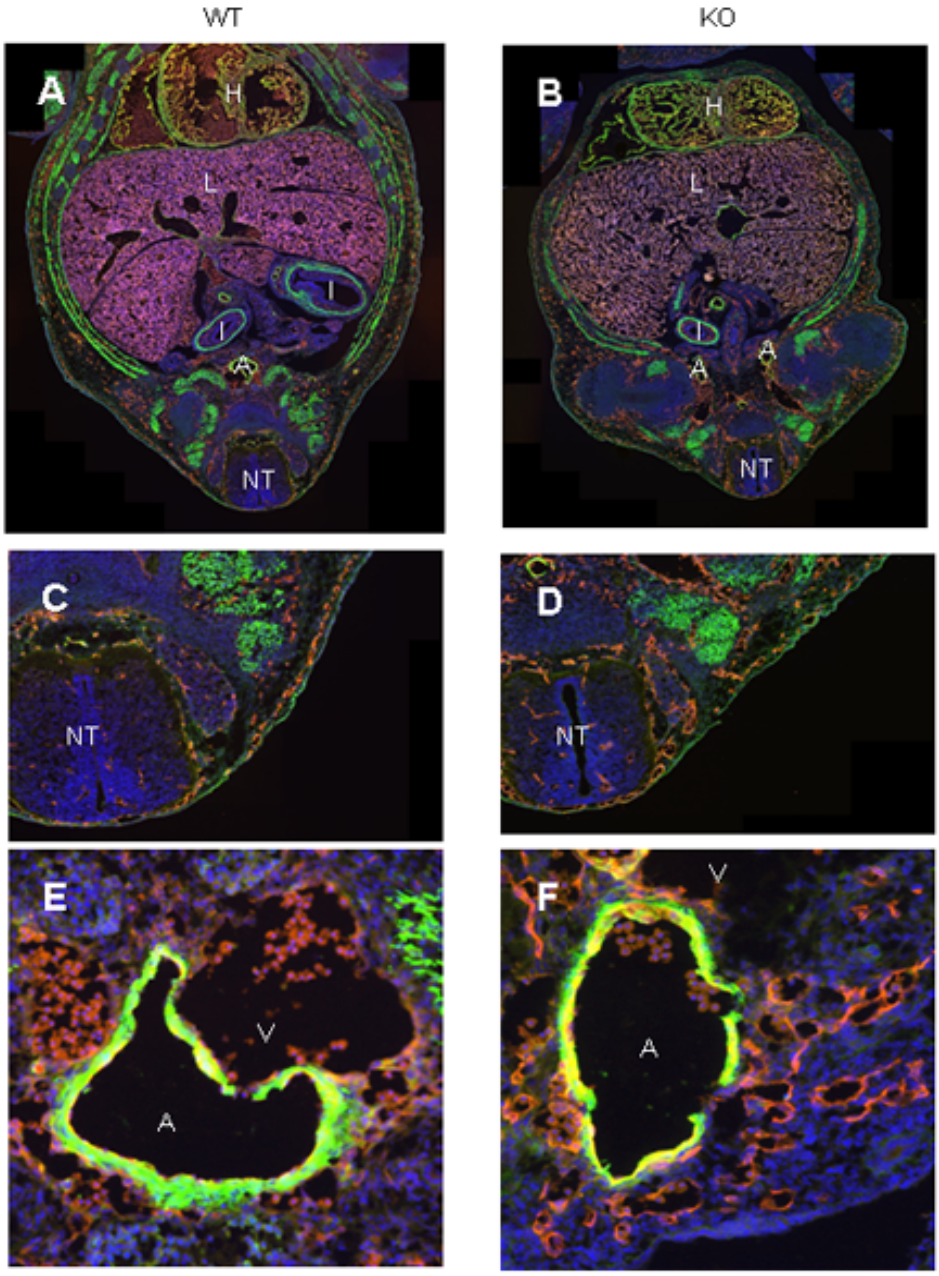


Figure 18: Irregular alignment of subcutaneous vessels and dilated small vessels in Memo knockout embryos

Immunofluorescent staining of frontal cryosections of wild-type and knockout embryos using CD-31 (red) and SMA (green) antibodies.

Low magnification reveals the correct presence of CD-31 positive cells (red) in the heart, liver and intestine of knockout embryos (A and B). Higher magnification reveals an irregular pattern of subcutaneous blood vessels in Memo knockout embryos (C and D). Higher magnification of the aorta reveals that it is well shaped and correctly surrounded by smooth muscle cells in the knockout embryos. However, smaller vessels appear dilated (E and F).

H=heart; L=liver; I=intestine; A=aorta; V=vein; NT=neural tube.

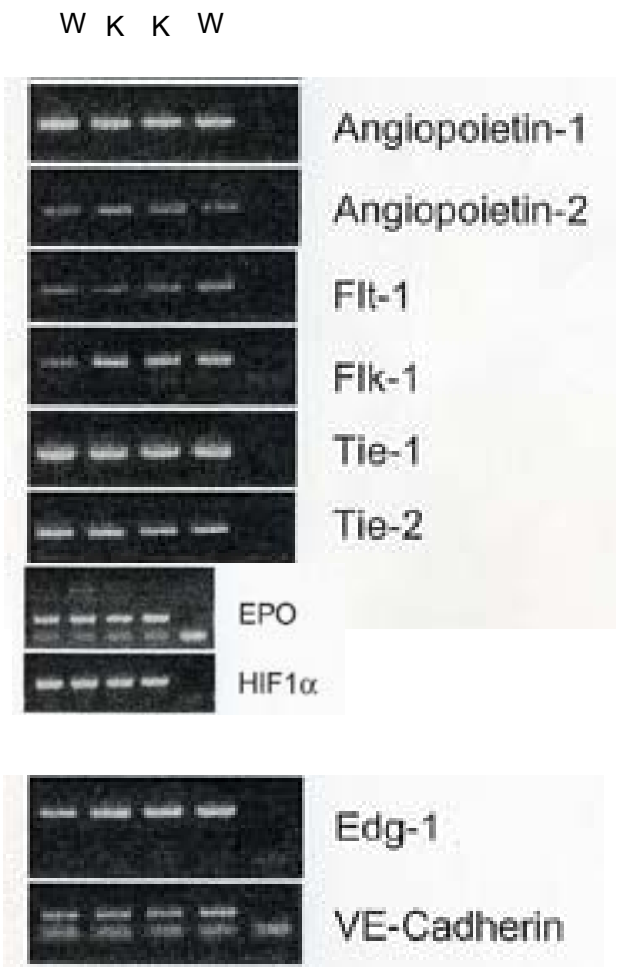


Figure 19: No change in the expression of genes involved in vasculogenesis, angiogenesis and maturation of blood vessels
RT-PCR of RNA extracts from wild-type and knockout embryos did not show any difference in the expression of Flt-1, Flk-1, angiopoietin-1 and 2 and their receptors Tie-1 and 2. There was also no change in the transcript level of EPO, HIF-1α, Edg-1 and VE-cadherin levels.

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3.2 Part II: Suppression of cell-cell contact followed by apoptosis and loss of integrity of mammary alveoli in mice with Memo deletion

Patrick Kaeser, Régis Masson, Francisca Maurer and Nancy Hynes

3.2.1 Abstract

Studies from our lab recently led to the discovery of Memo (mediator of ErbB2-driven cell motility), a novel 297 amino acid protein shown to be required for ErbB2- and other receptor tyrosine kinase-driven cell motility. Inhibition of Memo expression had consequences on the microtubule network which could not grow towards the periphery of the cells upon heregulin stimulation. It also had consequences on the actin cytoskeleton, since more actin stress fibers were seen. To study the in vivo role of Memo in the mammary gland, $Memo^{fl//fl}$ mice were crossed with WAPiCre transgenic mice, which led to specific ablation of Memo in luminal alveolar epithelial cells. As shown by pup weight measurement, the conditional knockout mice were unable to correctly nurse their pups. During lactation the mammary gland weight was smaller in CKO females compared to control females. H&E staining indicated the presence of shed cells in the lumen of CKO glands in the first days of lactation. It also revealed a progressive loss of alveoli which were replaced by adipocytes. Increased apoptosis was measured in the CKO glands by cleaved caspase-3 immunohistochemistry. Consistent with this apoptosis, an increase in the levels of pro-apoptotic P-Stat3 and Bax was seen at protein level. Improper localization of the adherens junction proteins E-cadherin and ß-catenin was seen in the conditional knockout glands, which probably is the reason for the observed increase of apoptosis.

