Genetic modeling of the Hippo pathway in hematopoietic stem cells

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2012

GENETIC MODELING OF THE HIPPO PATHWAY IN HEMATOPOIETIC STEM CELLS

LUNDS UNIVERSITET

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ISBN 978-91-8687-85-7

ISSN 1652-8220

Lund University, Faculty of Medicine Doctoral Dissertation Series 2012:23

Printed by: Media-Tryck, Lund 2012

Good tests kill flawed theories;
we remain alive to guess again

- *Karl Popper*
(1902-1994)

ABSTRACT

Hematopoiesis is the process of blood formation from a limited pool of hematopoietic stem cells (HSCs). These rare stem cells can both self-renew to maintain the HSC pool, and differentiate to continuously replenish lost blood cells. The mechanisms of HSC regulation are not fully known. The aim of this thesis was to study the role of the Hippo signaling pathway in HSCs. The Hippo pathway is a newly discovered signaling pathway, which regulates organ size in *Drosophila*. Hippo signaling has further been implicated in regulation of mammalian stem cells. In Article I we developed a new way of modeling genetic changes by combining genetic engineering of murine ES cells with blastocyst complementation. This approach avoids the cost and time constraints associated with the creation of standard transgenic mouse strains while taking advantage of the sophisticated site-directed manipulations that are possible in ES cells. In Article II we studied YAP1, the downstream effector in the Hippo pathway. We created a transgenic model with inducible YAP1 expression exclusively within the hematopoietic system using the blastocyst complementation approach developed in article I. When investigating the effect of overexpressing YAP1 in HSCs we detected no effect on HSC function during steady state or regenerative stress. This is contrast to effects seen in other tissue stem cells and suggests tissue specific functions of YAP1 in regulation of stem cells. In Article III we investigated a knockout model for the other Hippo effector Taz. Adult mice deficient in Taz display no changes in hematopoietic parameters but are born below mendelian ratios. Taz thus seems dispensable for adult hematopoiesis but may influence embryonic development.

Taken together, using both novel and traditional genetic engineering approaches in mice, we have taken the first steps to understand the role of the Hippo pathway in hematopoiesis.

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ABBREVIATIONS

AGM	-	Aorta-Gonad-Mesonephros
ALL	-	Acute Lymphoblastic Leukemia
aPKC	-	atypical Protein Kinase C
CFU-S	-	Colony Forming Unit Spleen
CLP	-	Common Lymphoid Progenitor
CMP	-	Common Myeloid Progenitor
Crb	-	Crumbs
Dco	-	Discs overgrown
dHCS	-	definitive Hematopoietic Stem Cell
Dlg	-	Discs large
Dpp	-	Decapentaplegic
Ds	-	Dachsous
Ex	-	Expanded
EX.Y	-	Embryonic day X.Y
EC cells	-	Embryonal Carcinoma cells
ES cells	-	Embryonic Stem cells
FGF	-	Fibroblast Growth Factor
Fj	-	Four-jointed
FL	-	Fetal Liver

GMP	-	Granulocyte Macrophage Progenitor
Hpo	-	Hippo
HSC	-	Hematopoietic Stem Cell
HSPC	-	Hematopoietic Stem and Progenitor Cell
ICM	-	Inner Cell Mass
iPS	-	induced Pluripotent Stem cell
Kbr	-	Kibra
KO	-	Knock Out
LATS	-	Large Tumor Suppressor
Lgl	-	Lethal giant larvae
MEP	-	Megakaryocyte Erythrocyte Progenitor
Mer	-	Merlin
MST	-	Mammalian Ste20-like kinase
MSC	-	Mesenchymal Stem Cell
NF2	-	Neurofibromatosis 2
NSC	-	Neural Stem Cell
RNAi	-	Ribo Nucleic Acid interference
ROS	-	Reactive Oxygen Species
Sav	-	Salvador
SCF	-	Stem Cell Factor
Scrib	-	Scribble
siRNAs	-	small interfering RNAs
TEAD	-	TEA Domain family of transcription factors
TGF-β	-	Transforming Growth Factor β

TPO	-	Thrombopoietin
YAP1	-	Yes-associated protein 1
Yki	-	Yorkie
Wg	-	Wingless
Wts	-	Warts
Wwtr1	-	WW domain Transcription factor 1

BACKGROUND

STEM CELLS

Definition and concepts

Stem cells are unspecialized cells present in all multicellular organisms set apart by their potential for generating multiple other specialized cell types. The criteria used to define stem cells are self-renewal, i.e. the indefinite production of a daughter cell with the same potential, and maturation into specialized cells. In the strictest sense of the definition the stem cells of the early embryo are able to give rise to all tissues in an organism including all extra-embryonic tissues and is therefore said to be totipotent from the latin word "***totus***" meaning "*whole*" (Cauffman et al., 2009; Pan et al., 2002; Rossant, 1976; Suwinska et al., 2008; Tarkowski and Rossant, 1976; Tarkowski and Wroblewska, 1967).

In practical terms the definition of a stem cell is represented by its functional abilities; the ability to rescue an organism deficient in stem cells by self-renewing and generating daughter cells throughout a lifetime. The idea of the *stemness* of a *cell* as a deterministic characteristic was turned on its head with the advent of the cloning of Dolly the sheep (Estrov, 2009). Removing the haploid DNA from an egg, and replacing it with the genetic material from a mature differentiated cell resulted in the birth of Dolly, healthy and normal (Campbell et al., 1996). This raises the question of what actually confers potency to a cell and how permanent the presence or absence of stemness in a cell can be. Despite Dolly not being the only example of the plasticity of cell potency (we will discuss other examples

later) the basic definition of a stem cell, self-renewal and differentiation, remains the same.

Pluripotency - Embryonic stem cells

After just a few divisions of the initial zygote, totipotency is lost. At the blastocyst stage, cells in the embryo located in the inner cell mass (**ICM**) are now specialized to give rise to embryonic tissues, whereas the hypoblast cells of the primitive extra-embryonic endoderm and the outside cells of the trophectoderm give rise to extra-embryonic tissues such as the yolk sac and the placenta (Brinster, 1974b; Chen et al., 2010; Senner and Hemberger, 2010; Tarkowski et al., 2010).

Since the cells of the inner cell mass can give rise to all the tissues of the embryo and the adult organism, they are considered pluripotent stem cells, from the latin word "***plures***" meaning "*several*". Cells from the ICM are today one of our most important tools we have for studying genetics at the organism level in mammals (Bockamp et al., 2002; Clarke, 2000).

During the 50s' and 60s' some researchers were exploring the potential of teratocarcinomas, serially transplantable tumors, in mice. Scientists at the time had noticed that there existed mouse strains with a high incidence of spontaneous testicular carcinomas, which were shown to be derived from the primordial germ cells in the testes. Furthermore, transplantation of cells isolated from such tumors produced teratomas. The teratomas contained not only differentiated cells but also undifferentiated cells that highly resembled the cells isolated from the original tumor and used in the first transplantation (Stevens and Little, 1954). It was further demonstrated that a *single* cell from a teratocarcinoma tumor, ectopically transplanted, could actually give rise to an array of different specialized tissues representing all germ layers, and these pluripotent cells were therefore aptly named embryonal carcinoma (**EC**) cells (Kleinsmith and Pierce, 1964).

Early on, researchers working in the field understood the inherent potential of being able to propagate EC cells in *in vitro* culture systems and a lot of

work was undertaken to map out factors governing the differentiation and pluripotency of these cells (Evans, 1972; Kahan and Ephrussi, 1970; Pierce and Dixon, 1959; Rosenthal et al., 1970). It was observed that EC cells in culture during the early stages of differentiation formed embryoid bodies with an outer covering of primary extra-embryonic endoderm, similar to that of an isolated ICM. With the comprehension that the differentiation pattern of EC cells actually mirrored that of normal embryonic development, and the additional findings that incorporation of EC cells into a blastocyst could contribute to chimera formation, came the realization that teratocarcinomas, although in principle malignant, can contain normal, non-cancerous, cells as well (Brinster, 1974a; Mintz and Illmensee, 1975; Papaioannou et al., 1975; Rossant, 1975). This also lead to the understanding that it was possible to isolate cells with the same *in vivo* potential as EC cells without having to go through the tumor stage (Evans and Kaufman, 1981). Cells isolated directly from the embryo and propagated in culture were termed embryonic stem (**ES**) cells to emphasize their normal embryonic origin and behavior (Martin, 1981).

ES cells are exploited today primarily in two ways in research. First, the directed differentiation of ES cells in culture serves both as a model system for gathering information on early embryonic development and cellular differentiation, and as a potential future source of material for cell replacement therapy (Odorico et al., 2001; Polak and Bishop, 2006). Specification to each tissue type presents with its own set of challenges to be able to generate functional mature cells. Additionally, all differentiation protocols share the common issue of obtaining a *pure* cell pool of differentiated cells for transplantation to be able to circumvent teratoma formation (Findikli et al., 2006; Heng et al., 2005; Hwang et al., 2006; Kaufman et al., 2001; Zhang et al., 2001). Second, since ES cells injected into blastocysts can contribute to germline chimerism, any genetic modifications that are introduced in the ES cells will also be present in the progeny and can be transmitted from one generation to another. Because of the ease of culture and genetic manipulation, murine ES cells have become the primary choice to establish models for studying genetic changes. The

many advances in genetic engineering of ES cells have paved the way for quicker and more sophisticated transgenic mouse models.

