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Regulation of Hematopoietic Cell Development and Hair Growth in Mouse Model: Expression Analysis of Tudor-SN and Polyamines Regulated Proteins

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ACADEMIC DISSERTATION
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the University of Tampere,
for public discussion in the Jarmo Visakorpi Auditorium,
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2. LIST OF ORIGINAL COMMUNICATIONS

I. Fashe T, Saarikettu J, Isomäki P, Yang J, Silvennoinen O. Expression analysis of Tudor-SN protein in mouse tissues. *Tissue and cell*, 45 (1), 21-31, 2013

II. Saarikettu J, Fashe T, Arretxe E, Pesu M, Junttila I, Partanen J, Sipilä P, Poutanen M, Yang J, Silvennoinen O. Transcriptional coactivator and RNA binding Protein Tudor-SN is required for normal myeloid maturation (Submitted for publication).

III. Fashe T, Keinänen T, Grigorenko N, Khomutov A, Jänne J, Alhonen L, Pietilä M. Cutaneous application of alpha-methylspermidine activates the growth of resting hair follicles in mice. *Amino Acids*. 38(2):583-90, 2010

3. ABSTRACT

Model organisms or reference organisms are non-human species, which are used to explore a given biological phenomenon deliberately to deduce the anticipated experimental findings to other organisms particularly to humans. Hypothetically, a given organism can be considered as a model organism when its size is small and it is competent to represent a certain specific organism by predicting myriads of biological and molecular processes related to genetics, development, physiology, evolution and ecology. By taking genetic conservation into account, a model organism with small size or simple form of life is expected to represent a larger organism including humans with complex genome and biological processes. Although no single organisms fulfill these theoretical criteria, most of our current knowledge of heredity, development, physiology and underlining molecular and cellular processes is obtained from studies based on model organisms. In this study, by using mouse as a model organism expression of Tudor-SN and polyamine regulated proteins were analyzed.

Tudor-SN staphylococcal nuclease (Tudor-SN) is a 100 kDa protein that was initially identified as a transcriptional co-activator. It has also been shown to function as a modulator of RNA metabolism and biogenesis and a component in the RNA-induced silencing complex (RISC). Tudor-SN protein is highly conserved through evolution from unicellular organisms such as yeast to higher organisms and it exists as a single gene without any close homologs. However, comprehensive analysis of the expression of Tudor-SN has not been investigated and the physiological function of Tudor-SN is poorly understood. In this study the expression of Tudor-SN was investigated in mouse tissues and organs by immunohistochemistry, fluorescent immunostaining, Western blotting and RT-qPCR. Up-regulated or high level expression of Tudor-SN was observed in rapidly dividing and progenitor cells such as in spermatogonial cells of testis, in the follicular cells of ovary, in the cells of crypts of Lieberkühn of ileum and basal keratinocytes of skin and hair follicle when compared to more differentiated or terminally differentiated cells in the respective organs. Analysis of Tudor-SN knockout bone marrow and peripheral blood cells indicated decrease in granularity of granulocytes. The wide expression pattern of Tudor-SN and high expression in proliferating and self-differentiating cells suggests that the protein serves functions related to activated state of cells. Besides, decreases in the numbers of eosinophils and granularity in bone marrow granulocytic cells in Tudor-SN knockout mouse suggests that the protein has an important role in immune system.

Natural polyamines putrescine, spermidine and spermine are crucial for mammalian cell differentiation and proliferation. In mammals, the growth of hair is characterized by three main cyclic phases of transformation, including a rapid growth phase (anagen), an apoptosis-driven regression phase (catagen) and a relatively quiescent resting phase (telogen). A high pool of polyamines is reported during the anagen phase compared to telogen and catagen phases. Hair cycle-associated fluctuation of polyamine concentrations and the skin abnormalities in the SSAT transgenic animals prompted us to investigate whether the growth of hair during the resting stage could be triggered by artificial elevation of the polyamine pool. However, natural polyamines are not stable molecules. α-Methylspermidine, a metabolically stable polyamine analog, has been used as surrogate molecule to elucidate the specific role of polyamines. The application of α-

Methylspermidine in telogen mice induced hair growth after 2 weeks of daily topical application. The application site was characterized by typical features of anagen and the result suggests that polyamines play an important role in the regulation of hair cycle.

4. ABBREVIATIONS

ADARs	Adenosine deaminases
AdoMet	S-adenosyl methionine
AEG-1	Astrocyte elevated gene-1
Ago	Argonaute
AgRP	Agouti related proteins
APC	Adenomatous polyposis coli
AT1R 3'-UTR	Angiotensin II type 1 receptor 3' untranslated region
AZ	Antizyme
BMP4	Bone morphogenic factor
BRE	TFIIB recognition element
bZIP	Basic leucine zipper
CART	Ccocaine- and amphetamine-regulated transcript
CCR5	Chemokine receptor 5
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CPSF	Cleavage and polyadenylation specificity factor
CTD	C terminal domain
CXCR4	CXC chemokine receptor 4
DC	Dendritic cell
DHT	Dihydrotestosterone
dcAdoMet	decarboxylated S-adenosyl methionine
dsRNA	Double stranded RNA
DGCR	DiGeorge syndrome critical region gene
EBNA 2	Epstein-Barr virus antigen 2
EBV	Epstein-Barr virus
eIF	Eukaryotic initiation factor
EPO	Erythropoietin
ES	Embryonic stem cells
esiRNAs	endogenous siRNAs
EXP 5	Exportin 5
GMP	Guanine mono phosphate
GMP	Granulocyte macrophage proginator
GM-CSF	Granulocyte-macrophage colony-stimulating factor
G-CSF	Granulocyte colony stimulating factor
GTF	General transcription factor
HCC	Hepatocellular carcinoma
HIV	Human immunodeficiency virus
HSC	Hematopoietic stem cell
IL	Interleukin
IL2rγc	IL-2 receptor gamma chain
M-CSF	Macrophage colony-stimulating factor
MEP	Megakaryocyte erythroid progenitor
αMSH	α-Melanocyte –stimulating hormone
miRNA	microRNA

mRNA	Messenger RNA
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells
NK	Natural killer cells
NPCs	Nuclear pore complexes
NPY	Neuropeptide Y
ODC	Ornithine decarboxylase
PAO	Polyamine oxidase
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCD	Programmed cell death
PIC	Preinitiation complex
P-PEFb	Positive transcription elongation factor
piRNAs	Piwi-interacting RNAs
POMC	pro-opiomelanocortic
P-PEFb	Positive transcription elongation factor
QTLs	Quantitative trait loci
RBPs	mRNA binding proteins
RISC	RNA induced silencing complex
RNA Pol II	RNA polymerase II
RUNX1	Runt-related transcription factor 1
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SG	Stress granule
SLE	Systemic lupus erythematosus
SMO	Spermine oxidase
Spd	Spermidine
Spm	Spermine
SSAT	Spermidine/spermine N^1-acetyl transferase
snRPs	Small nuclear ribonucleoprotein
ssRNA	Single stranded RNA
STAT	Signal transducer and activator of transcription
siRNA	Small interfering ribonucleic acid
TAFs	TBP associated factors
TPO	Thrombopoietin
TSS	Transcription start sites
TBS	Tris-buffered saline

5. REVIEW OF LITERATURE

5.1. Model Organisms

Model organisms also known as reference organisms are non-human species that are commonly employed to dissect certain biological process so that the anticipated findings and discoveries will be inferred to other organisms most notably humans (Fields & Johnston 2005). However, all experimental organisms are not model organisms. Model organisms possess two distinct epistemological features that underline the difference between a model organism and experimental organisms. The first characteristic feature required from an organism to be considered as a model organism is its ability to represent larger group of organisms and an organism should be smaller in size or simpler form of life. By taking genetic conservation into consideration, the smaller organisms are predicted to represent the higher organisms with complex genome. Therefore experimental findings from smaller classes of model organisms can be extrapolated to the biological process of interest in the higher organisms. However, many model organisms do not fulfill these hypothetical criteria. Nevertheless, these hypothetical criteria present a base to define and select a given model organism in order to articulate the common biological and molecular processes shared in other organisms particularly in human beings (Ankeny & Leonelli 2011). Apparently the principal purpose of analyzing model organism is to investigate a given biological or molecular process in order to generalize the findings beyond the organism itself: in other words, model organisms are not being investigated because they are interesting (Kimmel 1989). Another epistemological characteristic that defines model organisms is their ability to represent certain specific organism where they can serve as a model for various systems and myriads of biological processes related to as genetics, development, physiology, evolution and ecology. This feature is crucial for large-scale comparative investigation across different species, which can be achieved by combining various interdisciplinary research strategies. Although these two criteria are helpful tools to separate model organisms from experimental organisms, there are no clear boundaries that delineate these two models (Ankeny & Leonelli 2011).

Most of our current knowledge of heredity, development, physiology and underlining cellular and molecular processes are obtained from studies that have been carried out in model organisms. Therefore, model organisms serve as *in vivo* references and are very crucial tools to investigate physiology, biological processes and diseases of human. Very important experiments but difficult to carry out in humans because of ethical problems and unfeasibility are often conducted in model organisms (Müller & Grossniklaus 2010). Besides, human diseases research has become the principal genetic research owing to multitudes of research projects that have been carried out to understand the genetic bases of diseases in the last several decades and thorough strategy have been employed to discover the effective pharmacological compounds. The pursuit of profound understanding of physiological and pathological role of genes and novel therapeutic drugs has led to the discovery of several advanced genetic technologies. Consequently, the cutting-edge genetic technologies have facilitated the discovery and analysis of details of molecular mechanisms of several thousands of human diseases and disorders. Conceivably, in the past two decades the knowledge and approaches to investigate the functional role of gene both in normal and pathological conditions have been expanded. The rapidly expanding knowledge of the function of

a gene has increased our prospect and expectation and hence it necessitates mimicking the conditions of humans in model or reference organisms (Dow & Lowe 2012; Müller & Grossniklaus 2010).