3.2.2 Introduction

The mammary gland represents an attractive organ for developmental studies (Hennighausen and Robinson, 2001; Hens and Wysolmerski, 2005; Hinck and Silberstein, 2005; Oakes et al., 2006; Sternlicht, 2006; Watson, 2006). While pregnancy occurs, the gland begins to form side buds which elongate perpendicular to existing ducts. Alveolar growth and proliferation accompanied by migration occurs during pregnancy. This is followed by functional differentiation of the alveolar epithelium at the end of gestation with the onset of milk secretion at parturition.

Among other roles, hormones temporally control the tight junction closure during the transition from pregnancy to lactation (Nguyen et al., 2001). Some additional alveolar proliferation occurs during the first few days of lactation. In the absence of suckling or at cessation of nursing, the differentiated mammary epithelial cells are removed by apoptosis and by basement membrane degradation and the gland is remodeled to a duct system similar to that in the mature virgin. This process, named involution, occurs in two phases (Lund et al., 1996). In the first phase, the lack of suckling and milk stasis leads to the accumulation of local factors that result in a rapid but reversible induction of apoptosis within the differentiated mammary epithelial cells (Li et al., 1997; Marti et al., 1997). When the lack of suckling is prolonged, the involution goes into its second, irreversible phase. Apoptosis is accompanied by a tissue–remodeling phase involving the induction of matrix-degrading enzymes (Green and Lund, 2005) and inflammatory cell infiltration (Stein et al., 2004). The drop of circulating hormones might affect the cell-cell junctions (Zettl et al., 1992)

Memo (mediator of ErbB2-driven cell motility) is a novel molecule identified in our lab as a protein binding to the phospho-Tyr 1227 of the ErbB2 receptor (Marone et al., 2004). A single Memo protein is encoded in the human and mouse genome, but Memo homologs are found in all branches of life including in C.elegans (Lai et al., 2000).

With the help of transwell chamber assay, it has been shown that in vitro Memo is required for efficient heregulin-driven motility of breast carcinoma cells (Marone et al., 2004), hence its name Memo. It has also been shown that in vitro Memo is required for efficient FGF2- and EGF- induced cell migration, indicative of a widespread role for Memo in receptor tyrosine kinase-induced cell motility.

Inhibition of Memo expression markedly reduced the network of microtubules growing to the periphery. It has also been shown that the actin stress fibres appeared to be increased and it has been hypothesized that this effect is a consequence of an improper microtubule network.

To probe the *in vivo* function of Memo in the mammary gland, it was necessary to generate a tissue-specific, conditional knockout of Memo to overcome the embryonic lethality of Memo disruption. This result was achieved using the Cre-lox recombination system in which expression of Cre recombinase is directed

specifically to mammary luminal epithelial cells by the promoter of the milk protein gene whey acidic protein (WAP) (Wintermantel et al., 2002). These WAPiCre transgenic mice were crossed with mice harboring two floxed Memo alleles in which the lox P sites were inserted around exon 2. Without exon 2, any potential protein generated would have aberrant amino acid sequence. The mice exhibited epithelial apoptosis leading to abnormal morphogenesis and inability to nurse their pups. The apoptosis probably results as a consequence from improper adherens junction between the luminal epithelial cells.

3.2.3 Results

Analysis of Memo expression at various stages of mammary gland development and in the mammary glands of conditional knock out mice

A study of Memo expression in adult organs and in embryos has revealed a ubiquitous expression of Memo mRNA and protein. To investigate whether Memo is specifically required in mammary gland development, we first performed analysis of protein extracts at various stages of mammary gland development. Western blot analysis revealed a ubiquitous expression of Memo during gestation, lactation and involution (Figure 1A).

To study the role of Memo during mammary gland development, we conditionally knocked out Memo by crossing WAPiCre transgenic mice with mice harboring two floxed Memo alleles (Figure 1B). WAP (whey acidic protein) is a milk protein, that's why WAPiCre is specifically expressed in the secretory luminal epithelial cells of the gland starting at midpregnancy and reaches a maximum at day 3 of lactation. WAPiCre is not expressed in the ductal tree of the virgin gland, nor is it expressed in the basal myoepithelial cells of the alveoli. Littermates with the genotype $Memo^{fl//fl}$/$WAPiCre^{-/-}$ (referred to as control mice) or $Memo^{fl//fl}$/$WAPiCre^{+/-}$ (referred to as conditional knockout mice) were used for all analyses. These mice reached adulthood with no apparent abnormalities. Mammary glands from 3-day lactating females were used to examine Cre-mediated recombination. Figures 1C and 1D show RT-PCR and Western blot at day 3.5 of lactation to characterize the WAPiCre recombination occurring at the floxed Memo alleles. Following RT-PCR, the floxed

Memo allele was detected as a 295 bp fragment (lane 1) and the null Memo allele as a 212 bp fragment (lane 2). The residual 295 bp fragment corresponding to the floxed Memo allele can result from unrecombined floxed allele of the luminal epithelial cells or most probably of the surrounding myoepithelial and stromal cells since WAP is not expressed in these cells.

Deletion of Memo in the luminal epithelial cells resulted in severe pup weight reduction

During lactation, the luminal epithelial cells of the mammary glands undertake their main function which is to produce milk for the pups. An assay to measure if this function is altered consists in measuring the body weight of the progeny. For this purpose, pups coming from control and CKO females were mixed and 6 pups were randomly given to each female. The pup weights were then monitored from day 1 to day 15 of lactation (Figure 2A). At all time points, the average weight of the pups nursed by CKO females was lower than the one of pups nursed by control females. After 6 days of lactation, it represented 83.46% of the weight from control, after 10 days just before it reached a plateau, 73.79%. After 12 days it started to decrease to finally reach only 51.89% after 15 days. The maximum weight of the pups nursed from CKO females is after 11 days. It corresponds to the weigh of a pup nursed from control females after 7 days. Figure 2B shows a picture from 1 pair of pups coming from control and 1 pair of pups coming from conditional knockout mothers after 14 days of lactation. The latter were visibly smaller. The proportional weight of the mammary gland itself compared to the total mouse weight was also measured after 6, 10, 12 and 15 days of lactation (Figure 2C).
The proportional weight of the mammary gland was 1.10 to 1.27% in the control mice. It was only two third of this range in the conditional knockout mice, reaching only 0.78 to 0.84%.

Deletion of Memo results in abnormal mammary gland morphogenesis during lactation

Figure 3 shows hematoxylin- and eosin-stained paraffin sections of $Memo^{fl//fl}$ (A,C,E,G,I) and $Memo^{fl//fl}$/WAPiCre (B,D,F,H,J) mammary glands during lactation. At

days 3.5 and 6.5 of lactation, there was no significant differences in either the development of the lobular-alveolar structures or their density in CKO (B and D small panel) compared to control mammary glands (A and C small panel). However, a higher magnification revealed a noticeable change in the alveolar integrity of the glands from Memo CKO females. Indeed luminally shed cells were detected (black arrows in B,D).

From 10.5 days of lactation, the lobulo-alveolar structure was less dense in the CKO mammary glands (E,G,I) compared to the control glands (F,H,J).

Figure 4 shows a oil red O staining of cryosections of $Memo^{fl//fl}$ (A,C,E,G,I) and $Memo^{fl//fl}$/WAPiCre (B,D,F,H,J) mammary glands during lactation. The red staining reveals that the alveoli continue to secrete milk in Memo CKO glands. But the majority of the staining in the Memo CKO glands actually does not stain lipids from milk, but lipids from adipocytes surrounding the alveoli.

The area of the gland occupied by alveoli was manually delimited as shown in figure 4K using the Image Access software. The area occupied by alveoli was measured and the results are shown in Figure 4L. A significantly smaller surface percentage was occupied by alveoli in glands from $Memo^{fl//fl}$/WAPiCre mice compared with $Memo^{fl//fl}$ mice. In the wild-type glands, the surface occupied by alveoli was 68-78%, depending from the stage, whereas it continuously decreased in the CKO glands during lactation: from 68% after 3 days of lactation, it went down to 53% after 10 days and to less than 20% after 15 days. This loss of milk-producing cells likely explains the associated pup weight decrease.