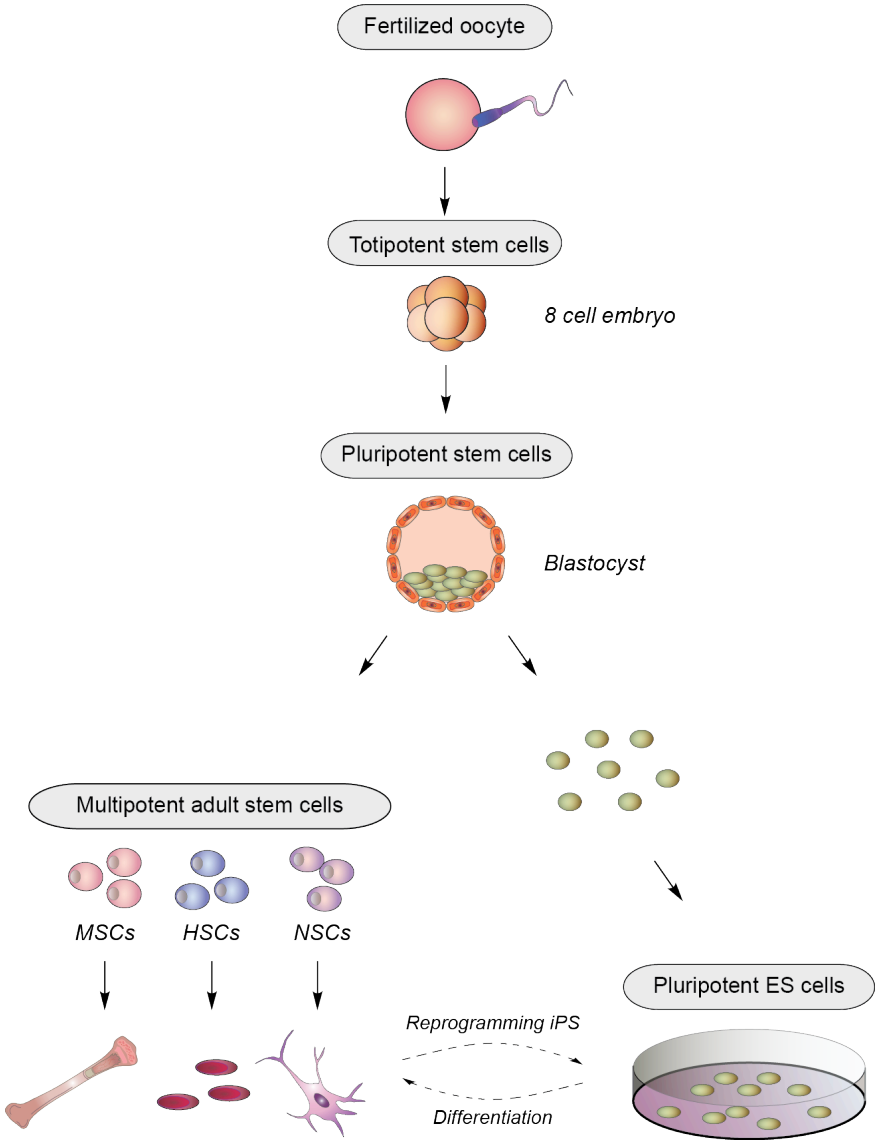


Figure 1. The stem cell potency ladder.

Multipotency - Adult stem cells

Further down the potency ladder are the stem cells that can give rise to several, but not all, cell types in an organism. The adult stem cell is sometimes also referred to as "*tissue stem cell*" or "*somatic stem cell*", from the greek word "***soma***" meaning "*of the body*". Adult stem cells are capable of self-renewal, same as stem cells of embryonic origin, but are more restricted in their capacity for producing specialized differentiated cells. They are usually limited to producing the cells of a particular tissue in the organism and are therefore referred to as multipotent from the Latin "***multus***" meaning "*many*" (Panchision, 2011).

Although adult stem cells all fulfill the basic criteria for stem cells they can differ a lot in terms of cellular behavior. Some are more or less regularly dividing to replenish cells in the tissue whilst others only divide under special conditions of cellular insult or tissue damage as a means of repair. For instance the epithelial stem cells located in the intestinal crypts and the epithelial stem cells in the skin are among the fastest dividing stem cells, continuously replacing cells in those tissues, since their barrier function necessitates a high cell turnover (Simons and Clevers, 2011; Tumbar, 2012). In contrast, stem cells in the liver and the pancreas are thought to only divide when an insult to the tissue triggers a repair response (Alison, 1998; Alison et al., 1998; Bonner-Weir and Sharma, 2002; Overturf et al., 1997).

Some dispute over the existence of true stem cells in different adult tissues still persists. For example the stem cell of the pancreas is hotly debated. Still, today stem cells or stem cell-like cells are thought to exist in the brain, bone, retina of the eye, hair follicle, muscles and several other tissues (Aubin, 1998; Coles et al., 2004; Cotsarelis et al., 1990; Dor et al., 2004; Gage, 2002; Goldring et al., 2002; Mauro, 1961; Pittenger et al., 1999; Reynolds and Weiss, 1992; Taupin and Gage, 2002; Tropepe et al., 2000; van Praag et al., 2002; Xu et al., 2008).

The disagreement on what cells are actually stem cells in adults is mainly due to limitations in the assays used to make sure the proposed cell meet the criteria for self-renewal and differentiation. In addition, the difficulty with assessing stem cell properties has been further complicated with the discovery that a certain degree of plasticity exists in some stem cells *in vivo*. Cells, previously described as differentiating into progressively more and more committed cells of a specific lineage have been revealed to, under special circumstances, cross tissue boundaries and differentiate to other lineages, so-called "*trans-differentiation*" (Gruh and Martin, 2009; Poulsom et al., 2002; Rovo and Gratwohl, 2008).

The controversy of potency and plasticity of stem cells was further augmented in 2006, when the Japanese scientist Yamanaka published his seminal paper on dedifferentiation of fibroblasts using a set of transcription factors to reprogram the cells into a cellular state that resembles that of the embryonic stem cell (Takahashi and Yamanaka, 2006). These cells, termed induced pluripotent stem (**iPS**) cells, have now been shown to have chimeric germline potential and today represent a massively expanding research field. iPS cells have been generated from differentiated cells from a wide spectrum of tissues and from several different species including humans(Loh et al., 2009; Lowry et al., 2008; Nakagawa et al., 2008; Okita et al., 2007; Ruiz et al., 2010; Tiscornia et al., 2011).

With regard to research and clinical applications, adult stem cells and iPS cells are a lot less controversial than their embryonic counterpart. Since adult stem cells and iPS cells in many cases can be collected from the adult source, without harm, less ethical objections are raised. However, this is not the case for all adult stem cell types; the neuronal stem cell for instance is not easily harvested without the use of invasive procedures (Haas et al., 2005; Liao et al., 2011; Prelle et al., 2002).

Finally, on the ladder of stem cell potency, there are also the oligo- and unipotent stem cells capable of giving rise to just a few or only one type of cells. The male germ stem cells giving rise to sperm is an example of a unipotent stem cell. The use of the word stem cell can vary quite a bit and

sometimes what would be described as a progenitor in one tissue is defined as a stem cell in another. In conclusion, although the theoretical definition of a stem cell has remained intact, the functional definition is more mottled today.

Cues governing stem cells – the niche

Stem cells generally represent only a very small portion of the cells of the tissue in which they reside (Morrison and Weissman, 1994; Tegelenbosch and de Rooij, 1993). This is linked to the need to protect these cells from genetic damage and also makes it easier to safeguard them from physical insult within the tissue. However, this low frequency is compensated by the enormous proliferation potential exhibited by the stem cells themselves and their direct progeny. In an obvious paradox, this ability is also what makes stem cells a potential danger. If stem cells were allowed to proliferate in an unrestricted manner they would cause disorganization of tissues and organs, reminiscent of the neoplastic invasive growth of tumors. It is therefore reasonable to assume that there exists an external, as well as an internal, way of regulating stem cell numbers and differentiation (Li and Xie, 2005; Lin, 2002).

The stem cell niche is a term that loosely signifies a microenvironment that stem cells reside in and that influences the stem cell fate choice. The theory of the stem cells niche has been around for decades but the first niche that was actually experimentally demonstrated was the one found in the *Drosophila* gonads. Stem cells in the gonads interact with the cap cells in the ovary and the hub cells in the testis. In fact these niche cells directly regulate stem cell numbers and behavior (Kiger et al., 2000; Spradling et al., 2001; Tran et al., 2000; Xie and Spradling, 2000).

Although the gonad niches are probably the most investigated, similar concepts are defined for a majority of stem cells, including those of the skin, intestine and the brain. Because the niche is usually located in a site that is relatively sheltered in those tissues, such as the bulge of the hair

follicle in the skin, it is also protected from external influences and tissue insults (Fuchs et al., 2001; Shen et al., 2004; Takeda et al., 2011).

Stem cell fitness and competition

The Darwinian concept of fitness and evolutionary competition has also been suggested to apply to cells in a multicellular organism. With the discovery of secreted growth factors it was hypothesized that cells compete for limiting space and nutrients in the niche (Purves, 1980; Raff, 1992; Ramón y Cajal, 1929).

In the *Drosophila* ovary, the cell fate of the stem cell daughter cells is determined by the relative position to the niche cells. If the progeny cell is adjacent to the cap cell it retains stem cell properties. But if the daughter cell has no direct contact with the niche cell, it instead becomes a differentiated cytoblast and moves away from the cap cells of the niche. Under normal circumstances this is however a passive, non-competitive process where intrinsic differences in cell fitness are never of consequence (Li and Xie, 2005). Another example of passive cell fate regulation is the self-sacrifice of cells in the *Caenorhabditis elegans* where cell autonomous programmed cell death occurs during development (Yuan and Horvitz, 1990).