In the early days of molecular biology, scientists addressed important questions of biology such as replication, transcription, and protein synthesis as well as gene regulation using simple unicellular organisms such as *E. coli* and *bacteriophages*. The information gathered from unicellular organism through time has led to complex questions of life. Addressing the complex questions of life requires higher model organisms (Philip Hunter EMBO reports (2008)). The most commonly used higher organisms are *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila*, *Arabidopsis*, zebra fish and rodents such as mouse and rat. These model organisms have become indispensable tools to study complex biological and clinical questions. The genomes of these organisms have already been sequenced, which allowed the large scale investigation of proteomics, transcriptomics and genomics (Müller & Grossniklaus 2010). Diversity and complexity of life has been a great challenge to choose the proper model organism. However, the common life principle shared among many organisms has provided flexibility to select and explore an appropriate model organism for a given experimental study (Müller & Grossniklaus 2010). Historically model organisms are chosen as resource material for research because of low cost of breeding, short life span, less demanding to "tame", cheap to transport and experimental manipulation can be carried out with relative ease (Ankeny & Leonelli 2011). During selection of model organisms, the advantages and limitations of choosing certain model organisms for a given experimental study need to be carefully scrutinized and some of important traits such as genetics, life span, generation time etc. of most commonly used model organisms are listed in **Table I.**

Model organisms in biomedical research play a crucial role; however, the predictive ability of model organisms has been serious source of contentions among scientific community and regulatory authorities. The controversies on predictive ability of the outcome of studies based on animal models become apparent as many highly regarded and successful studies that are carried out in model animals failed to be repeated in human clinical therapeutic trials (Ankeny & Leonelli 2011; Knight 2007). The possible reasons for the failure of replicating successful animal experiments in human clinical trials are believed to be interspecies differences, poor statistical design, poor experimental environments, unsuitable protocols and low quality experiments. To date there is no evidence that demonstrates the poor human clinical intervention studies can be upgraded by improving the quality of experiments. However, there is no easy way to overcome interspecies differences (Knight 2007). Prohibition of using model organisms has been suggested unless better alternative models such as *in vitro* or *in vivo* or *in silico* are available. Personalized medicine gives a clue that even humans are not responding equally to drugs. Therefore it is unrealistic to expect any model organism that flawlessly predicts other species including humans (Shanks *et al.* 2009).

Table I. Experimental benefits and limitations of different model organisms (Bier & McGinnis 2004)

Species of model organisms	Experimental benefits	Experimental limitations
Yeast (*Saccharomyces cerevisiae*)	Availability of powerful molecular techniques, easy cloning of genes, completed genome sequence (12 million base pairs of DNA with 6000 genes from which 31% have human equivalents) (Dujon 1996; Goffeau *et al.* 1996), contains all organelles of eukaryotic cells and cell cycle control, short life cycle (reproduce by budding and doubles every 90 minutes), very useful in cell cycle study	No organ- tissue system
Nematode (*Caenorhabditis elegans)*	Hermaphrodites, fast generation time (doubles every three days), self-fertilization, good genetics (99 million base pairs of DNA, 19,000 genes with 40% human equivalents), cloning of genes is relatively easy, RNAi effective, SNP mapping, transposon tagging and fully characterized morphology	Limited external morphology and no-organ system
Fruit fly (*Drosophila melanogaster*)	Genome sequence is completed, very good genetics (165 million base pairs of DNA, 3600 genes with 50% of human equivalents) (Adams *et al.* 2000), targeted gene disruption can be done with relative ease, effective RNAi and generation time is fast and availability of powerful molecular methods, generation of transgenic animal is relatively easy, SNP mapping, cloning and transposon mapping is easy. Very useful model of innate immunity (Lemaitre & Hoffmann 2007)	Embryonic manipulation and gene disruption has some difficulties and lacks humoral immune system
Zebra fish	Simplest vertebrate, genome sequence is available, manipulation of embryo is relatively easy, similar organ system to other vertebrates and rapid vertebrate development	Difficulty of generating transgenic animals and targeted gene disruption
Mouse (*Mus musculus*)	Mammal, similar anatomy and physiology to humans, excellent genetics (3 billion base pairs of DNA as humans), possible to generate knockout and transgenic animals, developmental process is similar to other animals and mouse is a source of primary cell cultures	Relatively expensive, early phenotype of a mutant mouse is difficult to analyse, relatively slow development and life cycle
Rat	Its anatomy, physiology, development and response to injury is very close to human, genetic manipulation possible	Prenatal experiment is difficult and high cost

5.1.1. The mouse as a model organism

Mouse has become the leading mammalian model for human diseases owing to its close physiological and genetic similarity to humans. Besides, manipulating mouse is easy, they are relatively cheap compared to larger mammals, they reproduce more quickly, and analyzing and manipulating the mouse genome is relatively simple. Flies *(D. Melanogaster)*, yeasts *(S. cerevisiae)* and worms *(C. elegans)* are indispensable tools to explore cell cycle and developmental biology. However, mice are by far a superior model to explore the complex organ system, tissues, behavioral traits and physiological systems of humans including cardiovascular, nervous, musculoskeletal, immune, endocrine, and others. Some of the most important diseases which humans acquire spontaneously including cancer, diabetes, osteoporosis, atherosclerosis, hypertension and glaucoma are also well documented to occur in mice. Other human diseases which do not occur naturally in mice such as Alzheimer's diseases, cystic fibrosis and others can be induced in mice by manipulating the mouse genome and environment. Besides, all functional genes of humans are reported to exist in mouse (Rosenthal & Brown 2007).

Furthermore, the availability of many inbred mice and the number of recently advanced genetic technologies to create human disease models has favored mouse as the principal model organism for complex or multigenic human diseases. Biochemical pathways that explain the mechanism of pathogenesis of multigenic complex traits such as histocompatibility, obesity, cancer, hypertension, diabetes and other diseases and disorders of human have been understood by utilizing multiple single mouse mutants (Moore 1999). The key characteristics and application of commonly used mouse strains in biomedical research is briefly summarized in **Table II**.

5.1.1.1. Evolutionary history of the laboratory mouse

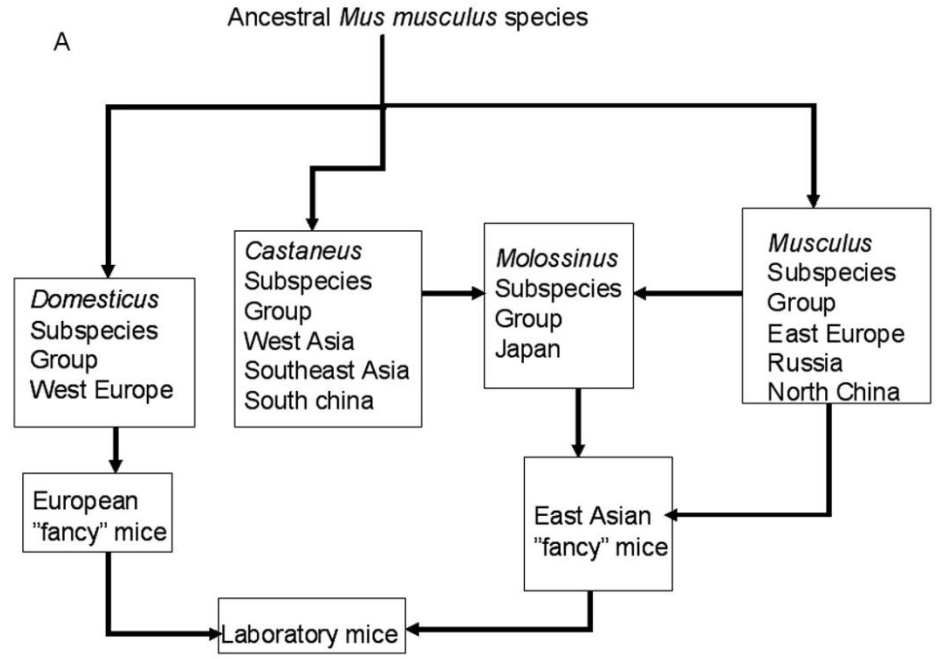
Knowledge of the evolutionary history of the laboratory mouse is essential to understand the nature and importance of phenotype of specific genes. The origin of "fancy" mice both in Asia and Europe is believed to be the wild mice in each of the respective regions. "Fancy" mice of Japan and Europe have been documented as origin of the current laboratory mouse (Yoshiki & Moriwaki 2006). The maternal origin of the most common laboratory strains was experimentally shown by analyzing mtDNA to be European domesticus subspecies group (Yonekawa H, Moriwaki K, Gotoh O, Hayashi JI, Watanabe J, Miyashita M, Petras ML, and Tagashira Y. 1980). However, analysis of the Sry gene revealed that Y chromosome of the laboratory mouse strains is inherited from the Japanese "fancy" mouse (Nagamine *et al.* 1992), which confirms early historical notes that describe the trade exchange between Asian and European fancy mouse breeders.