Increased epithelial apoptosis in the absence of Memo

The observed shedding of epithelial cells into the lumen at lactation 3.5 and 6.5 is likely to be responsible for the loss of alveoli visible from 10.5 days of lactation on. To check if the cells which were detached and shed into the lumen were apoptotic, we performed immunohistochemistry for cleaved caspase-3, an executioner caspase. Immunohistochemistry revealed that that the shed cells in the lumina were apoptotic. Some luminal epithelial cells in the lobuloalveolar structure were also positive for cleaved caspase-3 (see arrowheads in Figure 5H).

A significant increase in cleaved caspase-3 positivity was seen at day 3 (1.66%±0.1%) and 6 (1.69±0.15%) of lactation in the $Memo^{fl//fl}$/WAPiCre mice

compared to Memo$^{fl//fl}$ mice (0.03%±0.012% and 0.04%±0.07% respectively, mean ± S.E.M, n=4). A decrease in cleaved caspase-3 positivity was seen at day 10 of lactation in the CKO glands when the surface covered by alveoli has started to significantly decrease. A gradual increase in the number of cleaved caspase-3 positive cells was seen in the control glands.

Stage-specific changes of proliferation rates in the CKO mammary glands after deletion of Memo

The apoptosis detected in the shed cells and in lobuloalveolar structure could result from a proliferation defect. To investigate if there is aberrant proliferation, BrdU was injected intraperitoneally for 2 hours before mice were sacrificed and mammary gland prepared for sections. The ratio of BrdU-positive/total epithelial cells was determined for lactation 3.5, 6.5, 10.5, 12.5 and 15.5 (Figure 6). In the control mammary gland, the proliferation index constantly decreases during lactation. It goes from 2.31% at 3 days of lactation to 0.02% after 15 days of lactation. The proliferation index in the CKO mammary gland follows the one of the control gland, except at 6.5 and 15.5 days of lactation. It reaches a peak of 4.62% proliferation at 6.5 days. This stage-specific change in proliferation rate might represent a reaction of the mammary gland to try to compensate the loss of epithelial cells which undergo apoptosis and are shed into the lumen of alveoli.

Molecular analysis of mammary glands in the absence of Memo

The increased apoptosis seen in Memo$^{fl//fl}$/WAPiCre mammary glands prompted us to investigate some apoptosis regulatory proteins (Figure 7). Stat-3 protein level is increased at the onset of involution. Importantly it is specifically activated at the onset of involution through phosphorylation (Philp et al., 1996) and is necessary for the initiation of apoptosis and involution (Chapman et al., 1999; Humphreys et al., 2002). Western blot analysis of P-Stat3 showed an increase in P-Stat3 in Memo CKO mammary glands during lactation from L3.5 to L10.5.

Bax is an inducer of apoptosis (Adams and Cory, 1998) and KO of Bax delays the first phase of mammary gland involution (Schorr et al., 1999). An increase of Bax levels could therefore contribute to the increased apoptosis seen in

Memo$^{fl//fl}$/WAPiCre mice. Indeed, increased levels of Bax were detected throughout lactation in Memo CKO mammary glands.
P-PKB was undetectable in the mammary gland and P-Erk did not show any consistent levels.

Changes at the adherens junctions in the absence of Memo

To find the cause of apoptosis and presence of shed cells, we checked the integrity of myoepithelial cells by performing immunofluorescence with the smooth muscle actin (SMA) antibody. It revealed no change in the shape and distribution of myoepithelial cells surrounding the luminal epithelium (Figure 8A and B), indicating that knockout of Memo in the luminal epithelial cells does not impair the communication between these two types of cells.
We next looked at adherens junctions by performing immunofluorescence with E-cadherin and ß-catenin. In the control glands, E-cadherin was distributed basolaterally (Figure 8C), consistent with its role at adherens junctions. In the CKO glands however, E-cadherin was relocalized to the cytosol at 3.5 and 6.5 days of lactation (Figure 8D). ß-catenin is associated with E-cadherin at the cell-cell contacts. It was also relocalized from the lateral membrane in the control glands (Figure 8E) to the cytoplasm in the CKO (Figure 8F). At lactation 6.5, ß-catenin showed mislocalization at the apical membrane (not shown). Later at L10.5, no change in the distribution of E-cadherin and ß-catenin was seen (not shown).
We also examined the distribution of the tight-junction protein ZO-1 during lactation, but no major relocalization could be observed (Figure 8G and H).
We investigated the cell-substrate adhesions by performing immmunofluorescence with the α4ß1- (Figure 8I and J) and ß4- (Figure 8K and L) integrin, but no major difference in the contacts between the epithelial cells and the basement membrane was observed.
The relocalization of E-cadherin and ß-catenin could be a sign of epithelial-mesenchymal transition in the luminal epithelial cells. Therefore we performed immunofluorescence with the N-cadherin antibody. During epithelial-mesenchymal transition, N-cadherin is gradually expressed while E-cadherin levels decrease. However N-cadherin was not detected, revealing no sign of epithelial-mesenchymal transition (Figure 8M and N).

3.2.4Discussion

We have generated a conditional knockout of Memo to examine the role of Memo during proliferation, differentiation, apoptosis and involution of the mammary gland. In agreement with previous data using the WAPiCre transgenic strain (Wintermantel et al., 2002), we observed efficient deletion of loxP-flanked sequences in the lactating gland. Memo$^{fl//fl}$/WAPiCre mice were unable to feed their pups correctly throughout lactation. This effect was accompanied with a progressive reduction of mammary gland weight.

The morphology of the mammary gland was severely affected in the conditional knockout gland throughout lactation. It results in the fact that after 2 weeks of lactation, only 20% of the mammary gland is covered by alveoli, whereas 78% of it is covered by alveoli in the control mice. This decrease in alveolar density is explained by the earlier loss during the first week of lactation of luminal epithelial cells which are shed into the lumen, a place normally exclusively reserved for milk.

Apoptosis is revealed by performing immunohistochemistry with cleaved caspase-3 (Marti et al., 2000; Prince et al., 2002). Immunohistochemistry with cleaved caspase-3 to detect late apoptosis revealed that apoptosis was occurring in the lobuloalveolar structures before the cells were shed into the lumen. 1.69% of the cells were positive for cleaved caspase-3 after 6 days of lactation, whereas less than 0.04% was seen in the control glands. It has already been shown that apoptosis occurring in 1%-2% of cells at any time point can result in a 50% reduction of the total cell population over a 48-hour period (Howie et al., 1994).

In agreement with the histological data for apoptosis, molecular alterations in the levels of known regulatory apoptotic proteins could be observed by Western blot. The role of P-Stat3 in apoptosis has been investigated in a number of *in vitro* systems. In myeloid leukemia cells, Stat3 seems to induce apoptosis since its overexpression accelerated interleukin-6 (IL-6) or leukemia inhibitory factor (LIF)-induced apoptosis and a dominant negative blocked apoptosis induced by these cytokines (Minami, 1996). Conversely, in a pro-B cell line Stat3 seems to suppress apoptosis after induction of gp-130 receptor (Fukada et al., 1996). In T cells as well, Stat3 is required for the survival in response to IL-6 (Takeda et al., 1998). In the mammary gland, Stat

3 is specifically activated at the start of involution (Philp et al., 1996) and an impairment of its function reduces apoptosis and delays involution (Chapman et al., 1999; Humphreys et al., 2002). Stat3 is part of the primary axis regulating apoptosis in the mammary gland (Green and Streuli, 2004). In this study, we could show that the deletion of Memo resulted in an increase of P-Stat3 during lactation at levels comparable to the ones of the beginning involuting mammary gland.

Bax is a member of the Bcl-2 family of proteins (Adams and Cory, 1998). It plays a role in apoptosis by either forming pores in the mitochondrial outer membrane or by acting on voltage-dependant activated channels (Cheng et al., 2003). The result is a damage of the outer membrane of mitochondria which causes the release of apoptotic mediators in the cytosol. It has been shown that knockout of Bax delays the first phase of mammary gland involution (Schorr et al., 1999). Here we report an upregulation of Bax in lactating mammary glands from $Memo^{fl//fl}$/WAPiCre mice. This is in agreement with the measured increase of apoptosis in histological sections and with the increased activation of Stat3.