Active competition for survival however, requires a measure of the fitness of a cell and a way of sensing the fitness of adjacent cells. In *Drosophila* mosaics it was shown that when cells of two different metabolic rates were mixed, cells with the reduced metabolic rate disappeared completely in the presence of cells with a normal metabolism. This was surprising since the cells with a reduced metabolic rate are viable on their own. These flies are the so-called *Minutes* mutants, with changes in different ribosomal genes leading to altered rate of protein synthesis. Homozygous *Drosophila Minutes* are lethal but heterozygotes are viable and normal, although with a longer developmental period. However, in wing mosaics with both wildtype and heterozygous cells, wildtype cells detect the decreased fitness of

Minute cells and actively kill the mutant cells by sending a signal to trigger apoptosis (Morata and Ripoll, 1975; Moreno et al., 2002; Simpson and Morata, 1981). A similar process with actively competing cells is believed to exist in mammalian development (Oliver et al., 2004).

Not only cells with reduced fitness can induce competition. Genetic mutations exist that can transform cells into super-competitors. When they are mixed with wildtype cells, super-competitors outcompete the normal cells. The *Myc* and *Hippo* family of genes can acquire mutations that can transform cells into super-competitors. Recently, the presence of active cell-cell competition between stem cells in their niche has been demonstrated in the *Drosophila* ovary. Intriguingly, the process of stem cell competition in the niche seems to trigger differentiation of the loosing cell rather than apoptosis (de la Cova et al., 2004; Jin et al., 2008; Johnston et al., 1999; Moreno and Basler, 2004; Rhiner et al., 2009; Tyler et al., 2007; Zhao and Xi, 2010). It is appealing to consider a possible relevance for active cell competition in adult mammalian stem cell niches, such as the one in the hematopoietic system (Domen, 2001; Gaudin et al., 2004).

HEMATOPOIETIC STEM CELLS

While a lot attention these days is given to the therapeutic potential of ES and iPS cells, the stem cell of the blood system, the hematopoietic stem cell (**HSC**), has already been used in treatment of disease for several decades. It is also by far the most explored and well defined of the adult stem cells.

Hematopoiesis

The process wherein the cells of the blood and immune system are continuously replenished is termed hematopoiesis. The word derives from the Greek word "***haima***" meaning "*blood*" and "**poieō**" meaning "*to make*". Under homeostatic conditions 10^{11}-10^{12} new cells are formed each day in an adult human and under situations of stress the output is even more numerous (Ogawa, 1993).

The function of the blood and immune system

Hematopoiesis generates several distinct cell types that are responsible for the function of the blood and immune system. The most abundant cell is the *red cell*, or erythrocyte, which is responsible for transporting oxygen from the lungs to all tissues in the body. Erythrocyte production responds to low levels of oxygen, e.g. due to loss of blood, chronic anemia or other situations of stress (Hattangadi et al., 2011). *Platelets*, or thrombocytes, are produced from megakaryocytes and respond to hemorrhage or inflammation by helping with clotting. All other cells produced in hematopoiesis are said to be *white blood cells* and are part of the immune system (Widmaier et al., 2004).

The immune system is generally divided into one innate and one acquired portion. Innate immunity, in the broadest sense, consists of all the elements that we are born with and that can be used for fighting against challenges

from foreign invaders. This includes the skin and mucous membranes as well as for example the cough reflex. The hematopoietic contribution to innate immunity is phagocytic cells including the *granulocytes*, *macrophages*, and *dendritic* cells. Phagocytic cells recognize foreign particles, such as bacteria, and ingest and digest them. *Natural-killer cells* instead use cell-cell contact to distinguish non-self cells and kill them by releasing various cytotoxic molecules (Aderem, 2003; Allen and Aderem, 1996).

Acquired immunity is more specialized than innate immunity and as such acts as a second line of defense. From an evolutionary perspective it is a rather late development, present only in vertebrates. *Lymphocytes* are the essential cells in this specific immune defense. The adaptive response of the *B* and *T-lymphocytes* is slower than that of the innate response and it can take up to a week for the clonal expansion necessary to occur, before *effector cells* can start eliminating an infection. On the other hand the *memory cells* can, after that first contact with a foreign substance, persist for the whole lifetime and prevent reinfection (Coico et al., 2003).

Discovery of the hematopoietic stem cell

Most people are familiar with the culmination of the World War II on the eastern front in Asia – the nuclear bomb. After the ending of the war the threat of a nuclear war spurred interest in radiation protection research and it was noticed early on that the bone marrow was particularly sensitive to irradiation. Early attempts in the 50's to cure victims of radiation injury with bone marrow transplantation failed because the cells were recognized as foreign and rejected. When researchers learned to tackle the problem of the allogeneic nature of the transplants faster progress was made and in 1968 the first successful bone marrow transplant was performed (Congdon, 1962; Ford et al., 1956; Gatti et al., 1968; Main and Prehn, 1955).

This strong relevance of radiation research also fueled an interest in the understanding of blood formation and progressed the idea that one cell was

the origin of all blood cells, the blood stem cell. In their seminal papers James Till and Ernest McCulloch first demonstrated in mice the multi-lineage potential of injected bone marrow and then later the self-renewal capacity of secondary transplants. In the first paper published in 1961 (Till and Mc, 1961) they showed that the spleens of irradiated mice transplanted with bone marrow contained colonies consisting of cells from multiple blood lineages (colony forming units-spleen, (**CFU-S**). In the research published two years later (Becker et al., 1963; Siminovitch et al., 1963) they demonstrated that upon re-injection, such colony forming spleen cells resulted in new spleen colonies and each colony was formed from one cell in a clonal manner. So, somewhat ironically, one of the greatest fears of the 20th century was also the fuel for a field of research that may in the future solve some of the toughest medical challenges of cancer, tissue repair and aging.

The hematopoietic hierarchy

The cell that Till and McCulloch discovered was of course not what we today consider a proper HSC but rather something approaching a multipotent progenitor cell (Baines et al., 1982; Jones et al., 1990; Magli et al., 1982). The true HSC sits at the top of the hematopoietic hierarchy and has the potential to sustain hematopoiesis throughout a lifetime. Functionally this is defined by the ability to reconstitute an ablated hematopoietic system in several sequential transplantations (Harrison et al., 1978). Using retroviral integration analysis to trace the clonality of transplanted bone marrow cells, later work has delivered more robust support for HSC self-renewal and single-cell transplants generating long-term multi-lineage reconstitution provided the definitive evidence (Dick et al., 1985; Jordan and Lemischka, 1990; Keller and Snodgrass, 1990; Osawa et al., 1996).

Between the HSC and the mature blood cells are a series of more restricted progenitors. Early on there is a division between a common myeloid progenitor (**CMP**) and a common lymphoid progenitor (**CLP**) and later

also a separation of the CMP into a bipotent megakaryocyte erythrocyte progenitior (**MEP**) and a granulocyte macrophage progenitor (**GMP**) (Orkin, 2000).

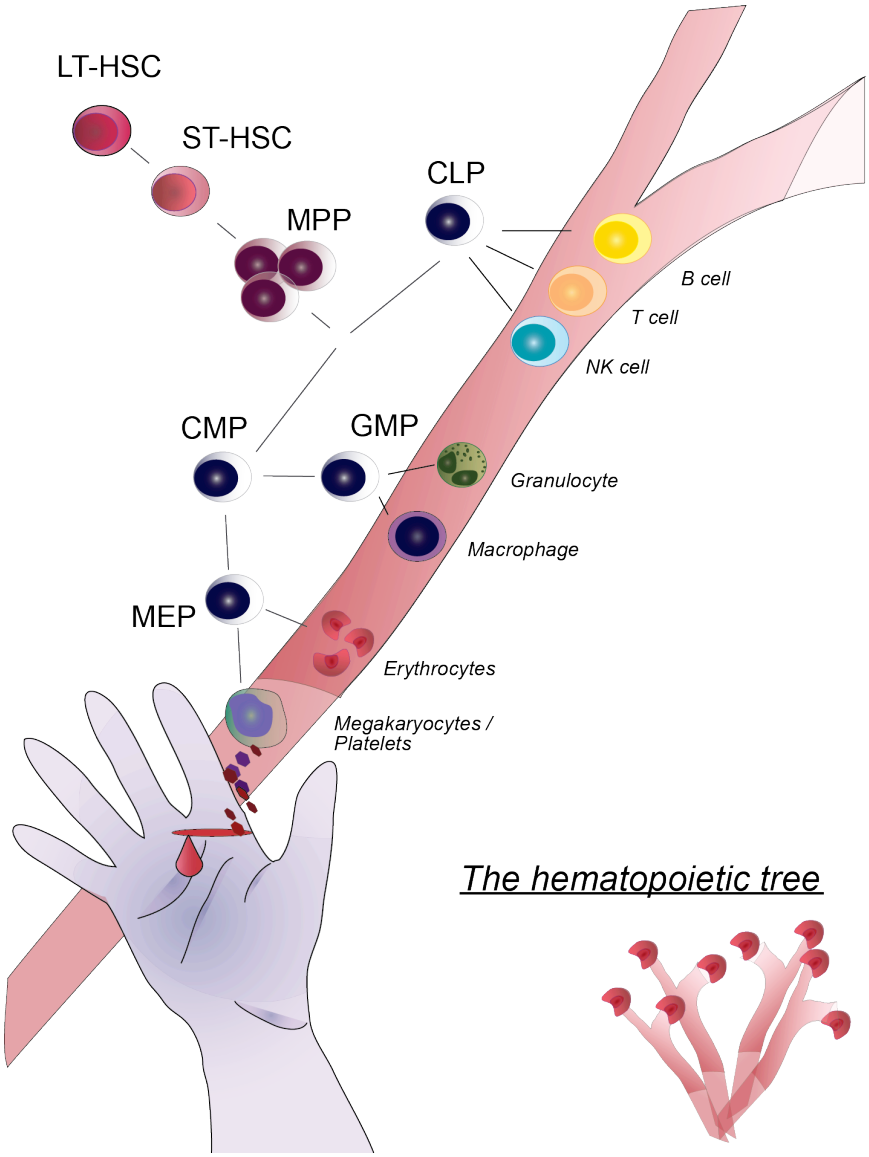


Figure 2. The hematopoietic hierarchy and function of the blood system.