Table II. Advantages, characteristic feature and main applications of some of commonly used laboratory mouse strains (adapted from http://www.labome.com/ and references there in)

Strain(key characteristics)	advantages and main application	Examples of physiological process and diseases to which the strain has been used as model
C57BL/6 (in bred, black)	Stable, easy to breed, fully sequenced genome, model of physiological and pathological *in vivo* experiments, commonly used mouse to generate transgenic and knockout models, serve as background strain for congenics and transgenics	T cell development, inflammation, fibrosis (Chaudhry *et al.* 2009) and cardiomyopathy (Spurney *et al.* 2008), heart failure (Hughes Jr *et al.* 2008), pneumonia (Ventura *et al.* 2008) and adaptive immunity (Sanders *et al.* 2009)
BALB/C (Inbred, albino and immunodeficient	Relatively easy for breeding, are tumor prone, important for production of monoclonal antibodies, hybridoma generation and serve as a model for cancer research and immunology	Cytokines(immunity) (Nurieva *et al.* 2008), cerebral malaria (Oakley *et al.* 2008) and cholestatic liver diseases (Takeda *et al.* 2008)
CD-1(out bred and albino)	Positional cloning, genotype selection and toxicological testing	Endogenous gene regulation in uterine biology(Gao *et al.* 2011) and serves as source for mouse embryos (Shih *et al.* 2012)
CB17/SCID (in bred, albino)	Lacks both T and B cells, tumor transplantation, for testing new cancer treatment and can serve as “humanized” mice by hosting tissues of human immune system.	PTEN/Akt/PI3K signaling (Dubrovska *et al.* 2009) (Shih *et al.* 2012), subcutaneous tumorogenesity (Miyagawa *et al.* 2009) and breast cancer regulation (Gonzalez *et al.* 2008)

The *M. musculus* subspecies are distinctly distributed across geographic areas: *musculus* group are known to reside in Eastern Europe and North Asia, the *castaneus* group inhabits Southeast Asia and South China, and the *domesticus* in North Africa and Western Europe. *M. musculus* is classified into three subspecies such as *domesticus, casteneus*, and *musculus* (Yoshiki & Moriwaki 2006). The genetic divergence time between Asian and European *domesticus* is estimated to be approximately 1 million years (Moriwaki *et al.* 1979). Furthermore, the microsatellite DNA marker aided scanning of the whole genome of eight strains of laboratory mice including A/J, C57BL/6J, CBA/J, DBA/2J, SM/J, SWR/J, NC/Nga, and 129/SvJ, and eight wild-derived inbred strains BGL2/Ms, CAST/Ei, JF1/Ms, MSM/Ms, NJL/Ms, PGN2/Ms, SK/CamEi, and SWN/Ms validated the pedigree divergence in mouse subspecies (Sakai *et al.* 2005). Discovery of genome-wide shotgun SNP by Mouse Genome Sequencing Consortium

(Chinwalla *et al.* 2002) and analysis of the available sequence information has been useful to describe the genetic variation among inbred laboratory strains (**Fig. 1**) (Wade *et al.* 2002).



B

	Strain	Locus 1	Locus 2	Locus 3
Laboratory mice	129/SvlmJ	TT GG TT GG GG TT GG	CC TT DEL CC TT CC TT	GG TT AA TT AA GG
	A/J	AA GG CC GG AA CC TT	TT TT T TT GG TT CC	GG TT AA TT AA GG
	AKR/J	AA GG CC GG AA CC TT	TT TT T TT GG TT CC	GG TT AA TT AA GG
	BALB/CByJ	AA GG CC GG AA CC TT	TT TT T TT GG TT CC	GG TT AA TT AA GG
	C3H/HeJ	TT GG TT GG GG TT GG	CC TT DEL CC TT CC TT	CC AA TT TT GG AA
	C57BL/6J	AA GG CC GG AA CC TT	CC TT DEL CC TT CC TT	GG TT AA TT AA GG
	DBA/sJ	TT GG TT GG GG TT GG	CC TT DEL CC TT CC TT	GG TT AA TT AA GG
	FVB/NJ	AA GG CC GG AA CC TT	TT TT T TT GG TT CC	GG TT AA TT AA GG
	SJL/J	AA GG CC GG AA CC TT	TT TT T TT GG TT CC	GG TT AA TT AA GG
Ancestors	*domesticus*	AA GG CC GG AA CC TT	TT TT T TT GG TT CC	GG TT AA TT AA GG
	musculus	TT GG TT GG GG TT GG	CC TT DEL CC TT CC TT	CC AA TT TT GG AA
	castaneus	AA AA CC AA GG CC GG	CC CC DEL CC TT CC TT	GG TT AA AA AA TT
	molossinus	TT GG TT GG GG TT GG	CC TT DEL CC TT CC TT	CC AA TT TT GG AA

Figure 1. A) European (*domesticus*) and Asian (*musculus* and *castaneus*) are predicted to donate genetic contributions as mice are originated from Asian and European fancy mice breeders. B) Two haplotypes in several loci match with wild type derived representative (WSB/Ei) (yellow), *musculus* (CZECHII/Ei) (gray) or *molossinus* (MOLF/Ei) but not *castaneus* (CAST/Ei) (green). Chromosome 14:121 Mb (locus 1), chromosome 6:139 Mb (locus 2) and chromosome 2:107 Mb (locus 3) are represented by polymorphisms from 1-kb windows re-sequenced in nine strains (Wade *et al.* 2002).

5.1.1.2. Mouse genetic history

In the beginning of the last century (1902), a French scientist Cuénot was the pioneer to demonstrate the Mendelian inheritance ratio of the coat color of mice (Cuénot 1887). Three years later in 1905, albeit with strange segregation ratio Cuénot further experimentally demonstrated what was later verified as the first lethal mutations in mice, A^y allele of agouti locus. However, in 1910 Castle and Little found that the missing unexpected segregation ratios in previous work of Cue´not that did not fit the expected Mendelian ratios were due to embryonic mortality (Castle & Little 1910). Besides, Cuénot also reported the first multiple alleles at A^y locus (Cuénot 1887). Though the existence of more than one allele at a single locus was proven by advancing the studies of Cuénot within a decade, it took almost half a century to recognize the fact that dominant and recessive traits are merely a physiological consequence of a gene action but not a genetic phenomenon (Paigen 2003). Two key historical events of mouse genetics were documented following the study by Cue´not and subsequent follow-up studies by other investigators: 1) Tyzzer demonstrated that the rejection of transplanted tumor in mice is a inheritable phenomenon (Tyzzer E. E. 1909) 2) the first inbred mouse strain was made by mating in attempt to generate the repeatable genetic material to study genetics of resistant traits that underline the rejection of transplanted tumor in Tyzzer's study. Tumors from Japanese waltzing mice were shown to be successfully transplanted to the same strain by J. Loeb but completely rejected when the same tumor was transplanted to common mice.

To understand the underlining genetics bases of J. Loeb's study, Tyzzer cross bred common mice with Japanese waltzing mice and found that all F1 hybrid mouse were prone to tumor but all of F2 generation rejected tumor transplant. Based on this finding Tyzzer concluded that tumor susceptibility trait did not follow Mendelian trait. However, Little thought that Tyzzer's observation could follow the Mendelian mechanism of inheritability and constructed DBA as the first inbred strain of mice. Little came up with alternative theoretical explanation about genetic bases of rejection of tumor transplant in the cross bred mouse in Tyzzer's study. His alternative explanation is that multiple genes direct rejection and acceptance of tumor and for a given gene there are two alleles, one allele for the dominant and another for the recessive trait. The mouse that accepts a tumor transplant must have at least one dominant allele in each locus involved. F1 animals in Tyzzer's study were accepting tumors because they all inherited the dominant allele at each locus from tumor donor of Japanese waltzing mice. But F2 mice rejected tumor transplant in Tyzzer's study because of indiscriminate mixture of large number of genes, which subsquently led very few animals to accept tumor transplant (Little 1914). Both Little and Tyzzer confirmed the inheritability of tumor susceptibility trait in F2 generation by using large number F2 animals (Little & Tyzzer 1916). Little was not only the father of genetics but also the founder of Jackson memorial laboratory, which is now called Jackson laboratory (Paigen 2003). The immunological reason for tumor rejection was elegantly explained by Gorer and he was able to demonstrate that the genes which determine resistance for tumor transplant functions through the presence of a cellular antigen and tumor rejection is mediated by an antibody formed against that specific antigen. His study led to discovery of the major histocompatibility complex, H2 (Paigen 2003).