Abnormal proliferation can result in apoptosis in the mammary gland (Lam et al., 2004). Since Memo has been implicating with changes in the microtubule cytoskeleton (Marone et al., 2004) and microtubules are necessary for cell division, a change in the proliferation index at beginning of WAPiCre activation could be the cause of the apoptosis observed in the early lactation time points (3.5 and 6.5). At 3.5 days however, measurement of BrdU incorporation indicates no change between the proliferation index of control compared to CKO glands. There was however a significant increase of proliferation observed after 6.5 days of lactation and as well after 15.5 days of lactation. But since this increase of proliferation in the CKO mammary glands did not happen from the beginning of lactation, when the Wap-Cre is mostly active, and is not maintained throughout lactation, it is unlikely that the CKO of Memo results in abnormal proliferation causing apoptosis. These bursts of proliferation likely are a reaction of the mammary gland to try to compensate the loss of epithelial cells which undergo apoptosis and are shed into the lumen of alveoli. This reaction of proliferation is in vain though, since at the end the epithelial cells are not any more in sufficient quantity to produce enough milk for the pups.

Evidence points to the important role of ß-integrin mediated cell-extracellular matrix adhesion in alveolar integrity (Li et al., 2005; Naylor et al., 2005). However no change in α4β1 integrin or in β4 integrin localization could be observed in the lactating

mammary gland after Memo deletion. Rather, a change in the cellular distribution of E-cadherin and ß-catenin could be observed in cryosections from lactating mammary glands at 3.5 and 6.5 days of lactation. During lactation, milk accumulates into the lumen of the alveoli and proper adhesion between the luminal epithelial cells is required to maintain the integrity of alveoli. E-cadherin mediated cell-cell adhesion affects the epithelial alveolar formation (Delmas et al., 1999). Moreover, it has been shown that E-cadherin gene inactivation under the MMTV promoter in the mammary gland results in massive alveolar apoptosis (Boussadia et al., 2002) and that the mutant mothers are unable to nurse their pups. In-vitro work also supports a critical role for E-cadherin in the function and architecture of the mammary gland (Daniel et al., 1995). It has been suggested that reduced cell-cell adhesion is an early event in the onset of apoptosis (Vallorosi et al., 2000).

Problems at the adherens junction can result in relocalization of tight junction proteins and occasionally canonical adherens junction proteins can be associated with tight junction proteins (Nunes et al., 2006). However we did not see any relocalization of ZO-1 in the Memo conditional knockout mammary gland.

The loss of E-cadherin at the adherens junctions prompted to investigate if N-cadherin was upregulated, a sign of epithelial-mesenchymal transition. But immunofluorescence did not reveal any sign of N-cadherin upregulation.

Here we show that Memo is pivotal for integrity of the alveolar epithelial cells during lactation. The mechanism of action of Memo remains unclear, but it has been shown in vitro to be implicated in cytoskeleton microtubule stability to the periphery of the cell. Knocked-down of Memo has also been associated to altered actin structure (Marone et al., 2004). Immunofluorescence of Memo on cryosections revealed a staining localized close to the membrane.

The calcium-dependant cell adhesion molecule E-cadherin has five extracellular comains and a conserved intra-cellular domain with motifs binding catenins. The resultant complex binds α-catenin and assembles other peripheral cytoplasmic proteins to connect E-cadherin to the actin cytoskeleton. We propose that the downregulation of Memo results in perturbation of the cytoskeleton, which in turn affects the adherens junction of the epithelial cells as seen by improper localization of E-cadherin and ß-catenin. This unability to form proper cell-cell contact then finally leads to apoptosis of the epithelial cells.

Further study will focus on in vivo and in vitro models to further unravel the mechanism of action of and targets of Memo in this process.
The improper localization of E-cadherin and ß-catenin suggests an implication of Memo in the correct formation of adherens junction. Whether this role is direct or indirect through an effect on the cytoskeleton remains to be elucidated.
All these events happening after birth make the mammary gland an organ of choice to study the in vivo role of a newly discovered protein.

3.2.5 Materials and methods

Generation of mice and tissue preparation for analysis

Mice with Memo deleted specifically in the luminal epithelial cells of the mammary gland were generated by crossing mice with two floxed Memo alleles with mice expressing Cre under the control of the WAP milk gene promoter. Mice were maintained in an outbred background. Genotyping was confirmed by tail tipping with the primers forward 5'-CCTGCTAGAGCCATTATTGCACC-3' and reverse 5'-GGATCGAGAAACTTTCATACTACAGC-3'to detect wild-type, heterozygous floxed and homozygous floxed Memo. WAPiCre expression was confirmed with primers 5'-GAAAAGCACCAGGAGAAGTCAC-3' and 5'-GACACAGCATTGGAGTCAGAAG-3'. Adult female mice were mated and following parturition, litters were maintained with 6 pups. Pups were removed after 10 days to initiate involution. The mice were maintained and handled according to the Swiss guidelines for animal safety.
For immunofluorescence, inguinal (fourth) mammary glands were dissected and frozen in optimal cutting temperature compound (OCT, Tissue Tek) for preparing 10um cryosections.
For immunohistochemistry, inguinal (fourth) mammary glands were dissected, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4 then embedded in paraffin for preparing 5um sections.
For BrdU labeling, females were intraperitoneally injected with 100 □g BrdU (Sigma)/g body weight 2 hr prior to sacrifice.
For mammary gland whole mounts, inguinal (fourth) mammary glands were dissected, spread onto a glass slide and fixed overnight in Tellyesnicky's fixative. The

slides were rinsed in water, the tissue was defatted with acetone, hydrated through graded alcohol, and stained with Iron-haematoxylin for at least 1.5 h, then washed in water, dehydrated, and mounted.

Extraction of RNA and RT-PCR analysis of Memo

RNA was prepared by the Trizol method (GiBCO) and purified using the RNAeasy kit (Qiagen). Purified RNA was reverse transcribed and PCR amplified by standard procedures using the specific oligonucleotide primer Fw 5'-CATTCATCCTCGTGCACCATAG-3'in exon 1 and Rw 5'-ACAGGGGCACATGATGGGAA-3' in exon 4 of Memo.

Pup Weight Analysis

The same number of pups (6) was left to each control and CKO mother. The body weight increase was documented for each pup from lactation day 2 to 20. The average body weight of the litters was calculated as means±SD.

Lysate preparation and Western blot analysis

To prepare lysates from mammary glands, the frozen tissue was ground to a powder in liquid nitrogen and homogenized in lysis buffer containing 1% Nonidet P-40, 50mM Tris (pH7.5), 120mM NaCl, 5mM EDTA, 1mM EGTA, 2mM Na-vanadate, 20mM ß-glycerophosphate, 10µM/ml aprotinin, 10µM/ml leupeptin, 0.5mM PMSF, 50mM NaF and 1mM DTT. Cell lysates were prepared in NP40 lysate buffer. Cell lysates were subjected to SDS-PAGE, transferred to PVDF membranes, which were blocked in 10% horse serum (GIBCO) or 5% nonfat milk for 30 minutes and incubated overnight at 4°C with specific antibodies. Membranes were then incubated with the specific secondary antibody (Amersham) coupled to horseradish peroxidase. Signals were detected by enhanced chemiluminescence (ECL; Amersham) and recorded by Kodak LS-OMAT film.

Antibodies used for western analyses were: Memo polyclonal antibody (blocked in 5% milk), □tubulin (Neomarkers), P-Erk (Cell Signalling), Erk (Cell Signalling), P-PKB (Biosource), PKB (Cell Signalling), Bim (Chemicon), Bax (Biosource), P-Tyr705Stat3 (Cell Signalling), Stat3 (Transduction Labs).

Immunohistochemistry (cleaved caspase-3, BrdU)

Immunohistochemistry on paraffin sections was performed with the Ventana Discovery XT system. BrdU antibody (Roche 376 001), was used 1/50 in combination with the MoMap kit (Ventana, 760-137) and sections were deparaffinized and subjected to antigen retrieval for 36 minutes in Tris-EDTA pH 8. Cleaved caspase-3 antibody (Cell Signaling no 9661) was used 1/100 and sections were pretreated 64 minutes in Tris-EDTA pH 8.