Hematopoiesis during development

In mammalian development, hematopoiesis begins in the blood islands of the yolk sac. In the mouse this transpires at embryonic day 7.5 (**E7.5**). Extra-embryonic mesoderm crosses the posterior primitive streak and then establishes the islands (Cumano and Godin, 2007; Silver and Palis, 1997). Moore and Metcalf demonstrated more than 40 years ago the existence of hematopoietic myeloid progenitor cells, capable of forming clonal hematopoietic colonies *in vitro* and also some cells resembling adult HSCs and cells capable of forming CFU-S(Moore and Metcalf, 1970). Later, more stringent studies have challenged the existence of true HSC and CFU-S in the yolk sac (Medvinsky and Dzierzak, 1996; Medvinsky et al., 1993; Muller et al., 1994). Furthermore, because of the nucleated nature the of erythroblasts produced in the yolk sac, we now refer to this immature form of hematopoiesis as *primitive hematopoiesis* to distinguish it from the *definitive hematopoiesis* that can generate a fully formed blood system (Palis et al., 1999). Instead, *definitive hematopoiesis* is first detected in the embryo proper at E9.5-10.5, in the aorta-gonad-mesonephros (**AGM**) region, and includes the presence of both lymphoid progenitors and definitive HCS (**dHSC**) activity (Ivanovs et al., 2011; Medvinsky and Dzierzak, 1996; Medvinsky et al., 1996; Medvinsky et al., 1993). This is in agreement with our knowledge of hematopoietic development in other species, such as the well-studied chick embryo (Dieterlen-Lievre, 1975). However, alternative descriptions of the origin of the dHSC, including a placental, umbilical cord or later stage (E10.5-11) yolk sac model, continue to be debated and are reviewed by Medvinsky et al (Medvinsky et al., 2011).

HSCs from the AGM region enter circulation and proceed to seed the liver of the embryo where a massive expansion of the HSC pool ensues between E12.5-E15.5 (Ema and Nakauchi, 2000; Morrison et al., 1995). The fetal liver (**FL**) is considered the major hematopoietic organ during development. Following the expansion in the FL, starting at E16.5 HSCs then proceed to seed the bone marrow where they then reside until

parturition and into adulthood. Lastly, three to four weeks after birth HSCs go through a switch where the rapid cycling behavior, begun in the FL, culminate, and they become primarily quiescent cells (Bowie et al., 2006). This is also how the HSCs will remain in the niche for most of their remaining life.

The hematopoietic stem cell niche

When Schofield in 1968 proposed the existence of a specific microenvironment, a niche, which the hematopoietic stem cell resides in it was a pioneering idea (Schofield, 1978). Although studies in invertebrates would be first to experimentally prove the existence of a stem cell niche, it was studies of the hematopoietic system that generated the idea. Even before Schofield proposed his theory Lord and his colleagues showed that hematopoietic progenitor cells were located closer to the endosteal surface of the bone marrow than were differentiated cells (Lord and Hendry, 1972; Lord et al., 1975). During the years since, a series of studies have defined many of the components that constitute a niche that influence HSC behavior.

Osteoblasts have the potential to produce many of the cytokines that are necessary for myeloid progenitor expansion and were an early contender for a niche constituent (Taichman and Emerson, 1996; Taichman et al., 1996). Several studies have provided strong evidence in support of that idea. Dye-labeling experiments have followed the homing of injected hematopoietic stem and progenitor (**HSPC**) cells to their endosteal location were they tend to accumulate (Nilsson et al., 2001). In the beginning of the millennia, it was demonstrated in two different mouse models that either changing the volume of bone, and consequently the number and total surface of available osteoblasts, or increasing the number of osteoblasts by their over-activation, both lead to a corresponding increase in HSC number (Calvi et al., 2003; Zhang et al., 2003). More specifically, it seems to be the osteoprogentior rather than the mature osteoblast that is important for normal HCS function (Raaijmakers et al., 2010). The publication of these

two mouse models is considered the first definitive proof of the existence of a HCS niche, 25 years after it was first proposed (Calvi et al., 2003; Zhang et al., 2003).

Another proposed location for the niche is the perivascular area in the bone marrow cavity. The endothelial cells surrounding the sinusoids have been shown to be indispensable for engraftment in irradiated recipients (Hooper et al., 2009). Furthermore, over-activated endothelial cells result in increased number of HSCs, similar to over-activated osteoblasts do (Butler et al., 2010; Kobayashi et al., 2010). Finally, recent work with cytokines and chemokines necessary for HSC maintenance indicate that vascular niche cells secrete these factors and that HSCs reside in close proximity to a vascular niche (Ding et al., 2012; Sugiyama et al., 2006). However, there is also conflicting evidence indicating that the vascular areas have an active role in differentiation and that HSPC are located on a gradient away from the vessels (Avecilla et al., 2004; Winkler et al., 2010). Live imaging has tried to merge the opposing theories by indicating that the niches might not be physically separated but rather two parts of one niche (Lo Celso et al., 2009; Xie et al., 2009). Other important components of the niche, or niches, include osteoclasts, mesenchymal cells, adipocytes, and monocytes (Park et al., 2012; Purton and Scadden, 2008).

Finally it has been postulated that the HSC niche is hypoxic in its nature and the area close to the endosteum is indeed low in oxygen (Dello Sbarba et al., 1987; Harrison et al., 2002; Levesque et al., 2007). There is also strong evidence that HSC reside in a low oxygen environment; in vitro culture of bone marrow cells in hypoxia result in increased reconstitution (Cipolleschi et al., 1993) and HCS enriched cell fractions are positive for hypoxia markers (Parmar et al., 2007) and contain lower levels of reactive oxygen species (**ROS**) (Jang and Sharkis, 2007). Additionally, there exist evidence that instead implies that LT-HSCs, with a low oxygen content, are located close to the sinusoids (Kubota et al., 2008).

Regulation of hematopoietic stem cells

One of the mouse models of the osteoblastic HSC niche described previously also, identified the Notch signaling pathway as a major regulator of stem cell maintenance (Calvi et al., 2003). Other ways the osteoblast has been suggested to influence the HSC includes, cell-cell interactions via N-cadherins (Nakamura et al., 2010); secretion of the chemotactic agent CXCL12 (Schajnovitz et al., 2011); Thrombopoietin (**TPO**) expression (Qian et al., 2007); Angiopoietin-1 secretion (Arai et al., 2004; Hirao et al., 2004) and through production of the extra-cellular matrix protein osteopontin (OPN) (Nilsson et al., 2005).

HSCs express the tyrosine kinase receptor **c-kit** on their cell surface and its ligand, stem cell factor (**SCF**), is thought to be present in the extra-cellular matrix of the endosteal microenvironment and is secreted from vascular niche cells. The presence of SCF is necessary for the survival and retention of HSCs in the bone marrow (Ding et al., 2012; Driessen et al., 2003; Heissig et al., 2002). SCF is also vital for HSC survival and function in vitro and is one of a few that have the ability to support HCS maintenance in cultures (Keller et al., 1995). There exist a plethora of naturally occurring kit deficient mouse models with varying phenotypes (Miller et al., 1996; Nocka et al., 1990). Depending upon the severity of the mutations hematopoiesis is more or less effected. If partial receptor activity remains the HSC compartment seems to be functional unless rigorously challenged (Thoren et al., 2008). The W^{41}/W^{41} mouse strain has a point mutation in the c-kit receptor, which negatively affects the numbers and function of HSCs (Thoren et al., 2008).

Other important extrinsic regulators of HSCs include the transforming growth factor β (**TGFβ**)(Karlsson et al., 2007; Langer et al., 2004) and fibroblast growth factor (**FGF**)(de Haan et al., 2003) signaling pathways.

Signals imposed on HCS by the microenvironment will naturally impact on intrinsic signaling pathways, determining cell fate options such as cell cycling, differentiation, apoptosis, cytoskeleton organization and migration.

There are many such intrinsic factors shown to be indispensable for HCS activity including but not limited to cell cycle regulators such as p21 (Cheng et al., 2000), Gf1 (van der Meer et al., 2010) and Pbx1 (Shimabe et al., 2009) and transcription factors such as Bmi-1 (Park et al., 2003) and Prdm16 (Aguilo et al., 2011; Chuikov et al., 2010).