5.1.1.3. Mouse models of simple, Mendelian inherited diseases

There are number of human monogenic diseases reviewed by (Carter 1977) including Huntington's chorea, neurofibromatosis, myotonic dystrophy, multiple polyposis coli, diaphysical aclasia, dominant form of blindness, polycystic kidney disease, dominant forms of early childhood onset deafness, monogenic hypercholesterolaemia and congenital spherocytosis in the blood. Many phenotypes of mouse mutations are similar with rare genetic human diseases and consequently the discovery of mutated mouse genes helped to detect respective orthologs of human diseases (Spritz *et al.* 1993) including Piebaldism (Spritz *et al.* 1993), Usher syndrome (Well *et al.* 1995), Chediak-Higashi syndrome (Nagle *et al.* 1996) and Hermansky-Pudlak syndrome (SWANK *et al.* 2006). Besides, the disorder in the biochemical pathway that underlines the complex human diseases has been identified by using single gene traits of mouse mutants. The best example is the obesity model of single gene mouse mutants including *obese (Ob,* chr 6, *diabetes (db),* chr 4, *fat(fat),* chr 8, *tubby(tub),* chr 7 and *agouti lethal yellow(A^Y, A^Y)* chr 2 (Naggert *et al.* 1995). Combination of the five mutant genes helped to understand the underlining pathological mechanism of obesity and provided several important therapeutic targets of obesity and metabolic syndrome (Moore 1999). Leptin and its receptor (db gene) present a very crucial signaling cascade that regulates adipose tissue, food intake and energy expenditure by their action in the central nervous system (Spanswick *et al.* 1997). Central action of leptin focuses on two major neuronal pathways. 1) Catabolic pathway of pro-opiomelanocortic (POMC) and cocaine- and amphetamine-regulated transcript (CART) neurons are activated by leptin. 2) Anabolic pathway mediated by neuropeptide Y (NPY) and agouti related proteins (AgRP) neurons are inhibited by leptins. α-Melanocyte stimulating hormone (αMSH) is secreted in response to the stimulation mediated by leptin on POMC neuron in hypothalamic arcuate nucleus. αMSH is a potent agonist of melanocortin receptors (MC-3R and MC-4R) located in the paraventricular nucleus of the hypothalamus. Leptin has also been shown to inhibit the release of agouti-related protein. With these two mechanisms leptin regulates the food intake and energy expenditure (Fellmann *et al.* 2012).

Excess availability of agouti protein antagonizes the binding of αMSH to melanocortin receptors. A strong yellow hair in A^y mice is due to antagonistic effect of agouti like protein on melanocortic receptor, MC-1R as a consequence of the ectopic expression of the agouti protein in A^y mice whereas the obese, hyperphagic, hyperglycemic and hyperinsulinemic phenotype of A^y mice (Miller *et al.* 1993) is due to antagonistic effect of agouti protein on melanocortic receptors (MC-3R and MC-4R) (Lu *et al.* 1994). Carboxy peptidase E (CPE) activity has been shown to be decreased 20-fold both in islets and pituitary glands of *fat/fat* mouse compared to wild type littermates. Moreover, missense mutation has been shown in the *CPE* gene of *fat/fat mouse.* Thus both biochemical and genetics studies suggest that CPE is a candidate gene for fat. Indeed, CPE is shown to activate αMSH by cleaving two C-terminal amino acids from cleavage products of POMC (Naggert *et al.* 1995). Interestingly, CPE is reported to be expressed in obese individuals. Thus an important lesson is learnt about the complex human diseases such as obesity and metabolic syndrome using the information obtained from the phenotypes as well as the underlining genetic and biochemical mechanism of the above five single gene mouse mutants (Moore 1999).

5.1.1.4. Mouse models of complex multigenic diseases

Our understanding of complex human diseases such as diabetes, autoimmunity, obesity, etc. and other complex traits is expanded recently owing to availability of advanced genetic throughput technology. Conceivably, these technologies have been crucial tools to investigate the inheritance of complex traits of very common and complex human diseases such as cancer, metabolic syndrome, obesity, diabetes and heart diseases. In early days of molecular biology critical foundation to the construction of linkage genetic maps was established by using information obtained from cloning of genes and DNA sequence of model organisms. The cloned genes were investigated for any responsible Mendelian trait exclusively based on their genomic position and these types of studies include identification of locus, sequencing the regions to define any mutations and analyzing molecular as well as biochemical function of the genes identified (Spritz *et al.* 1993; Altshuler *et al.* 2008). Linkage analysis was initially unfeasible in humans but the early progress was limited to blood type antigen and hemoglobin β protein (Altshuler *et al.* 2008). The progress to high density genetic map has expanded the simplicity not only for positional cloning of monogenic traits but has also become a very helpful tool to discover important genetic loci in complex heritable multigenic traits in higher mammals including human and mouse (Altshuler *et al.* 2008).

A multitude of quantitative trait loci (QTLs) of complex human diseases such as diabetes, atopy, autoimmunity, CNS disorder, and alcohol sensitivity has been discovered in mouse. A quantitative trait refers to a strongly variable phenotype as a consequence of two or more gene products and the environment whereas QTL is a stretch of DNA sequence containing genes that underline polygenic phenotype (Moore 1999). For example, about 48 QTLs were identified in a mouse obesity model. These loci were obtained by combining different strains of inbred mouse pairs to measure the factors which influence obesity including whole body weight determination, high fat diet induced obesity, weight of specific fat pads and total obesity (Suto *et al.* 1998). The availability of different kinds of breeding strategies provided an opportunity to probe each QTL and this has been achieved by generating a mouse strain that carries one QTL (congenic strains) (Hunter & Crawford 2008). For example, QTL analysis of variability in alleles which determines Ucp1 level and stimulation of brown adipocytes in A/J vs. B6 has provided a crucial perspective on the pathways that regulate brown fat induction in abdominal fat storage of the mice. The interactions which underline the regulation of PPARα, PGC-1α and type II iodothyronine (DIO2) have been demonstrated to control the expression level of UCP1 mRNA (Kozak 2011).

The monogenic strains obtained through different breeding techniques to analyze each QTL can be exploited by simple cloning strategies and have recently become very popular. Therefore, this reductionist strategy provides a platform to clone some of QTLs. However, the daunting lesson learnt from this strategy is the lack of disease phenotype or sub phenotype in mice generated by breaking the components of complex diseases. Moore (Moore 1999) discussed the following possible reasons why phenotype or at least components of phenotype were missing in newly generated mice that carry one QTL of the complex diseases. I) Possible epistatical interaction of a given QTL with other QTLs and in the absence of this interaction a given QTL lacks functional ability to confer the desired phenotype. II) Isolated QTL provides too low contribution to be detected as a component of the original diseases. III) Failure of breeding strategy to transfer the desired genomic fragment into congenic strains. IV) The assays used to measure the original

phenotype do not fit to measure a given QTL; however, assay for subphenotype is needed to detect a given locus. V) QTL mapping may generate false positive QTLs and therefore all QTLs are not always true.

Nevertheless, generation of a congenic mouse strain through the expansion of QTLs studies has presented an interesting platform to logically explain some of the complexity of human diseases. The previously discussed five genes of obesity, Ucp1 and the four genes to be discussed below in systemic lupus erythematosus (SLE) represents the best examples of complex human diseases where mouse QTLs studies have been crucial to elucidate the contribution of each locus to the pathogenesis of complex human multigenic diseases (Moore 1999; Kozak 2011). SLE is a clinically heterogeneous systemic autoimmune disease of connective tissues that damages almost all organs including heart, nervous system, lungs, liver, kidneys, joints and blood vessels (Kozak 2011). Both human and murine lupus studies revealed the complex mechanism of pathogenesis of SLE. Murine lupus models helped to identify at least nine distinct loci which in turn present susceptibility to lupus upon mutation (Vyse & Todd 1996), most notably Sle1 on chr1, Sle2 on chr4, Sle on chr 7 and H2 locus on chr17 have been shown to be susceptibility loci of lupus in Sle prone NZM2410 mouse model (Morel *et al.* 1994). Congenic strain of mouse model for each of the above mentioned locus in a different chromosome has provided a distinct contribution of each locus to the pathogenesis and progression of the complex SLE diseases in human. For example, congenic strain of Sle1 is shown to be susceptible for break of immune tolerance to chromatin. Sle2 congenic has been shown to be susceptible to B-cell hypersensitivity. A Sle3a congenic strain is prone to end-organ sensitivity and a Sle3b congenic strain is susceptible to T-cell hyper reactivity (Morel *et al.* 1997). The history of SLE clearly demonstrates the fact that the complex human disease phenotype is a consequence of a cumulative effect of multiple genes where each gene has its own share. QTLs murine model serves as an indispensable tool to elucidate the pathogenic mechanism of other complex multigenic human diseases.

5.1.1.5. Genetic technologies to generate mouse models

The vast expansion of molecular biology and genetics conferred us very important knowledge about genes and their function both in human and models. From the humble beginning of Gregor Mendel, the founder of modern genetics, to recent cutting-edge achievements including sequencing of human genome (Venter *et al.* 2001), scientists have successfully overcome technological hurdles by inventing many novel technological tools to dissect the functions of genes and their products. While other novel technological tools are expected to come, in post genomic era the major remaining confrontation is the elucidation of the biochemical and physiological function of each gene. The availability of advanced genetic mapping strategies have also greatly facilitated the identification and analysis of a gene in case of monogenic diseases or set of genes in case of complex diseases both in human and model organisms, most notably in the mouse (Mak 2007). Furthermore, comparative genomic studies revealed high homologies in mouse and humans genomes. In addition to similarity between mouse and human anatomy, physiology and cellular functions, relatively low cost of breeding as well as fast generation time, genetic homology and the availability of advanced mapping techniques made mouse the foremost mammalian experimental model, which can be engineered with relative ease and precision. The justification to engineer the mouse genome is to study the function of human orthologous gene and the engineering of the mouse principally aims at gain of function of a given

gene through generation of transgenic mouse and loss of function through generation of knockout or knock in mouse. Besides, the mouse has also been employed as a model for various human diseases including infectious diseases and cancers (Hunter 2008).