Quantification of proliferation, apoptosis and surface covered by alveoli

The number of cells positive for BrdU or cleaved caspase-3 was established in a minimum of 8 randomly chosen fields per mammary gland taken with the 20x objective of the Nikon Eclipse E600 microscope and the Leica DFC 420 camera. All counts were calculated as a percentage of the total cell count which was performed using the Imaris software. Area occupied by alveoli was scored from hematoxylin and eosin-stained slides. The area delimited by alveoli was surrounded manually using the Image Access software. Area was measured by the software and calculated as a percentage of the total area of the field of view. The average of four representative fields was used for each section.

Immunofluorescence

Antibodies used for immunofluorescence on unfixed cryosections, postfixed in acetone-methanol (1:1) were: E-cadherin 1/200 (BD Transduction Laboratories), □̃smooth-muscle actin 1/400 (Sigma, product no A 5228), ZO-1 1/200 (Zymed 61-7300), ß-catenin 1/200 (Transduction Laboratories), α4ß1integrin 1/200 (gift from Prof. Charles Streuli), N-cadherin 1/200 (Transduction Laboratories).

Secondary antibodies were AlexaFluor (all from Molecular Probes). They were diluted 1/400.

3.2.6 Figures

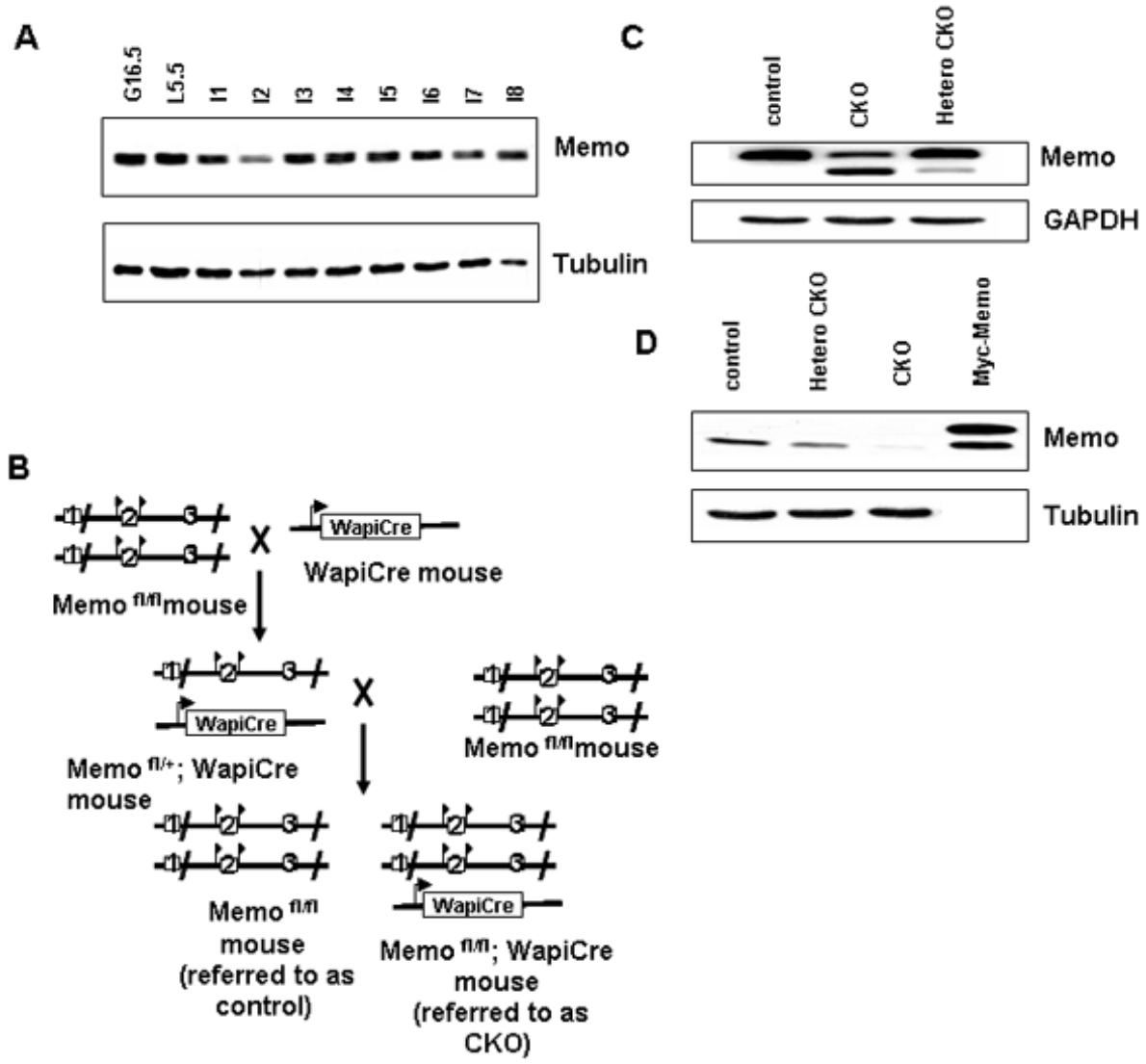
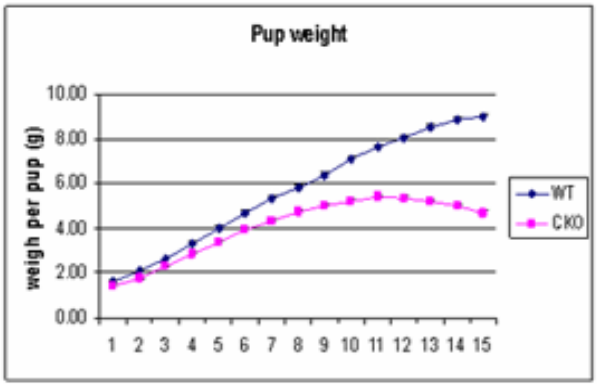
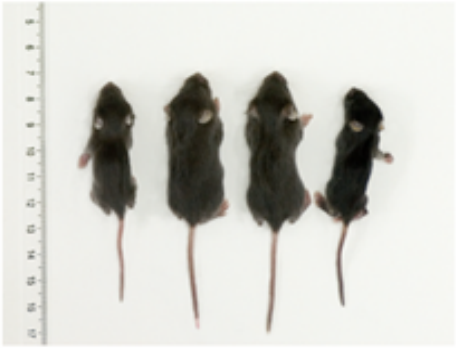


Figure 1

A


Pup weight
weigh per pup (g)
10.00
8.00
6.00
4.00
2.00
0.00
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
WT
CKO

B



C

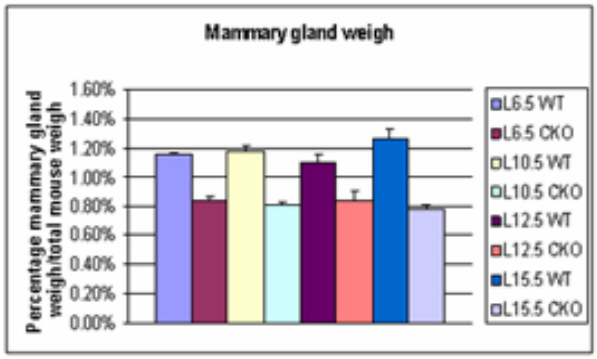

Mammary gland weigh
Percentage mammary gland weigh/total mouse weigh
1.60%
1.40%
1.20%
1.00%
0.80%
0.60%
0.40%
0.20%
0.00%
L6.5 WT
L6.5 CKO
L10.5 WT
L10.5 CKO
L12.5 WT
L12.5 CKO
L15.5 WT
L15.5 CKO