Functional assays for HSCs

The CFU-S assay described by Till and McCulloch was the first in vivo assay used in the study of HSCs (Till and Mc, 1961). The CFU-S is based on the ability of injected cells to form visible colonies in the spleen 7 to 12 days after transplantation. The number of colonies is easily determined and correlates to the number of progenitors injected (Jones et al., 1990). Many more assays have been developed since then and the gold standard today is long-term serial bone marrow transplantations. Recipients are conditioned, usually by irradiation, to remove endogenous cells and then the hematopoietic to be evaluated are injected intra-venously. HSPCs have the ability to home to the bone marrow and engraft the recipient. Progenitor and stem cells are then read out in CFU-S assays or by their contribution to hematopoiesis in peripheral blood and bone marrow at different time points. LT-HSC are generally considered to be present if multi-lineage contribution is detected in the bone marrow after 12 weeks or more and can be further challenged by harvesting bone marrow from the first recipient and injecting into a second host. To compare the quality of two different cell populations competitive transplantations are performed. Two cell populations are competed against each other to discern possible differences in reconstitution capacity. To complement the in vivo assays, and dissect progenitor function, colony formation in vitro is usually studied. Cells are seeded in a semi-solid medium supplemented with growth factors supporting differentiation to distinctive lineages. Each progenitor with the proper lineage potential will form a colony and the number of colonies can be counted. The size and cell composition of the colonies give further

information on proliferation capacity, and possible multi-lineage potential of the originating cell (Purton and Scadden, 2007).

THE HIPPO PATHWAY

Discovery of the Hippo pathway

In the middle of the 90's a set of mosaic studies in *Drosophila* unveiled a tumor suppressor gene that resulted in an irregular, warts-like, surface phenotype with clonal cell outgrowths. Loss of only one allele led to massive over proliferation of the fly's imaginal discs. The gene was aptly named *warts* (***wts***) (Justice et al., 1995; Xu et al., 1995). Later a similar phenotype was observed in other mosaics when the genes *salvador* (***sav***) and *hippo* (***hpo***) were mutated and the new pathway was pieced together and titled the Hippo pathway (Harvey et al., 2003; Jia et al., 2003; Kango-Singh et al., 2002; Tapon et al., 2002; Udan et al., 2003; Wu et al., 2003).

During the course of the *Drosophila* mosaics studies, it was established that the pathway suppresses overgrowth, at least in part, by the transcriptional regulation of *cyclin E* and *diap1* and it seemed natural to assume the existence of a downstream transcriptional regulator (Lai et al., 2005). Using Wts as bait in a yeast hybrid screen the Hippo pathway effector Yorkie (**Yki**) was discovered. Yorkie was shown to be negatively regulated by Wts. Wts is in turn activated by Hpo and Sav, linking the pathway together in a cascade that ultimately inactivates Yki (Huang et al., 2005) (Figure 3).

A large body of work in the past years have extended the pathway and uncovered several elements that may be acting upstream of Hpo.

The Hippo signaling cascade

Directly upstream of Hpo two cytoskeletal-binding proteins, Merlin (**Mer**) and Expanded (**Ex**) and their associated protein Kibra (**Kbr**), activates the Hippo pathway (Baumgartner et al., 2010; Genevet et al., 2010; Hamaratoglu et al., 2006; Yu et al., 2010). Further upstream the protocadherin Fat in conjunction with Dachsous (**Ds**), Discs overgrown

(**Dco**) Four-jointed (**Fj**) and Lowfat (**Lft**) act at the cell surface to regulate the pathway in an as yet unknown way (Bennett and Harvey, 2006; Cho et al., 2006; Mao et al., 2009; Silva et al., 2006; Simon et al., 2010; Sopko et al., 2009; Tyler and Baker, 2007; Willecke et al., 2006). The activity of Fat is also influenced by gradients of the morphogens Wingless (**Wg**) and Decapentaplegic (**Dpp**) (Rogulja et al., 2008).

Much less is known about the constituents and biochemical interactions of the mammalian Hippo pathway. There is however great evolutionary conservation of the signaling cascade and homologs of the core proteins and their interactions are known (Callus et al., 2006; Chan et al., 2005; Graves et al., 1998; Hao et al., 2008; Oka et al., 2008). Expression of the human Yes associated protein (**YAP**, a Yki homolog), large tumor suppressor (**LATS1**, a Wts homolog) and mammalian sterile twenty kinase 2 (**MST2**, a Hpo homolog) have all been shown to rescue their corresponding *Drosophila* mutants (Huang et al., 2005; Lai et al., 2005; Wu et al., 2003).

In mammals, Yap is subjected to the same inhibitory signaling cascade as in *Drosophila* and its phosphorylation by Lats results in increased retention of Yap in the cytoplasm and consequently less transcriptional regulatory activity in the nucleus (Zhao et al., 2007). As a transcriptional co-activator Yap has been coupled to numerous transcription factors in different tissues. However, complex formation between the TEA domain (**TEAD**) family of transcription factors and Yap is the only one preserved from the fly to humans, and is responsible for mediating a major part of the Hippo induced proliferation (Vassilev et al., 2001; Wu et al., 2008; Zhao et al., 2009; Zhao et al., 2008).

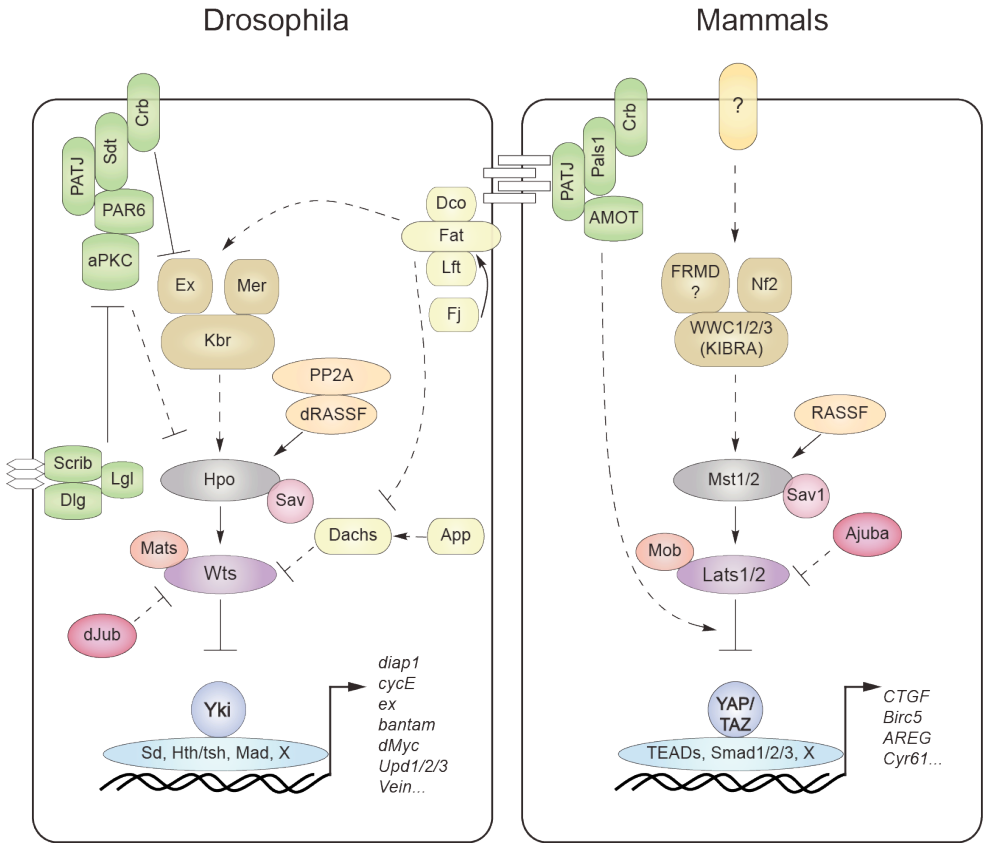


Figure 3. The Hippo signaling pathway in *Drosophila* and mammals. Boxes with the same color represent homologous proteins in *Drosophila* (left panel) and mammals (right panel). Several proteins involved in cell polarity, reviewed in (Grusche et al., 2010), such as dJub, Scrib, Dlg, Lgl, aPKC and Crb have all been implicated in upstream regulation of the *Drosophila* Hippo pathway. Adapted from Zhao et al (Zhao et al., 2011).

Hippo signaling redundancy - Wwtr1

In the mammalian Hippo pathway there exists a certain redundancy. Hpo and Wts both have two homologs, Mst 1/2 and Lats1/2 respectively. More important however, may be the existence of a Yap paralog, WW domain containing transcription regulator 1 (**Wwtr1**) also known as transcriptional co-activator with PDZ-binding motif (**Taz**) (Hong and Yaffe, 2006; Oh and Irvine, 2010; Wang et al., 2009). Taz and Yap have very similar structure, with 46% amino acid sequence identity, and they interact with many of the same proteins in the pathway. Both effectors, for example, can regulate transcription by binding to TEAD transcription factors (Li et al., 2010; Mahoney et al., 2005; Zhang et al., 2009a). However, there are also functional differences. Yap, but not Taz, can initiate pro-apoptotic transcriptional programs via interaction with p73. Taz on the other hand also has specific, distinct, binding partners, such as Pax3, which is involved in the embryo limb formation (Murakami et al., 2006). Additionally Taz has been shown to regulate osteoblastic differentiation of mesenchymal stem cells by binding Runx2, while simultaneously binding Pparγ to inhibit adipocyte differentiation (Hong et al., 2005). Thus, though mainly described as promoting proliferation, Yap and Taz can also promote cell cycle exit, cell death and differentiation. Moreover, they act in a highly cell and tissue dependent manner (Downward and Basu, 2008; Strano et al., 2005; Strano et al., 2001; Zhang et al., 2009b).