5.1.1.5.1. Transgenic mouse models

A method that enables to stably insert foreign DNA into the germ line of mice was developed in 1980s and allows the generation of transgenic mice (Hogan *et al.*; Rulicke & Hubscher 2000; Gordon *et al.* 1980). The generation of transgenic mice effectively transformed the investigation of functional role of genes both during development and pathogenesis. For example, transgenic mice over-expressing genes associated with human diseases, such as cancers including oncogenes, presented a well-built support that explains the disease-linked genes directly contribute to the pathogenesis of diseases. Furthermore, transgenic mice have been a useful tool to study pharmacological compounds and to investigate extragenic enhancers and suppressors (Bedell *et al.* 1997). There are different ways of generating transgenic mice. Babenet (Babinet 2000) and (Rulicke & Hubscher 2000) reviewed different methods of generating transgenic animals including pronuclear injection of cloned DNA into a zygote, transduction of early embryos with recombinant retrovirus carrying a gene of interest, and transfer of a gene of interest into pluripotent embryonic stem (ES) cells which are derived from the inner cell mass of blastocysts. Co-injection of foreign DNA with sperm cell head, whose membrane is disrupted, into the cytoplasm of unfertilized mouse oocytes has also been reported to generate transgenic progeny (Perry *et al.* 1999). Both lentivirus and retrovirus-mediated insertions of naked DNA are solely based on the addition of the sequence of the gene of interest into the virus genome whereas the ES cell-based method targets the endogenous gene via homologous recombination. Each method is useful for specific applications but all of them have certain drawbacks (Perry *et al.* 1999). However, the microinjection-based mouse transgenesis has become popular and nowadays it is widely used in many laboratories (Rulicke & Hubscher 2000; Gama Sosa *et al.* 2010).

5.1.3.5.1.1. Mouse transgenesis by pronuclear injection

The introduction of a foreign gene to mouse embryos was first demonstrated by Gordon (Gordon *et al.* 1980) and since then the method has been adapted by several laboratories. Briefly, first the fertilized oocytes are obtained from the oviduct of superovulated female mice, where superovulation is achieved by hormonal stimulation followed by mating female mice with male mice. The mouse pronuclei are easily noticeable that makes the microinjection a relatively easy task. It is easy to notice early pronuclei from near distance to polar body. Six hours following fertilization both pronuclei are located in the middle of oocyte and their size also increases. The size and transparency of both pronuclei depends on the strain of the female mouse, however, because of its large size, the male pronuclei are usually favored for microinjection (Rulicke & Hubscher 2000). Nevertheless, there have not been any significant variations in the number of offspring whether microinjection is done in male or female pronuclei (Brinster *et al.* 1985).

The transgene construct intended for pronuclear injection contains usually cDNA of the gene of interest under the control of a promoter that regulates both temporal and spatial expression of the gene (Haruyama *et al.* 2009), Kozak sequence (GCCGCC(G/A)NN) followed by translation start codon (ATG) (Kozak 1987) and translation stop codon (UGA, UAG, UAA). Besides, insertion of

reporter genes such as *β-galactidase (lacZ)* or *green fluorescent protein (GFP)* under the control of the gene's promoter and/or with enhancer elements confers an additional tool to analyze the regulation of expression of the gene. Furthermore, the introduction of a natural intron such as Simian virus 40 (SV40) or intron of rabbit β globin or an artificial intron at 5' or 3' end provides stability and efficient translocation of mRNA from the nucleus. Finally, to avoid the intrusive effect of vector sequence over the trangene expression, the prokaryotic plasmid backbone is cut from the transgene insert used for microinjection (Gama Sosa *et al.* 2010).

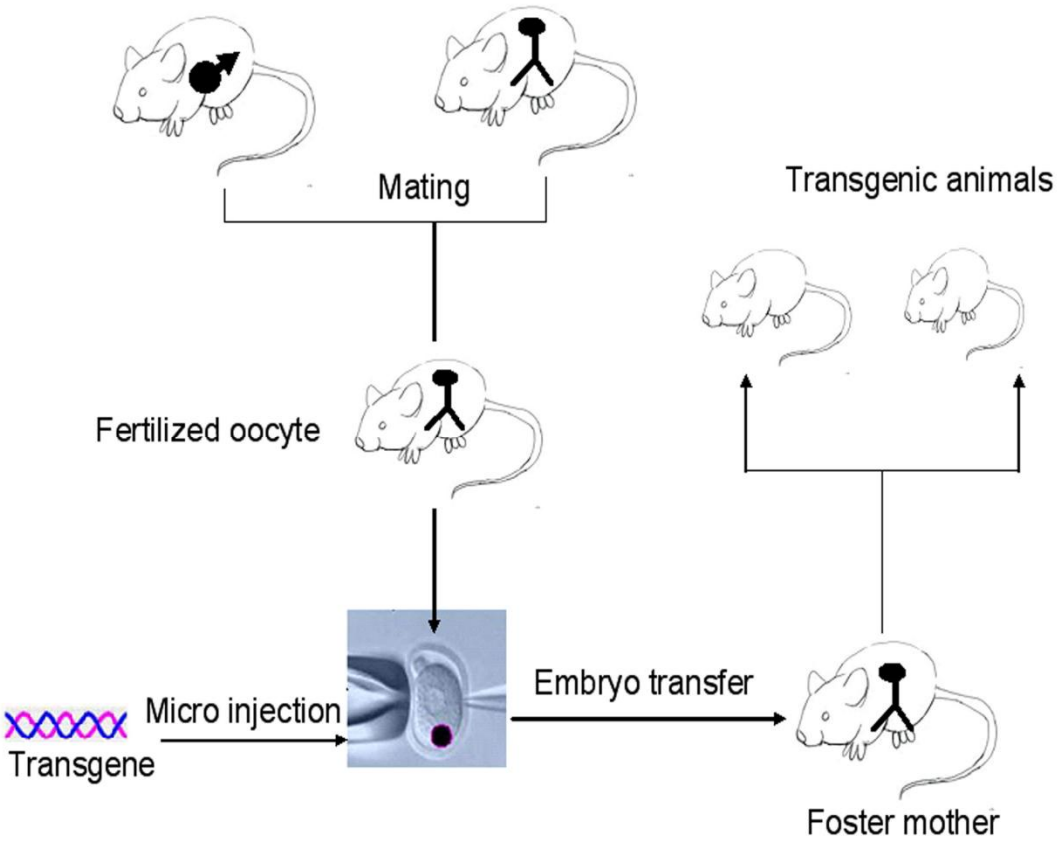


Figure 2. Pronuclear microinjection based transgenesis.

The microinjection of the construct cDNA into the fertilized pronuclei of mouse results in the generation of transgenic progeny quite often with several copies. However, there are some setbacks in the generation of transgenic mice. Usually, the integration of the transgene is random and this makes the expression of transgene prone to positional site-dependent effects. The consequences of the position site-dependent effect due to random integration of transgene include low expression of transgene, completely silenced transgene, alteration of tissue or cell specificity of transgene expression and thus, affects general expression of transgene from no phenotype to unexpected phenotype (Rulicke & Hubscher 2000). The expression of an endogenous gene may also be affected at the integration region of the transgene. Analysis of multiple lines of mice from different founders helps to identify the insertion site-dependent effect from random insertion of transgene. An insertion of an insulator sequence in the transgene construct may

eliminate or reduce the insertion site effect. Following successful microinjection of pronuclei, the zygotes are often allowed to progress to 2-cell stage embryos, which are subsequently transferred to a foster mother **(Fig. 2)** (Bushey *et al.* 2008).

5.1.1.5.2. Knockout mouse models

The knockout mouse model has become the leading tool to dissect the function of a gene of interest in post genomic era and has been extensively utilized to study the functional role of a gene during development, physiology and pathophysiology of diseases and for pre-clinical trials for pharmacological molecules. Knockout mice can be generated basically by two independent technologies, which are called gene trapping and gene targeting in ES cells where the former technology is random, based on tagged sequence and high throughput while the latter technology confers a unique know-how to generate specific gene targeted knockout mice (Guan *et al.* 2010). Both technologies are based on homologous recombination and ES cells. Homologous recombination has long been identified both in yeast and bacterial cells as a process of self-repair of DNA damage in both organisms (Mak 2007). Later it has been shown that homologous recombination is the genetic phenomenon that occurs in all forms of life. Somatic recombination of part of T and B cell receptor genes indicated the possible existence of the mechanism of homologous recombination in mammalian cells (Mak 2007). Indeed homologous recombination plays crucial role in several biological processes such as DNA repair, DNA replication fork rescue, meiotic chromosome segregation and telomere maintenance. For example, prominent homologous recombination is known to be triggered during programmed double-strand breaks of DNA at a time of mating type switching in *S. cerevisae* and meiosis. The functional abnormality in homologous recombination is also implicated to play an important role in pathogenesis of some diseases including Bloom's syndrome, Fanconi anaemia as well as in breast and ovarian cancer patients with mutated BRCA1 and BRCA2 genes (Mak 2007; Krejci *et al.* 2012).