Figure 2

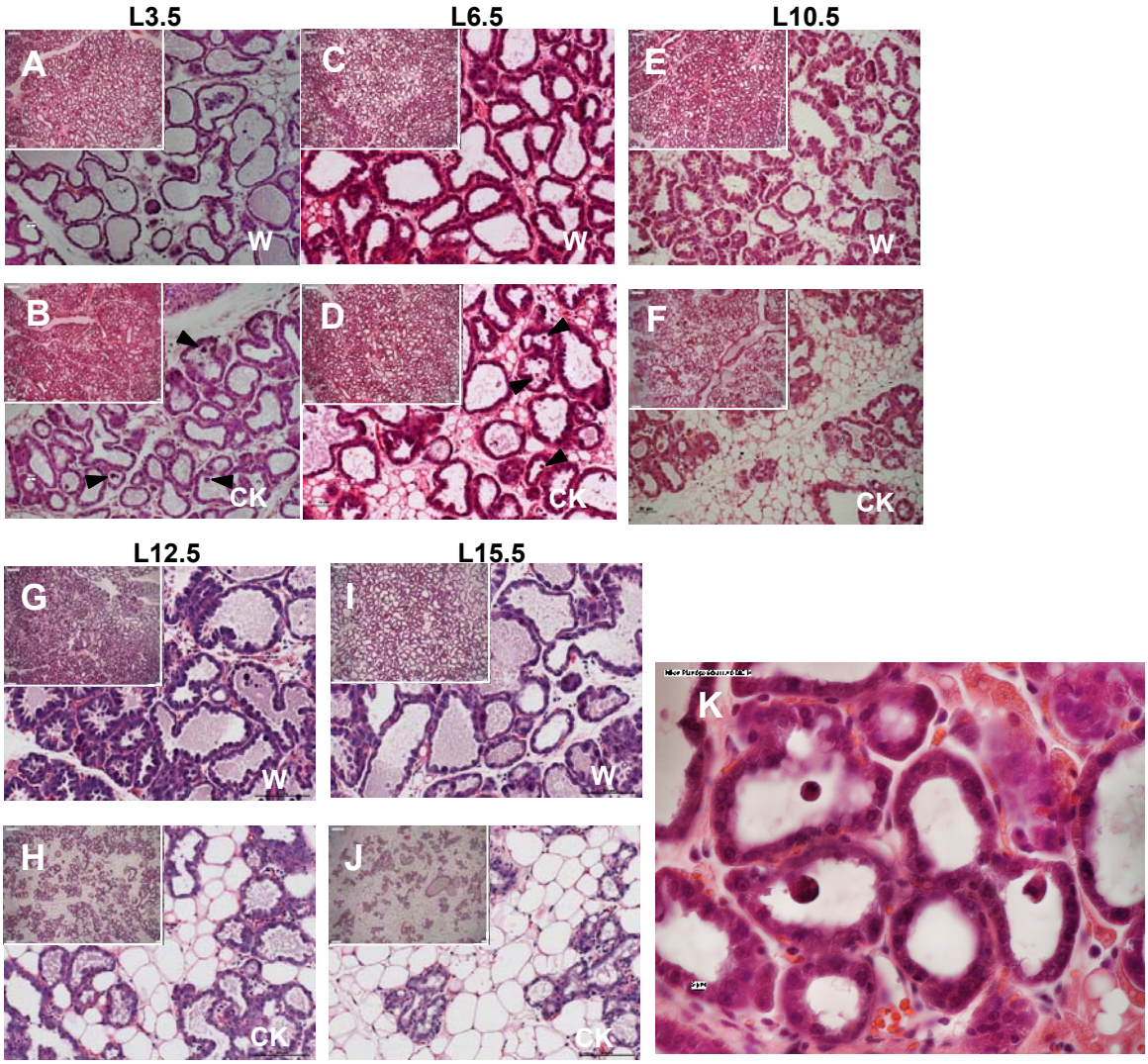


Figure 3

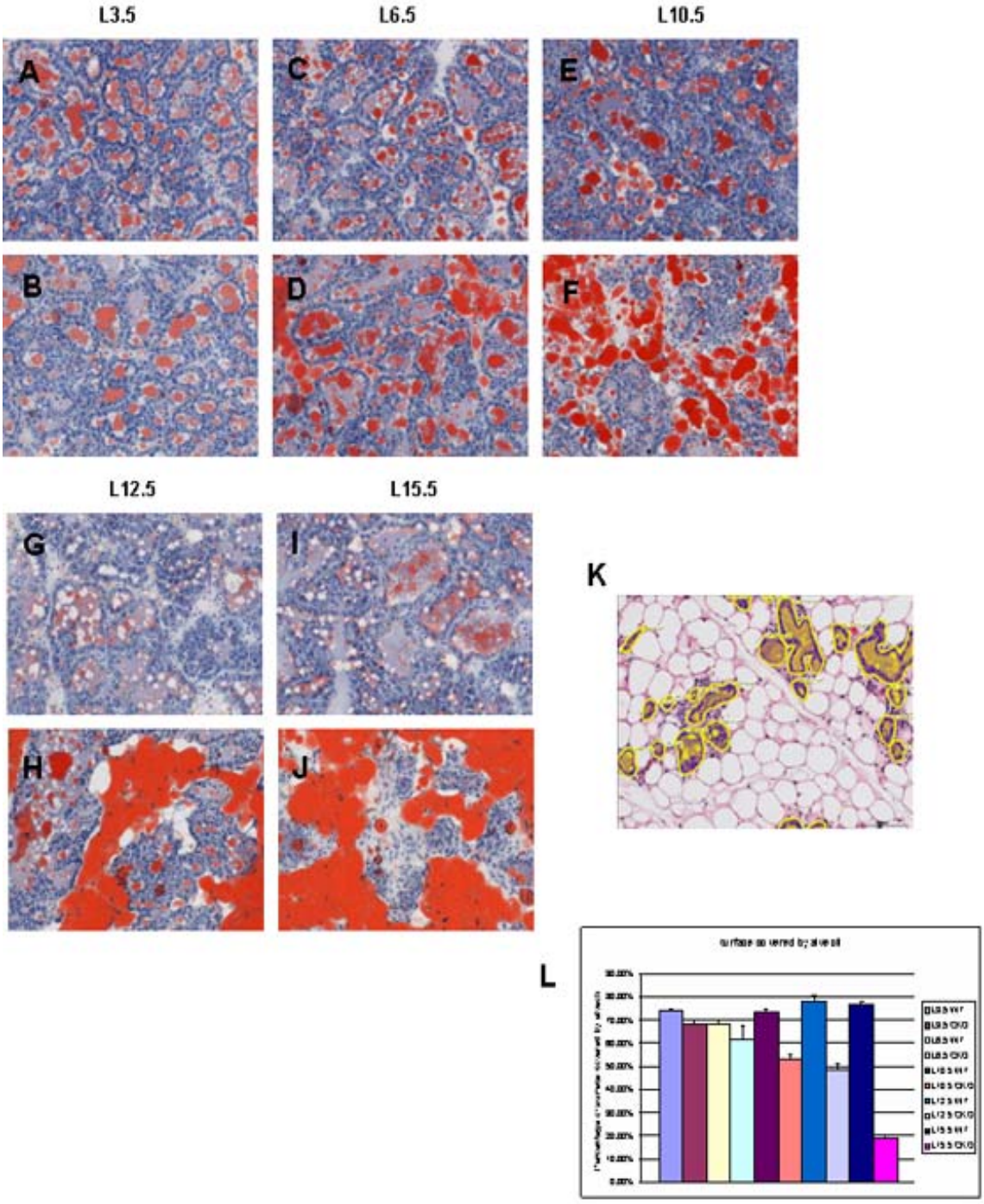
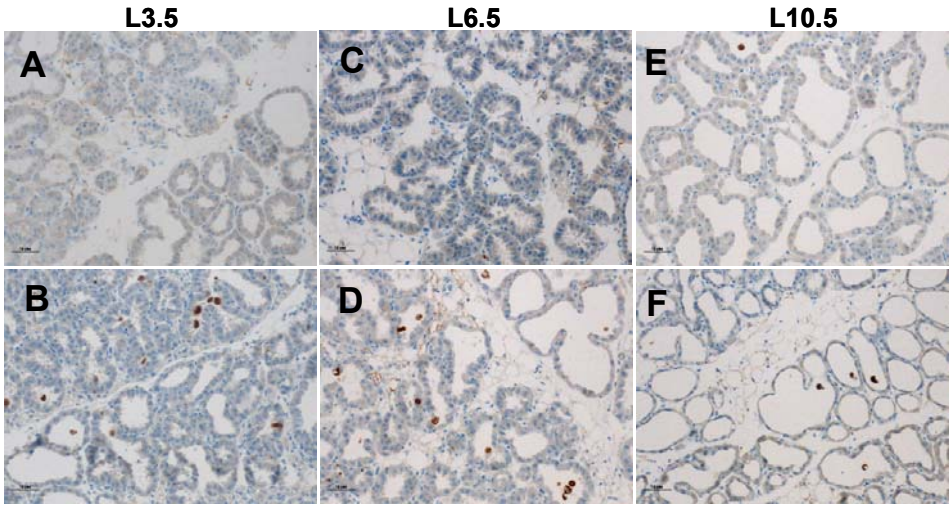
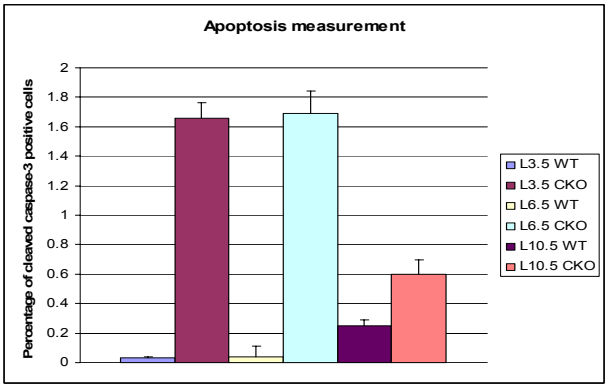