The role of Hippo signaling in disease and cancer

Most of the mammalian gene homologs in the Hippo pathway had been identified and studied before they were recognized as components of the pathway. The homolog of Merlin, **NF2**, has long been known as the gene that is responsible for neurofibromatosis 2, a genetically inherited disease that results in tumor-growth of nerve tissue (Rouleau et al., 1993; Trofatter et al., 1993). Mutations of NF2 are also found in some meningiomas and mesotheliomas (Hansson et al., 2007; Sekido, 2010).

LATS1 and LATS2 have been implicated in many different cancer forms. The promoters of both genes have been found to be hypermethylated in astrocytoma and breast cancer (Jiang et al., 2006; Takahashi et al., 2005). Low levels of LATS2 can even be an indication of a good prognosis in responding to treatment (Takahashi et al., 2007). Hypermethylated MST1 and MST2 promoters have similarly been found in soft tissue sarcomas (Seidel et al., 2007).

YAP levels are elevated in gastric adenocarcinoma (Lam-Himlin et al., 2006), medulloblastomas (Fernandez et al., 2009), as well as in cell lines from liver, colonic and lung cancers (Steinhardt et al., 2008). Additionally, YAP is located on chromosome 11q22, which is frequently amplified in various cancers (Overholtzer et al., 2006). In general, downregulation or reduced expression of upstreams components of the Hippo pathway, and conversely increased levels or increased nuclear localization of the downstream effector YAP, both correlate with a poor prognosis (Minoo et al., 2007; Xu et al., 2009). A summary of disease phenotypes in mouse models and patients for the different Hippo components can be found in Table 1.

Table 1. Summary of data on mouse models and disease pathology for different Hippo pathway components.

Hippo component	Mouse model phenotype	Human pathology	References
Fat4	Polycystic kidney disease	Potential pulmonary adenomas, breast cancer	(Saburi et al., 2008);(Berndt et al., 2011; Qi et al., 2009)
Sav1 (ww45)	Growth retardation, perinatal lethality	Renal cancer cell lines	(Lee et al., 2008);(Tapon et al., 2002)
Mst1	Viable, sarcomas, increased radiation and ROS sensitivity in T cells	Soft tissue sarcomas	(Choi et al., 2009; Zhou et al., 2009);(Seidel et al., 2007)
Mst2	Viable, mammary tumors	Soft tissue sarcomas	(Oh et al., 2009; Zhou et al., 2009);(Seidel et al., 2007)
Mst1/2	Embryonic lethal E 8.5. Growth retardation, impaired hematopoiesis		(Oh et al., 2009);
Lats1	Soft tissue sarcomas, ovarian tumors; predisposition T cell lymphomas	Astrocytoma, Breast cancer	(Cornils et al., 2010; St John et al., 1999);(Jiang et al., 2006; Takahashi et al., 2005)
Lats2	Embryonic lethal E12.5	13q12 deletion, Astrocytoma, breast cancer	(McPherson et al., 2004; Yabuta et al., 2007);(Jiang et al., 2006; Takahashi et al., 2005)
Yap1	Embryonic lethal E9.5	Liver, colonic, lung, ovarian tumors, 11q22, medulloblastomas	(Morin-Kensicki et al., 2006);(Fernandez et al., 2009; Overholtzer et al., 2006; Steinhardt et al., 2008)
Taz	Partial embryonic lethality, polycystic kidney disease	Breast and lung cancer	(Hossain et al., 2007; Makita et al., 2008);(Chan et al., 2008; Zhao et al., 2012; Zhou et al., 2011)

Hippo signaling in stem cells

Yap1 and Taz are both involved in the maintenance of *stemness*, usually in opposite manners. Yap1 tends to inhibit, while Taz induces differentiation. Whereas Taz was shown to induce differentiation in mesenchymal stem cells (Hong et al., 2005; Hong and Yaffe, 2006) Yap has been shown to instead impair differentiation in the intestinal crypt (Camargo et al., 2007), the sub ventricular zone in the developing brain (Cao et al., 2008) and in mouse ES cells (Lian et al., 2010). However, this may be an oversimplification; in human ES cells, TAZ is necessary for nuclear localization of the TGFβ signaling components Smad2/3/4, which are needed for self-renewal (Varelas et al., 2008).

Overexpression of Yap, specifically in the liver, neural tube and intestine, results in massive proliferation and an increased self-renewal of stem and progenitor cells (Camargo et al., 2007; Cao et al., 2008; Dong et al., 2007). Furthermore, Yap is highly expressed in dermal progenitors and controls hair follicle morphogenesis by expanding progenitors and inhibiting terminal differentiation, all in a TEAD dependent manner (Zhang et al., 2011).

A Hippo in hematopoiesis?

Although the Hippo pathway is well studied in Drosophila and mammalian tissues such as the liver and intestine, very little is known about is function in the hematopoietic system. A few studies have suggested that the pathway may be implicated in regulation of hematopoiesis. The promoter of the potential upstream Hippo pathway regulator Ras association domain family member 6 (**RASSF6**) is frequently hypermethylated in childhood B cell acute lymphocytic leukemia (**ALL**) and in almost half of T cell ALL (Hesson et al., 2009). Furthermore, the Mst1 knockout mouse display defective T cell homing and increased apoptosis. Finally, using a Nf2 knockout mouse, Hippo signaling has been implicated in HSC regulation through the niche (Larsson et al., 2008).

GENETIC MODELING

With the advent of the sequencing of the human genome came a whole new set of challenges in biomedical research; to translate all that sequence data into knowledge of gene function (Olivier et al., 2001; Venter et al., 2001; Venter et al., 1998). Fortunately, a tool was already established in the laboratory mouse. Sir Martin Evans, Mario Cappechi and Oliver Smithies consecutively discovered the potential of ES cells, isolated them from the mouse blastocyst, learned how they could be propagated in culture and finally developed techniques for homologous genetic recombination.

Gain of function models

A basic constitutive transgenic strategy involves injecting a DNA construct into a fertilized oocyte, also termed pronuclear injection (Gordon et al., 1980). A promoter and enhancer element is used to direct the expression, sometimes to a specific tissue or developmental stage. In theory, once the sequence has randomly integrated in the genome it directly produces the desired transcript and does so forever. In practice however, complications such as positional effects, gene silencing and insertional mutagenesis often occur (Rijkers et al., 1994; Wilson et al., 1990).

Another commonly used gain of function strategy is to introduce a gene of interest via a virus vector. This can be done in oocytes or ES cells in order to create a transgenic mouse or directly into cultured cell lines. Hematopoietic cells can be isolated from the bone marrow, targeted with retrovirus, and then injected into irradiated recipients (Haviernik et al., 2008).

Loss of function models

Building on the Nobel Prize winning work of Evans, Capecchi and Smithies the first traditional knockout (**KO**) mouse was generated in 1985 (Doetschman et al., 1987; Thomas and Capecchi, 1987). The chief advantage of gene targeting approaches such as homologous recombination over standard transgenic methods is that the integration locus can be clearly defined. Given that homologous recombination is a relatively rare event in ES cells some sort of selection strategy, negative and/or positive, is usually employed. More complex KO mice can have inducible deletion of the gene in a specific tissue or during a certain developmental stage. Combinations of approaches including mice with multiple targeted genes can also be generated through breeding strategies.

The careful studies done with gene targeting have revealed the influence of genetic background on phenotype. Mice with the same mutation on different genetic backgrounds have displayed quite varying symptoms (Phillips et al., 1999; Sanford et al., 2001).

With the discovery of RNA interference (**RNAi**) a new way of reducing gene expression became available. Using sequence specific post-transcriptional targeting of messenger RNA with so called small interfering RNAs (**siRNAs**), gene expression is down regulated instead of completely abolished (Fire et al., 1998). This approach has been successfully used both with virus delivery and in targeted mouse models (Jaako et al., 2011; Shi, 2003).

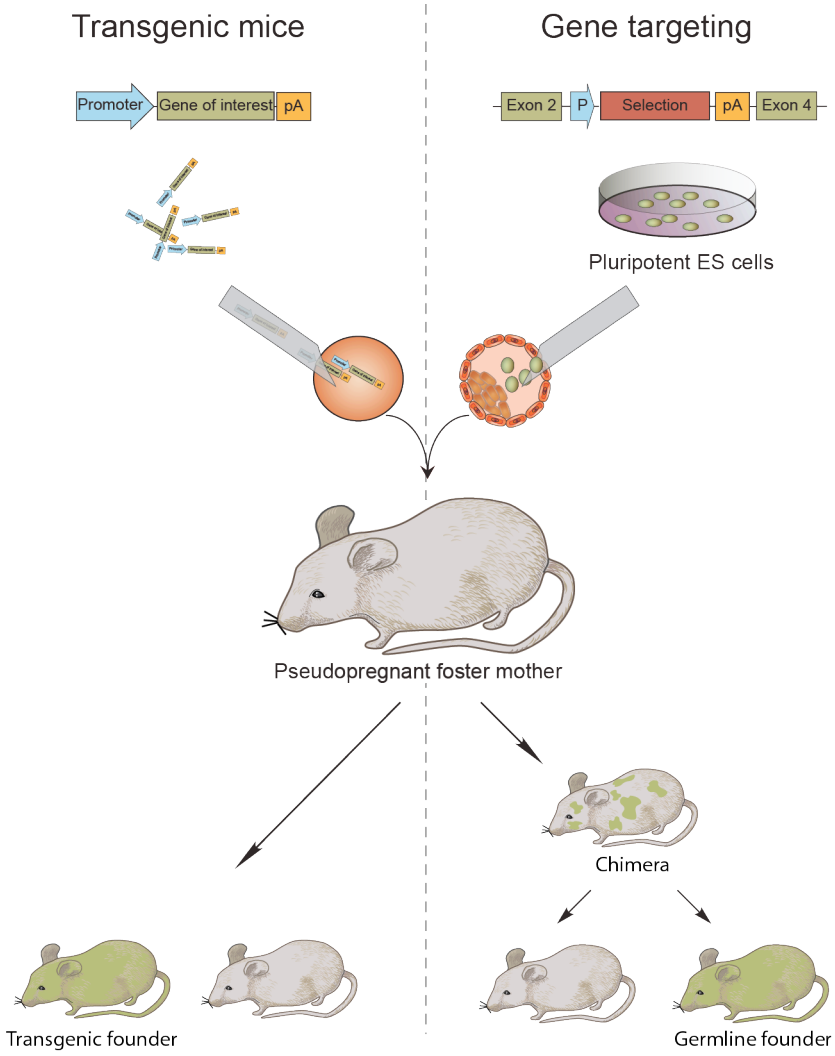


Figure 4. Basic transgenic versus gene targeting approaches for generating mouse models.