Many pathways are implicated in homologous recombination-mediated repairing of the double stranded DNA break. However, predominantly the repair of double-strand DNA break is accomplished by processing the broken end of chromosome to confer it a single stranded DNA tails. The single stranded DNA tail enables the broken chromosome to invade and copy the genetic information from the homologous chromosome. Besides, the homologous recombination of the double-stranded DNA break can also be achieved when it happens in closely repeated sequences by a process of single-stranded annealing. Recombinases are a strongly regulated class of enzymes known to catalyze the homologous recombination. By manipulating the mechanism of homologous recombination and the biology of ES cells Capecchi's and Smith's group (Thomas & Capecchi 1987) demonstrated and detected the homologous recombination between mammalian ES cell and plasmid DNA introduced to mammalian cells for the first time. Furthermore, both pioneered the incorporation of mutation in to mammalian cells by the mechanism of homologous recombination through introduction of a plasmid harboring foreign DNA. The independent efforts of the three groups of Mario Capecchi, Marti Evans and Oliver Smiths laid unprecedented foundation for homologous recombination-based generation of genetically modified mammalian model with specifically targeted gene such as knockout mouse. These achievements have led to the 2007 Noble Prize award in physiology or medicine.

The generation of conditional knockout mouse has been attained by sandwiching a conditional knockout allele between two palindromic sequence sites also called *loxp sites*, which are in turn inserted into two introns of a gene or at opposite end of the gene. When *cre recombinase* is expressed in mice harboring an allele sandwiched by palindromic sequence or *loxP* sites, it recognizes and subsequently cleaves the *loxp* sites including the conditional knockout allele. Consequently, it renders the deletion and inactivation of the gene in a tissue specific and time controlled manner **(Fig. 3)** (Hayashi & McMahon 2002). However, the most challenging part of the generation of conditional knockout mouse is time it requires and difficulty of designing the correct targeting vector. To generate the targeting vector, the traditional strategy has been the identification of suitable restriction enzyme sites in the proximity of the gene that facilitates the ligation of several DNA fragments including homology arms, positive selection marker, and negative selection marker as well as the *loxp* sites both in genomic DNA and cloning vectors. However, the drawback of this strategy is the potential absence of proper restriction enzyme sites at suitable locations. Nevertheless, a much easier strategy called recombineering presented a unique way of employing homologous recombination to build the targeting vector (Muyrers *et al.* 2001; Copeland *et al.* 2001).

5.1.1.5.2.1. Recombineering

Recombineering or recombinogenic engineering has shortened the time required to make a targeting plasmid construct and enabled the insertion of *loxp* sites and other previously mentioned DNA fragments anywhere in the gene. Yeast was the first organism where successful recombineering is reported (Baudin *et al.* 1993); however, recombineering has become more popular in *Escherichia coli,* as bacteria have an obvious superior advantage over the yeast system: maneuvering the recombinant DNA in E.coli is less laborious than in the yeast and the recombinant DNA from bacteria system can be directly used (Muyrers *et al.* 2001). The use of phage-encoded proteins such as proteins from the *red* genes of bacteriophage λ enabled homologous recombination-mediated insertion of linear double stranded DNA fragment such as *loxP* sites and selection markers into the DNA of cloning plasmids, BACS or PACS owing to competent establishment of homologous recombination in *E.coli* (Muyrers *et al.* 1999; Lee *et al.* 2001; Swaminathan *et al.* 2001; Zhang *et al.* 1998).

Exo and *bet* genes are the two indispensable *red* genes of bacteriophage λ needed for efficient recombination in *E.coli*. The product of *exo* gene, 5'-3' exonucleases, is essential to generate 3' single stranded DNA overhangs whereas the product of *bet* gene, pairing protein (beta), facilitates the annealing of 3' single stranded DNA overhangs generated by 5'-3' exonucleases to the complementary sequence of the 3' single strand DNA over hangs on the cloned DNA (Stahl 1998; Poteete 2001). Linear double stranded DNA can easily be degraded by RecBCD exonucleases in E. coli, however, λ encoded Gamma protein (Lee *et al.* 2001) blocks the exonuclease activity of RecBCD (Zhang *et al.* 1998; Murphy *et al.* 2000). The λ recombination genes are expressed from a defective prophage which has been integrated into the chromosome of *E.coli (Yu et al. 2000).* By expressing the recombination genes under control of a strong λ P_L promoter from defective prophage expression system Lui et al. (Liu *et al.* 2003) maneuvered *E.coli* recombineering and demonstrated a rapid, reliable and repeatable technique to construct conditional knockout targeting vectors.

To demonstrate the efficiency of *E.coli* recombineering system Lui et al (Liu *et al.* 2003) targeted the *Evi9* gene that encodes zinc finger transcription factor. They constructed the targeting vector through homologous recombination-mediated insertion of *loxP* sites, positive and negative selection markers into the DNA of BAC. To avoid difficulty that emanates from the introduction of *loxP* sites in the BAC because of the existence of many *loxP* sites, Liu et al (Liu *et al.* 2003) subcloned 10-15-kb fragment of BAC DNA into pblue-script (pSK blue) plasmid prior to insertion of the desired *loxP* sites.

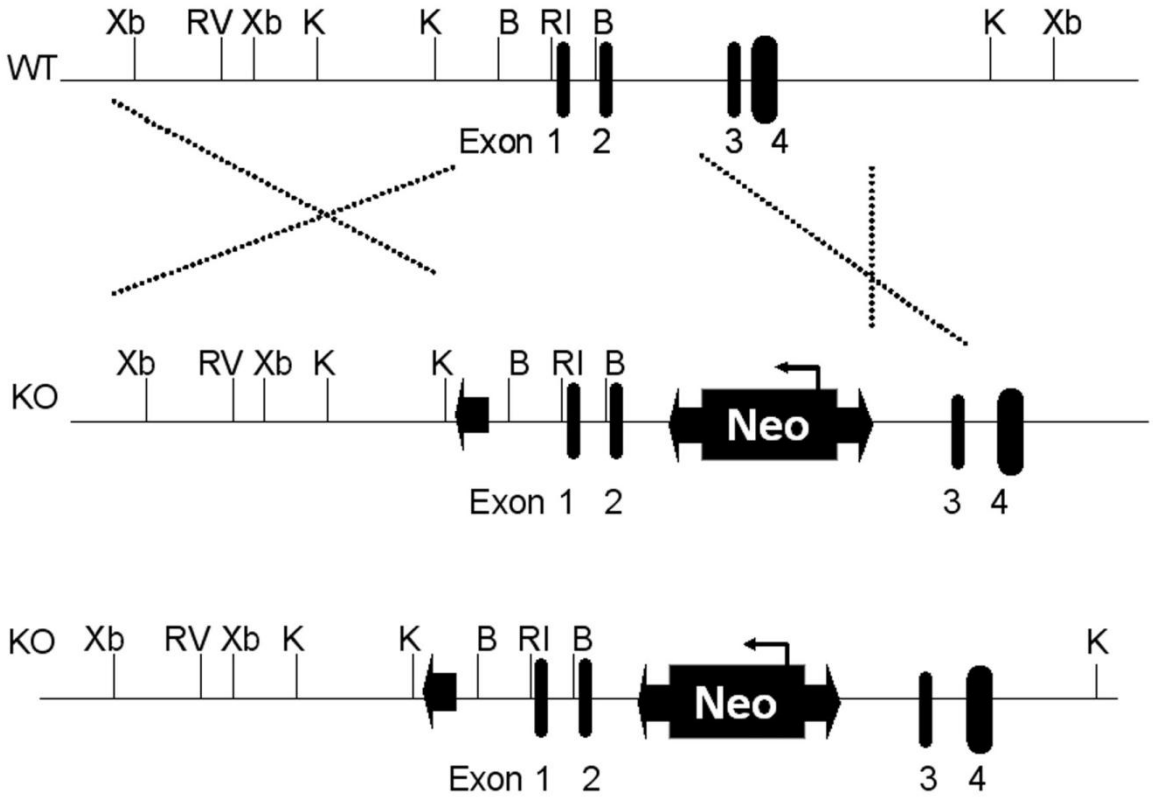


Figure 3. Schematic illustration of conditional knockout construct for a mouse gene X. A conditional knockout construct where exon 1 and 2 of a mouse gene X as well as different restriction enzyme sites are schematically depicted. RV, EcoRV; Xh, XhoI; Xb, Xbal; RI, EcoRI; K: Kpal, B, BamHI; Neo, Neomycin resistant gene; WT, wild type; KO, Knock out and black arrows lox P sites.

Subcloning of DNA from BAC to pBluescript plasmid has been achieved through gap repairing (Lee *et al.* 2001). The alternative gap repair approach that utilizes the longer homology arms comprises 200-500 bp increases the frequency of subcloning as opposed the gap repair method, which uses shorter base pairs. With two sets of PCR primers, two 200-500-bp regions of BAC are amplified and these regions highlight the ends of the section to be subcloned-region of BAC by alternative gap repair. Following successful subcloning, Lui *et al* (Liu *et al.* 2003) aimed at introduction of a *loxP* site into subcloned DNA. The introduction of the *loxP* site has been accomplished by homologous recombination-mediated insertion of floxed neomycin resistance gene (*Neo*) cassette (PL452) into subcloned plasmid DNA. To allow efficient expression of Neo gene both in bacterial and mammalian cells, floxed Neo gene is expressed from hybrid EM7

promotor (for bacterial cells) and PGK promoter (for mammalian cells). At the targeted site, a single loxP site is left after floxed *Neo* gene has been removed by cre-recombinase. The last step in the generation of conditional knockout vector is the introduction of the second *loxP* site. This has been achieved by constructing a new selection cassette (PL451). The construction of PL451 has been carried out by introducing *FRT* site upstream of *Neo*, *FRT* and *LoxP* site down stream of Neo in PGKneobpA (Liu *et al.* 2003). FLP recombinase recognizes a *FRT* site and DNA sandwiched between two a *FRT* sites can be removed by *FLP* recombinase in mouse ES cells (Buchholz *et al.* 1998). Thereafter, the expression of Cre recombinase allows the removal of the entire DNA located between the two loxP sites and the expression of Cre recombinase in mouse germ-line allows the generation of the germ-line null allele (Liu *et al.* 2003).