Figure 4


L3.5
L6.5
L10.5
A
C
E
B
D
F

G


Apoptosis measurement
Percentage of cleaved caspase-3 positive cells
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
L3.5 WT
L3.5 CKO
L6.5 WT
L6.5 CKO
L10.5 WT
L10.5 CKO

H

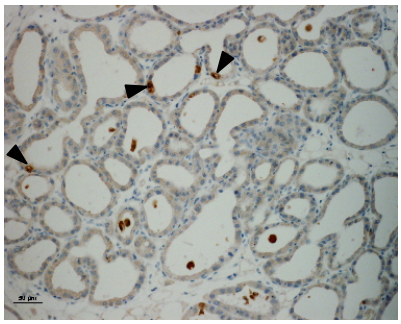
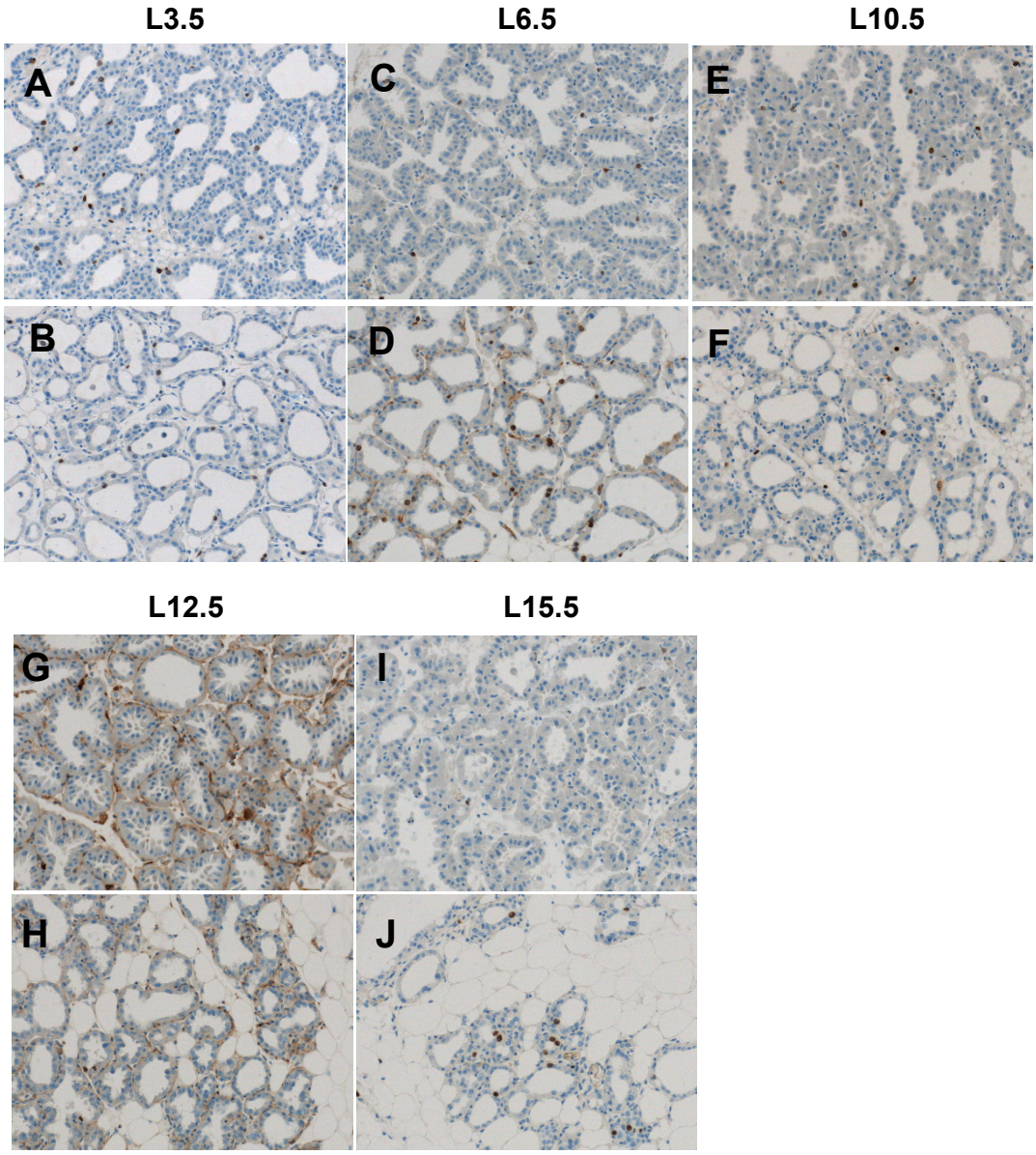


Figure 5


L3.5
L6.5
L10.5
A
C
E
B
D
F
L12.5
L15.5
G
I
H
J

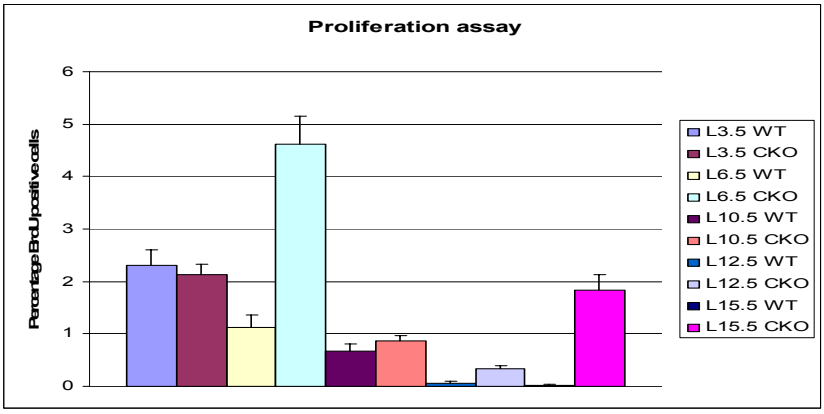

Proliferation assay
Percentage BrdU positive cells
0
1
2
3
4
5
6
L3.5 WT
L3.5 CKO
L6.5 WT
L6.5 CKO
L10.5 WT
L10.5 CKO
L12.5 WT
L12.5 CKO
L15.5 WT
L15.5 CKO

Figure 6

3.2.7 References

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4 Discussion

Memo has been discovered as a protein binding to ErbB2 in breast cancer cells and has been shown to play a role in the *in vitro* cell migration of these cells. This role of Memo in cell migration has been attributed to a function of Memo in the microtubule outgrowth towards the periphery of the cell. Memo also seems to play a role in the actin cytoskeleton, since more actin stress fibers were seen in Memo knock-down cancer cells.

In this report we could show that Memo's expression was neither restricted to cancer cells, nor to migrating cells. Indeed Memo's expression pattern was ubiquitous in adult organs as well as in embryos (figure 6 part I). Memo was not seen stronger in the migrating cells than in other cells.