AIM OF THE THESIS

It is not known whether the Hippo signaling pathway plays a role in hematopoiesis and hematopoietic stem cells. Many studies have underlined the importance of the pathway in organ development, cell proliferation and stem cell renewal. My main focus for this thesis was to through genetic modeling investigate if and how Hippo signaling effects mouse HSCs.

1. In the first part of the work I focused on improving the genetic models available for studying overexpression in HSCs.
2. In the second part of the work I employed the model generated in (1) to study effects of overexpressing the Hippo effector YAP1 in mouse HSCs.
3. In the final part of the work I am complementing the YAP1 studies with an investigation of the other downstream effector Taz using a traditional knockout mouse.

SUMMARY OF THE RESULTS

ARTICLE I

W41/W41 blastocyst complementation: a system for genetic modeling of hematopoiesis

In this work we wanted to improve upon available methods to study genetic changes in HSCs. We decided to create a new system for overexpression in the hematopoietic system to complement the use of transgenic mice and viral vectors. We injected GFP-labeled ES cells into blastocysts from the c-kit-deficient W^{41}/W^{41} mouse strain to give the ES cells an advantage in founding the hematopoietic system. Investigating the fetal liver (FL) hematopoietic cells from W^{41}/W^{41} blastocyst complementation embryos we showed that we could generate embryos with hematopoietic cells with an almost complete ES cell origin. We further demonstrated that the FL cells can be transplanted to establish large cohorts of bone marrow chimeras with hematopoiesis of pure ES-cell origin. Furthermore, using ES cells with tet-inducible GFP expression, we could show that expression, both *in vitro* and *in vivo* is doxycycline dose-dependent with low background levels and that expression in HSPCs is feasible.

ARTICLE II

Normal hematopoietic stem cell function in mice with enforced expression of the Hippo signaling effector YAP1

To study the role of Hippo signaling in HSCs, we used or W^{41}/W^{41} system to create a transgenic model with inducible YAP1 expression exclusively within the hematopoietic system. After transplanting engineered FL cells

into irradiated recipients we waited 6 weeks and induced YAP expression. Mice were then analyzed 12 weeks post-induction. In our examination of blood and bone marrow no changes were detected in the lineage distribution compared to control mice. Furthermore, colony formation was normal and using flow cytometry we determined that the number of HSPCs was not affected. To address whether YAP1 affects the quantity and function of HSCs we also performed competitive transplantation experiments. No difference in the reconstitution ability of YAP1 expressing cells was detected after transplantations. Taken together this indicates that YAP overexpression does not affect HSC function during steady state or during regeneration.

ARTICLE III

The role of Taz in fetal and adult hematopoiesis

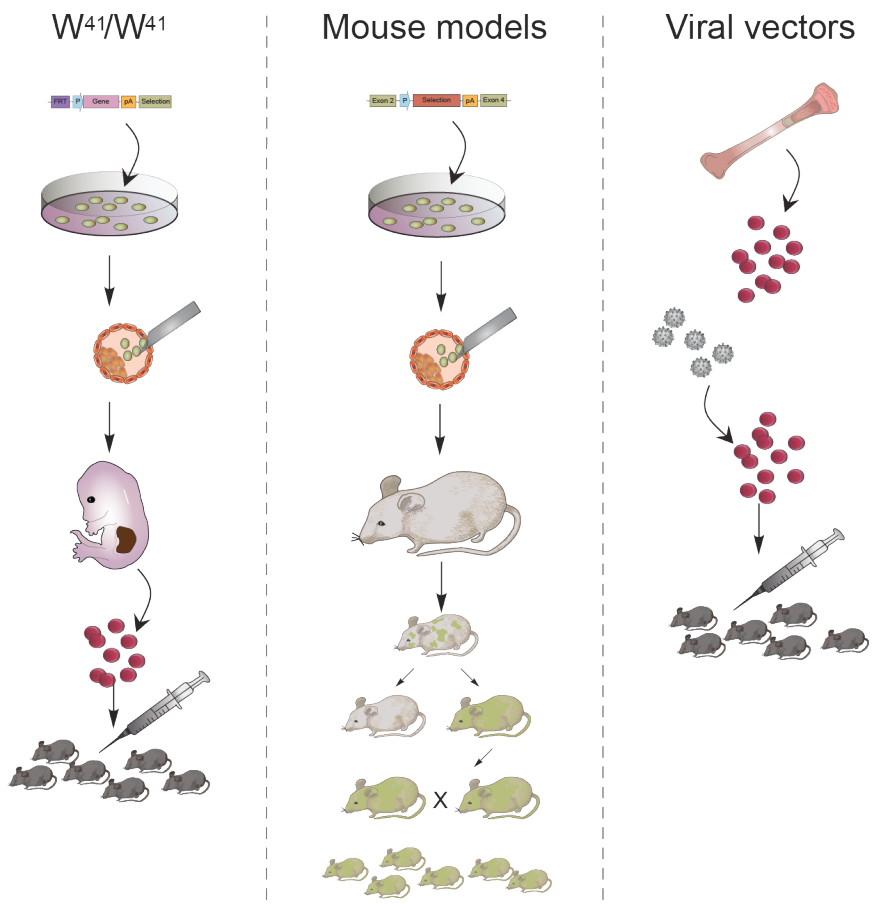
In the last project we are studying the effect of knocking out the other Hippo effector Taz on the hematopoietic system in mice. Taz KO mice are born well below the expected mendelian ratios and we have found no changes in steady state hematopoiesis in adult homozygous and heterozygous mice. At E14.5-15.5 the number of homozygous embryos are still below the expected mendelian ratio but are more frequent than post partum. Out of two homozygous embryos recovered, one had a reduced fetal liver cellularity while the other was normal. The first embryonic FL performed normal in colony assays but gave no short-term reconstitution when transplanted. The second embryo FL reconstituted irradiated mice comparable to wildtype cells. Although this is very preliminary data and more embryos have to be investigated, the divergent behavior of the homozygous FL cells correlates with the reduced penetrance observed and raises the question of why some homozygous embryos are viable while others succumb before birth. In summary, Taz seems dispensable for adult hematopoiesis but Taz deficiency leads to partial embryonic lethality; possibly by mechanisms that affect HSC function.

GENERAL DISCUSSION

GENETIC MODELING OF HEMATOPOIESIS

In **Article I** we generated a new model for studying genetic alterations in HSCs to complement already existing approaches. The benefit of using ES cells to generate FL cells for the production of bone marrow chimeras is twofold. First, it is considerably faster than generating a transgenic mouse model. Second, via available ES cell recombination techniques, it lets us control integration site, copy number and take advantage of an inducible system. Compared to using viral vectors this results in fewer confounding factors affecting the results and a more consistent level of expression. Pros and cons of the different models are summarized in Figure 5.

To achieve full ES cell chimerism in the fetal livers used for transplantations, we employed blastocysts from the W^{41}/W^{41} mouse. The idea is that hematopoietic cells from the W^{41}/W^{41} mouse are generally of normal viability until challenged, for example in a competitive transplantation assay. In such a situation they are outcompeted by wildtype cells (Thoren et al., 2008). This prompted us to assume that W^{41}/W^{41} blastocyst would not only be viable but will contribute to both embryonic and extra-embryonic tissues when ES cells are injected, but not be able to partake in establishing HSCs during development. Indeed other parts of the E14.5 embryo, excluding the fetal liver, displayed on average about 50% ES to W^{41}/W^{41} chimerism (data not shown). However, when it came to founding the hematopoietic system, W^{41}/W^{41} cells were outcompeted by the



	W41/W41	Mouse models	Viral vectors
+	Fast Clonal Inducible	Unlimited cell source Clonal Specific targeting	Fast Cheap
-	Access to W41 strain Only overexpression	Costly Time consuming	Unkown copy number Insertional mutagenesis Silencing
Time	~ 5 weeks	> 1 year	~ 1 week

Figure 5. A comparison between different approaches for studying genetic changes in the hematopoietic system.

ES cells. The nature and timing of this out-competition is unknown, as we did not investigate embryos at an earlier stage than E14.5. It is known that c-kit signaling is required in fetal liver HSCs and it is believed that dHSCs in the AGM are c-kit^{+} but the functional relevance of c-kit signaling for HSC formation is still unclear (Bowie et al., 2006; Miller et al., 1997; Nishikawa et al., 2001; Nobuhisa et al., 2012; Sanchez et al., 1996; Sasaki et al., 2010).