5.1.1.5.3. "Humanized" mouse model

The need for *in vivo* models to mimic the complex human conditions such as multifactorial diseases and biological processes has been previously discussed. Investigation of physiological or pathological process in human has been traditionally limited to non-invasive techniques, exvivo approaches and clinical trials. These approaches are subjected for ethical restrictions and very expensive. However, small animal models, most notably mouse models have become very important tools to overcome limitations of traditional approaches of investigating human diseases as well as complex biological processes (Brehm *et al.* 2010). Indeed, several research advancements have been made and many more breakthroughs are expected from mouse model systems. Nevertheless, mice vary in many ways from human being, plainly mice are not humans and experiments conducted in mouse are not easily or directly translated to human conditions. Another possible way to bridge the gap between mice and human is the use of monkeys and chimpanzees as model animals for biomedical studies. However, in Europe for example the use of chimpanzee as a model animal is prohibited and new financial support for chimpanzee research has been terminated in USA by US National Institute of Health (NIH). It has been long sought to craft an advanced mouse model such as humanized mouse or mouse-human chimeras that presents and enables mimicking and recapitulating the human physiological conditions and biological processes (Shultz *et al.* 2012; Shultz *et al.* 2007).

Humanized mice are produced either by expression of human genes in mice or through transplantation of human tissues into immunodeficient mice. The successful generation of humanized mouse models through human tissue transplantation such as hematopoietic stem cells (HSCs) or peripheral blood mononuclear cells (PBMCs) conferred an important opportunity to dissect complex biological process (Shultz *et al.* 2012; Shultz *et al.* 2007). The historical account that describes athymic or nude mouse (Flanagan 1966) deciphers the beginning of utilization of humanized mouse as a model for human diseases. The detection of mutations in severe combined immune deficiency (*scid*) led to further progress in the development of humanized mouse models (Bosma *et al.* 1983). The availability of knockout and transgenic technology facilitated both qualitative and quantitative expansion of the humanization of mice through engraftment of human tissues and cells (Brehm *et al.* 2010). Furthermore, the immunodeficient mouse which bears mutations on IL-2 receptor gamma chain (*IL2rγc*) brought the advancement in the generation and application of humanized mouse models for human diseases (Shultz *et al.* 2007). *IL2rγc* encodes a very important signaling component for the action of cytokines such as IL-2, IL-4, IL7, IL-9, IL-15 and IL-21(Shultz *et al.* 2007). For example, Natural Killer (NK) cell

development has been completely prevented by disrupting *IL-7* and *IL-15* genes, which in turn helped to engraft human tissues in these mutant mice. Severely immunocompromised mice have been generated by selectively breeding IL2rγc mutants with other mouse strains such as *SCID, NOD, Rag1* or *Rag2* gene mutations and consequently this strategy enabled successful and easy transplantation of human tissues. Some of the commonly used mutant mouse strains generated by these combination methods include *Rag1-/-γc-/-, Rag2-/-γc-/-, NOD/shi-scdi/ γc-/-,* and *NOD/scid/ γc-/-* (Shultz *et al.* 2011).

By using these mutant mouse strains, the advanced generation of humanized mice has been continued. Currently, the attempt is to generate transgenic humanized mice harboring human cytokine genes and HLA class I and II immune system with the objective of introducing strong adaptive and cellular human immune system into mouse by taking the advantage of the previously mentioned immuno-compromised mouse strains (Shultz *et al.* 2011; Willinger *et al.* 2011). Contribution of the humanized mice is important and it can serve as a crucial tool in unraveling the complex biological process and pathogenesis of human diseases such as hematopoiesis, immune system, cancer immunity, infectious diseases and autoimmunity (Shultz *et al.* 2011). Successful hematopoiesis has been achieved by engrafting HSCs in IL2rγ-/- immunodeficient mice with the aim to generate physiologically competent immune system that supports continuously available multiple lineage of cells, which are eventually needed to generate both innate and adaptive immunity. When HSCs derived from fetal bone marrow, fetal liver and adult bone marrow are transplanted into humanized mouse, the number of $CD4^{+}CD25^{+}FOX3P^{+}$ regulatory T (T_{reg}) cells in the thymus are higher in those mice which have received HSCs of fetal origin than HSCs of adult. This suggests that intrinsic differences exist between fetal and adult HSC populations and this difference is responsible for different immune systems at early stage and adult stage of development (Mold *et al.* 2010). With the different modeling approach that has utilized *IL2rg*$^{-/-}$ mice, efficient and reliable transplantation of human cells with low number of HSCs has been attained compared to efficiency and reproducibility of HSC transplantation achieved with other immuno-deficient mouse models (Shultz *et al.* 2007). Several lineages of hematopoietic cells **(Fig. 4)** such as red blood cells, platelets and T-cells are effectively differentiated from HSCs and progenitor cells using *IL2rg*$^{-/-}$ mice (Ishikawa *et al.* 2005). Furthermore, the *in vivo* function of HSCs derived from induced pluripotent stem (iPS) cells has been obtained from the humanized mouse in which HSCs are effectively transplanted (Tian *et al.* 2009).

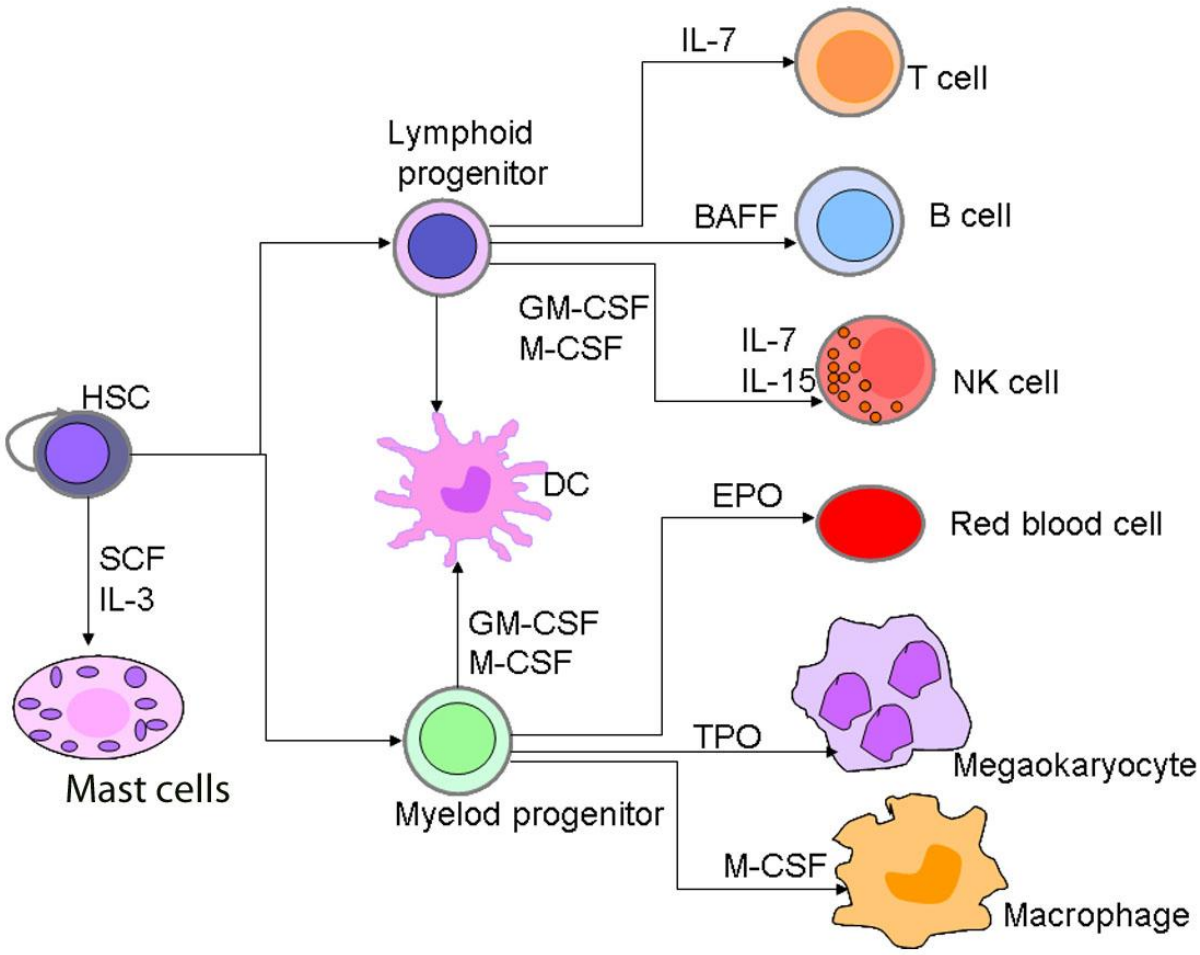


Figure 4. Expression of cytokines of human cells from immunodeficient *IL2rg*$^{-/-}$ mice engrafted with human hematopoietic cell and subsequent functional immune cell development. The picture depicts that the different stages of hematopoietic cell development, which is characterized by expression of specific cytokines. HSC, hematopoietic stem cell; DC, dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; EPO, erythropoietin; BAFF, B cell-activating factor; M-CSF, Macrophage colony-stimulating factor; NK natural killer cell; SCF, stem cell factor; TPO, thrombopoietin (Shultz *et al.* 2012)