We also generated a conventional knockout mouse for Memo. This knockout of Memo was lethal, since no living Memo homozygous knockout pup was seen (Table1 part I). The death of the embryos deficient for Memo occurred at around 13 days of embryonic development.
In vitro Memo has been shown to interact with ErbB2. However the phenotypes of ErbB2 and Memo knockout embryos are different. ErbB2 knockout embryos die by E10.75. They show a cardiac and a neuronal phenotype: they show absence of trabeculae in the heart ventricles and reduced size of endocardial cushions. They also have reduced Schwann cell number, abnormal cranial ganglia, hypoplasia of primary sympathetic ganglion chain. Memo embryos die by E13.0-E13.5. Their heart morphology is correct, but they show a vascular defect with hemorrhages. Some neural components also seem affected, since the neural tube is bent in majority of Memo knockout embryos.
Suprisingly, the death of embryos deficient for Memo did not seem to be due to a default of cell migration in the embryo. Indeed, cell migration is required for gastrulation, a process during which some ectodermal cells enter in contact with endodermal cells to give rise to a third germ layer, the mesoderm. Among other cell types, the mesoderm gives rise to blood and blood vessels. In the mouse, the

gastrulation occurs between E6 and E7.5. However Memo knockout embryos die between E13.0 and E13.5 (Table 2 Part I). At this time the complex events of gastrulation have already taken place and this indicates that Memo is not necessary for the migration events to occur at gastrulation. The neural crest is also prone to migration. However Memo knockout embryos contained dorsal root ganglia as well as cranial ganglia, indicating that these derivatives of the neural crest could properly migrate in the absence of Memo. The somitomeres of the head give rise to the branchial arches and musculature of the head whereas somites of the body give rise to ribs and vertebrae. The formation of these derivatives of the somites and somitomeres requires their migration. However Memo knockout embryos don't show any defect in the branchial arch system (Figure 14 K and L, Part I) and ribs and vertebrae are properly formed (Figure 13 C and D, Part I), indicating that the migration of the somites and somitomeres could take place in Memo knockout embryos.

However, we saw some vascular defects in the Memo knockout embryos, since hemorrhages could be observed.

Vasculogenesis is responsible for the formation of the primordia of the major blood vessels as well as of a homogenous capillary network. Formation of the blood islands occurr in the yolk sac at E7.5, when the ectoderm migrates through the primitive streak during gastrulation. But the formation of the dorsal aortae, the endocardium, and the cardinal and vitelline veins is also accomplished by vasculogenesis. Memo knockout embryos did not show any defect in the general pattern of the vasculature (Figure 14 Part I) and the dorsal aorta was properly formed (Figure 13 C and D, Part I). This indicates that the vasculogenesis occurs normally in Memo knockout embryos.

Angiogenesis involves the formation of new vessels from preexisting vessels of the primary vascular plexus. It involves both the proliferation and the migration of endothelial cells at the tips of the angiogenic sprouts. It is responsible for the growth of blood vessels into most developing organs. The intersomitic vessels for example are formed from the dorsal aorta via sprouting angiogenesis. The blood vessels in the head are also formed by angiogenesis. However Memo knockout embryos did not show any defect in the formation of intersomitic vessels, or of vessels of the head. Overall no sign of defective angiogenesis could be observed in Memo knockout embryos.

After remodeling of the vascular plexus, mesenchymal cells are recruited to give mechanical and physiological support to the endothelium. Pericytes are recruited to the small capillaries, and smooth muscle cells and adventitial fibroblasts are recruited to larger vessel to form their vascular wall. It is known that the smooth muscle cells migrate from ventral to dorsal when they surround the aorta. In Memo knockout embryos, the smooth muscle cells were properly recruited around the aorta though (Figure 16, Part I).
Hence, the hemorrhages observed in the Memo knockout embryos don't seem to originate from a misformation of the vascular plexus or from impairment in its stability. It is possible that they originate from a defective permeability between the endothelial cells. It is also possible that the cell-cell adhesion between endothelial cells is defective.

We also generated a conditional knockout (CKO) mouse where Memo disruption was restricted to the mammary luminal epithelium. We observed efficient deletion of Memo in the lactating gland (Figure 1 C and D, Part II). CKO mice were unable to feed their pups correctly throughout lactation as indicated by pup weight reduction (Figure 2 A, Part II). We saw severe defects in the morphology of the mammary gland in the conditional knockout gland throughout lactation. We observed a loss of luminal epithelial cells which are shed in the lumen (Figure 3 K, Pat II). This led to a progressive decrease in alveolar density (Figure 4L, Part II). Indeed, the epithelium is almost completely absent after two weeks of lactation, and the mammary gland becomes mostly filled with adipocytes. This explains why the pups are not correctly fed. Through immunohistochemistry with cleaved caspase-3 to detect late apoptosis we could see that apoptosis was occurring in the lobuloalveolar structures before the cells were shed into the lumen (Figure 5 H, Part II). In agreement with the histological data for apoptosis, we observed molecular alterations in the levels of known regulatory apoptotic proteins by Western blot. We observed an increase of P-Stat3 and of Bax levels during lactation in the Memo CKO as compared to the control glands (Figure 7, Part II).

We observed a change in the cellular distribution of E-cadherin and ß-catenin in cryosections from lactating mammary glands at 3.5 and 6.5 days of lactation (Figure 8 C,D,E,F, Part II). E-cadherin and ß-catenin are part of the adherens junctions,

which maintain cell-cell adhesion. During lactation, milk accumulates into the lumen of the alveoli and proper adhesion between the luminal epithelial cells is required to maintain the integrity of alveoli. It is probable that the shedding of cells in the lumen of alveoli is due to improper cell-cell adhesion in the Memo deficient mammary glands.

It is probable that the observed apoptosis is a consequence of improper adhesion between the epithelial cells of the mammary gland.
It is also possible that the apoptosis causes the mislocalization of E-cadherin and ß-catenin. However, other proteins such as ZO-1 seemed to be properly localized (Figure 8 G and H, Part II). Moreover, the mislocalzation of E-cadherin and ß-catenin was present in most of the cells of the mammary gland, whereas the apoptosis occurred in about 1.7% of the mammary gland. Even if this rate is high for apoptosis, it suggests that improper localization of E-cadherin and ß-catenin occurs before the cells undergo apoptosis.

The hypothesis of improper cell-cell adhesion is interesting. It could not only explain the phenotype of the shed cells in the mammary gland, but as well the hemorrhages observed in the knockout Memo embryos. The bending of the neural tube observed in the Memo knockout embryos could also be due to improper cell-cell adhesion. Indeed, adhesion is necessary for closure of the neural tube. Interestingly, both the epithelial cells of the mammary alveoli and the endothelial cells of the blood vessels are submitted to pressure, by milk and blood respectively. This pressure might be sufficient to reveal phenotypes due to a fragile adhesion between the cells knockout for Memo.

5 Acknowledgements

I would like to thank…

…Prof. Nancy Hynes, my supervisor, for giving me the opportunity to work in her international lab and support me to finish the thesis. She was very helpful in reviewing this manuscript, and throughout my Ph.D. studies she gave me the opportunity to lead a project and aquire independence.

…Prof. Patrick Matthias and Prof. Markus Affolter, the other two members of my thesis committee for the interest they took in my work and for the nice advices they gave me during the committee meetings concerning the orientation of the project.

…Dr. Régis Masson for his great technical support and for the lively atmosphere. Working with him really allowed me to learn molecular biology and to get into the benchwork.

...Dr. Sandrine Bichet for her expertise in histology and for creating a nice facility, as well as for her support.

…Dr. Patrick Schwarb and the members of the imaging facility for their competence and their disponibility to help.

…the members of the transgenic facility (Jean-François Spetz, Patrick Kopp, Bernard Kuchemann) for their nice contribution in generating the mice.

…the members of the mouse monoclonal facility (Dr. Susan Schenk, Michel Siegmann) for production of the monoclonal antibody.

… Dr. Thomas Schlange for his help with the monoclonal antibody purification and for his realism.

…Dr. David Cappellen and especially Dr. Ali Badache for their help concerning the directions of the project at its beginning.

…all my collegues in the lab, including former colleagues and Francie, for the very nice atmosphere. Thank you also to other members of FMI who helped me to make life more enjoyable.

…my family: many thanks to my parents and my sister who supported me during my Ph.D.