THE HIPPO PATHWAY IN HEMATOPOIESIS

In **Article II** we used the new system to investigate the effects of YAP overexpression in HSCs. Because of the site-specific and clonal nature of the genetic modification introduced using the W^{41}/W^{41} approach, there is less variability compared to the use of viral vectors. We therefore feel comfortable in concluding that no positive effect is seen from ectopic YAP expression in HSCs. Indeed if anything, there might be a small negative effect seen with the mutated version of YAP when enumerating HSCs by flow cytometry (Article II, figure 4D). However, this effect was not significant and did not read out in transplantation assays. We did notice a decrease in p21 levels that hints at another possible scenario, wherein active YAP signaling in HSCs might lead to a loss of stem cell potential through exhaustion. Serial transplantation assays could be done to examine this possibility.

The knockout model for Yap1 is embryonic lethal, and thus to continue investigating the Hippo pathway in hematopoiesis in **Article III** we chose to study the Taz KO mouse. There are two different models, targeting the same exon, but on different strain backgrounds. The model in our possession is on a pure 129 genetic background while the other is on a 129/B6 mixed background (Hossain et al., 2007; Makita et al., 2008). There are differences in the penetrance of the genotypes between the strains, with the first model having lower numbers of homozygotes born. The second model also reports homozygotes to succumb perinatally. Our findings

indicate that homozygotes at E14.5-15.5 are still not found at mendelian ratios. Therefore the exact stage where homozygotes die is yet to be determined. Out of the two null embryos examined the first was smaller, with a pale hypo-cellular liver. The other liver was normal. However, the first null liver performed on par with wildtype cells in colony assays but not when competitively transplanted. A possible explanation is that this is the result of a HSC specific defect but then the question arises why this only happens in some of the homozygous embryos. It is most likely not due to variations in the genetic background as the mice are on a pure inbred background. Rather, this points towards a multifactorial situation where small fluctuations in external environmental parameters at a certain stage of development in combination with the genetic lesion, sometimes tips the scale. Adult homozygotes had normal blood parameters. It therefore looks like Taz is dispensable for adult steady state hematopoiesis but it cannot be ruled out that it plays a role during embryonic hematopoietic development.

Solid versus liquid tissues

Hippo signaling has been demonstrated to be regulated in a highly cell and tissue context dependent manner. We can therefore not rule out that the pathway as a whole has no endogenous function in HSCs. Recent publications indicates a strong link between cell components coupled to cell polarity, such as Crumbs, and Hippo signaling in other tissues (Varelas et al., 2010). This then raises the question of how that would be related to our knowledge of the HSC niche. There is strong evidence for some sort of cell-cell interactions being involved in HSC regulation in the niche. On the other hand the hematopoietic system is a liquid tissue where cells are not in fixed position relative each other. HSCs do migrate to other parts of the body through the circulation upon tissue insult. It is also believed that a certain percentage of HCS at all times can be found in the circulation and that there is a continuous cycle of migration to and from the bone marrow. Evidence for the involvement of other mechanisms besides cell-cell contact in regulating cell behavior exists. For example gradients of growth factors

and chemokines are known to influence motility and differentiation. Furthermore, HSCs are thought to reside in a hypoxic area where an oxygen gradient may dictate stem cell potential.

Taken together this indicates that the anti-proliferative Hippo signaling cascade, believed to be influenced mainly by cell-cell contact, may be obsolete in the bone marrow, which is a liquid tissue. Moreover, differences in how cells react to cell-cell contacts As an example, epithelial cell lines grown as a monolayer will stop proliferating when becoming confluent in contact with neighboring cells though contact-mediated inhibition. Hematopoietic cells on the other hand, when grown in suspension, are not growth inhibited by cell contact. If similar mechanism apply in vivo, this could explain the differences seen when Yap is overexpressed in solid organs compared to hematopoietic cells.

It would further be interesting to investigate Hippo signaling in the niche to address any niche-mediated Hippo effect on HSCs.

Yap and Wwtr1 specificity

Since Yap and Taz have also been demonstrated to have partly redundant, yet divergent tissue or species specific function, one can of course not rule out Hippo involvement in HSCs without looking at both effectors simultaneously in gain of function as well as loss of function studies.

Characteristics of fetal liver HSCs

Since the FL is a place of enormous HSC cell activity, with a more than 50 fold expansion during a few days during development, it is easy to assume that a pathway such as Hippo, which regulates proliferation, might be involved. Moreover, as discussed above, hematopoiesis in a solid organ such as the liver may be regulated by different mechanisms compared to the liquid bone marrow. In depth studies of a potential FL niche is lacking and we don’t have a complete picture of how the massive HSC expansion is

regulated(Sugiyama et al., 2011). SCF signaling is necessary, but likely not solely responsible, as SCF/kit signaling has been demonstrated to have mainly survival- and maintenance functions in adult HSCs. Both Yap and Taz mRNA is expressed in FL cells, specifically in HSCs. Yap protein levels are also high in cell extracts from FLs. Since the Yap KO mouse is early embryonic lethal (E9.5) it would be interesting to study a conditional model where Yap/Taz is deleted later during development.

CONCLUSIONS

- W^{41}/W^{41} blastocyst complementation can be used as a method to model genetic modifications in HSCs. In this system we can use the sophisticated site-directed manipulations that are possible in ES cells to generate inducible genetic changes.
- The W^{41}/W^{41} approach can be used to avoid the costly and time consuming methods of standard transgenic mouse strains
- YAP overexpression does not influence *in vivo* HSC function during steady state or regeneration. This is contrast to effects seen in other tissue stem cells and suggests tissue specific functions of YAP1 in regulation of stem cells
- Taz is dispensable for adult hematopoiesis but may effect embryonic development.

ACKNOWLEDGEMENTS

First and foremost I want to thank my supervisor Jonas for taking a chance on hiring me when he started his group and then being an incredible supportive boss throughout my whole PhD studies.

I would also like to give a hand for my co-supervisor Stefan Karlsson who has managed to create a lab with such an exceptional positive atmosphere that I have never encountered before. It makes it a real pleasure to do science in this environment. Also big shout-outs to Karin, Eva and all the other PIs for having a large part in that…

With that said it is natural to start thanking all the wonderful co-workers at the Department of Molecular Medicine and Gene Therapy to name but a few; Aurélie for being a great mentor when I started my PhD; Pekka and Evan for endless hours of chattering; Justyna and Lyndon for wonderful times trekking across California (including bear scares!); Ida and Matilda for the most fascinating discussions on life, work and…well everything; Sauna-buddies Marianne and Carolina for lots of shared discussions (and sweat); Gym-buddies Alex and Jens for putting the b in bench; Kristian for teaching me *proper* ping-pong technique; Sofie, Göran and Kenichi for helping with, well science!; All Larsson group members past and present, Christine, Praveen, Natsumi, Karolina, Ann-Margreth, Mehrnaz, Roman and Ineke for making our meetings hilarious; and all you other guys you are the best!

I could go on forever, but I'll skip to the essential part…of course this would not have been possible with out the fantastic support from my friends and family! You know I love you! I should say it more often…

POPULÄRVETENSKAPLIG SAMMANFATTNING

I vår kropp genereras konstant ett stort antal nya blodceller för att ersätta de som går förlorade. Olika sorters blodceller är ansvariga för att transportera syre till alla våra vävnader, hjälpa till med blodets koagulering vid skador och att slåss mot infektioner orsakade av bakterier och virus. All blodceller härstammar från en liten population med blodstamceller som finns i benmärgen. Dessa stamceller har kapaciteten att både dela sig och bilda nya dotterceller som även de är stamceller, samt att dela sig och bilda dotterceller som mognar till de specialiserade blodcellerna. Man vet idag inte riktigt hur blodstamcellerna instrueras att välja mellan att bli dessa två typer av dotterceller och inte heller vad för faktorer som gör att de är stamceller. I mitt projekt har jag tittat på en signalerings väg, kallad Hippo, som finns i celler och som ännu inte har undersökts i blodceller. För att göra det så började jag med att komma på ett system för hur man kan titta på vad som händer i blodstamceller när uttrycket av en gen ökar. I våra laboratorier använder vi mest möss för att göra våra studier eftersom de liknar oss människor ganska mycket. Så mitt system går ut på att skapa ett uttryck av en gen som kan slås av och på bara i blodceller genom att ge möss en antibiotika i deras dricksvatten. Efter att ha kontrollerat så att systemet fungerade så använde jag det för att titta på vad som händer med blodstamceller om en gen i min signalerings väg, YAP, har mycket högre uttryck än normalt. Jag testade funktionen hos stamcellerna genom att transplantera dem till möss som fått hela sitt blodsystem utslaget med strålning och kunde därmed visa att YAP-cellerna varken hade bättre eller sämre förmåga att ersätta det gamla blodsystemet. Eftersom det finns två gener i Hippo signaleringsvägen som kan täcka upp för varandra, YAP och

Taz, så studerade jag sen också vad som händer om man tar bort allt genuttryck från Taz i blod celler. Att ta bort Taz i vuxna möss påverkar inte deras blodceller utan dessa förblir normala vilket indikerar att Taz inte behövs för blodbildning i vuxna. Sammantaget är detta de första studier som gjorts på Hippo signalering i blodsystemet och även om varken överuttryck av YAP eller borttagande av Taz verkar ha någon inverkan så måste man göra många fler studier för att se om Hippo signaleringsvägen reglerar stamcells processer i blodet.

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