Humanized mouse has also become an important tool to study infectious diseases. The study of infectious diseases using model organism has been very challenging because most of causative agents of infectious diseases such as human immunodeficiency virus (HIV), plasmodium, dengue virus and filariae distinctly cause human diseases. However, humanized mouse has become an important tool to investigate the pathogenesis of human specific infectious diseases. It is also used in preclinical trials in the investigation of efficacy trial of pharmacological compounds and it has also become a helpful tool in vaccine trials (Shultz *et al.* 2007). For example, humanized mouse which has been used as a model for HIV infection with either chemokine receptor 5 (CCR5) or CXC chemokine receptor 4 (CXCR4)-tropic strains demonstrated viral infection (plasma HIV RNA copy number 10^4-10^5/ml) that is comparable to the level of HIV viral load in human patient (Zhang *et al.* 2007). CCR5-tropic HIV strains are the main transmitted strains until

late phase of the diseases (Philpott 2003) whereas CXCR4-tropic strains usually appear in the advanced stage of HIV diseases and accelerate the immune deficiency (Arrildt *et al.* 2012). Transplantation of human CD34+ stem cells that lack CCR5 to treat myeloid leukemia is reported to completely cure a HIV infection (Hütter *et al.* 2009). CCR5 deletion using engineered zinc finger nucleases (ZFNS) in human CD34+ HSCs does not affect the engraftment capacity of HSCs. However, when CCR5 lacking HSCs are transplanted into *NOD/SCID/ IL2rγ*$^{-/-}$ mice HSCs differentiated into CCR5 lacking polyclonal mulitilineage progeny. The infection of this mouse with CCR5-tropic HIV-1 has showed dramatic loss of $CD^{+}4$ T-cells (Holt *et al.* 2010).

5.2. Transcription

The discovery of the structure of DNA at the beginning of 1950's has led to the understanding of the programming of hereditary information in the form of the nucleotide sequence in the DNA. After discovery of DNA structure, genome sequences of many model organisms and human were determined. These advancements in turn have given us an understanding of the process of formation of complex forms of life including humans. A proper execution of biological processes such as development, proliferation, apoptosis, differentiation and aging require an accurate and strict coordination of set of steps of gene expression. Gene transcription, the process of executing a genetic instruction in a cell by copying a particular portion of DNA nucleotide sequence also called a gene into another nucleotide sequence called RNA, is one of the most crucial cellular processes to regulate cell growth and differentiation. Transcription is mediated by enzymes called RNA polymerases (Pol) and general transcription factors (Maston *et al.* 2006; Hahn 2004).

Transcription is a more complex process in eukaryotes compared to prokaryotes such as bacteria; however, transcription mechanism and its basic regulation are conserved. For example bacteria have one Pol but eukaryotes have three RNA polymerase enzymes (Pol I, II and III) (Lee & Young 2000). Eukaryotic RNA polymerases have more subunits than bacterial Pol though subunits are homologous to bacterial Pol. Sigma (σ) factor has an equivalent function of general transcription factors of eukaryotic transcription system. All three forms of RNA polymerase have their own specific general transcription factors (Lee & Young 2000; Grummt 2003; Schramm & Hernandez 2002).

5.2. 1. The eukaryotic transcription machinery

Despite the structural similarity with each other, the three eukaryotic RNA polymerases transcribe distinct type of genes. RNA Pol I and III transcribe non-protein coding RNAs such as transfer (tRNA), ribosomal RNA (rRNA), and various small RNAs. RNA Pol II transcribes most genes including protein-coding genes (Alberts *et al.* 2002). The binding of RNA Pol II to the gene specific regulatory factors in the close proximity of transcription initiation site activates transcription. To carry out an accurate transcription of protein coding genes, RNA Pol II requires three important factors such as general (basic) transcription factors (GTFs), promoter specific activators, and co-activators. GTFs are indispensable for initiation of transcription and have been reported to initiate transcription *in vitro* (Orphanides *et al.* 1996). GTFs are comprised of RNA Pol II itself, TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH; however, for *in vivo* transcription an additional multi-subunit complex called a mediator is required (Malik & Roeder 2005; Conaway *et al.* 2005).

To form a transcription preinitiation complex (PIC), a bacterial equivalent of a closed complex, GTFs including Pol II assembles at core promoter (Alberts *et al.* 2002). During PIC formation, binding of TFIID to RNA Pol II is the first step and the subsequent binding of PIC to core promoter guides RNA Pol II to transcription start site (TSS) (Maston *et al.* 2006). Consequently, the binding of RAN Pol II to TSS leads to an important conformational change that results in the melting of 11-15 base pairs of DNA nearby TSS. The melting of the promoter at TSS facilitates the formation of an open complex by positioning the template strand of the promoter to the active cleft site of pol II (Wang *et al.* 1992). Formation of the first phosphodiester bond during synthesis of RNA underlines the initiation of transcription (Holstege *et al.* 1997). Before successful synthesis of full length RNA it is reported that Pol II synthesizes many short RNAs consisting of 3-10 bases. Following synthesis of first 30 bases of RNA it is believed that Pol II leaves core promoter and continues the next stage of transcription called elongation (Luse & Jacob 1987). Once RNA Pol II is released from the promoter, other components of PIC and the mediator remain on the core promoter. Current model suggests that transcription is cyclic where the initiation of PIC occurs only once. After the first cycle of efficient transcription, transcription is reinitiated through recruitment of RNA Pol II-TFIIF and TFIIB **(Fig. 5)** (Maston *et al.* 2006).

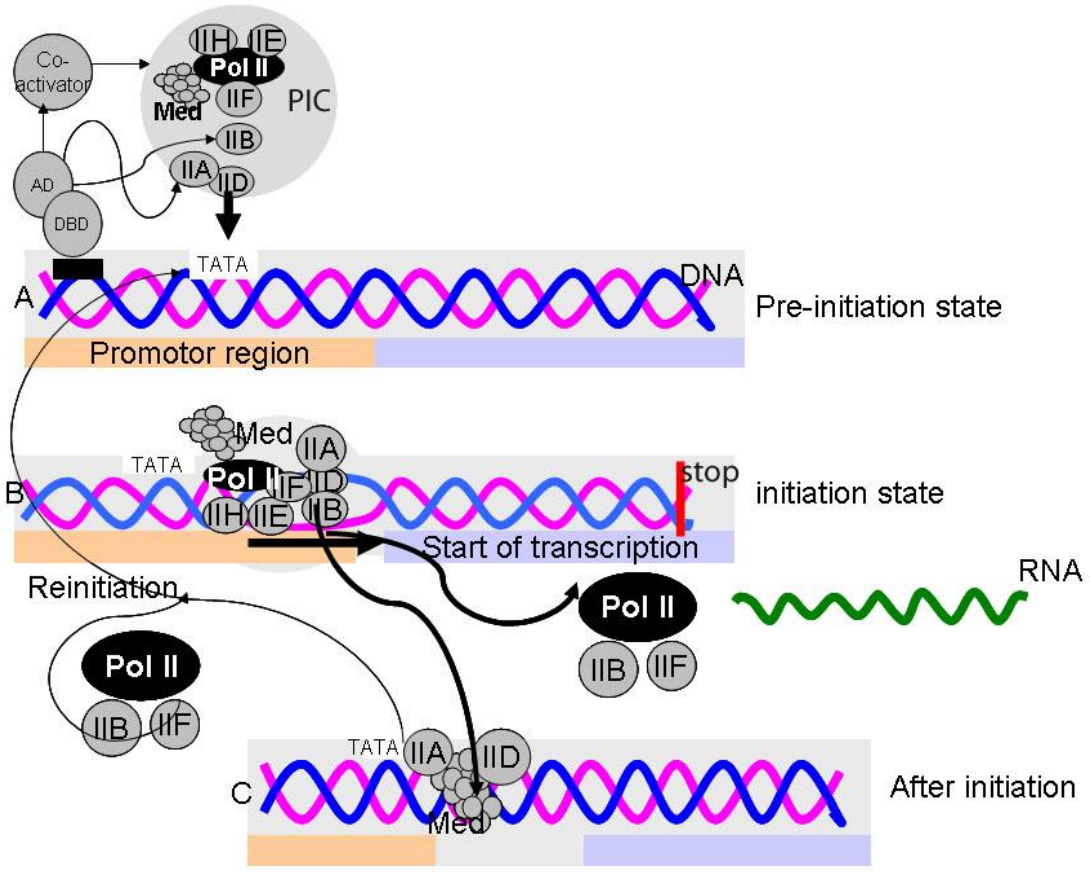


Figure 5. Schematic depiction of state of the eukaryotic transcription machinery: before, during and after transcription initiation. To trigger successful transcription three different

factors such as general transcription factors (GTFs) including RNA Pol II, activators and co-activators are important in eukaryotic cells. GTFs include RNA polymerases itself and transcription factor IIA, IIB, IID, IIE, IIF and IIH. PIC is formed when GTFs bind core promoter region of a gene. After PIC binds the core promoter, RNA Pol II will be released from the complex and activates transcription by binding transcription start site (TSS). Activators, which usually bind specific regions of DNA upstream to the core promoter, further stimulate formation of PIC. Activators are proteins comprised of DNA binding and activating domains. Stimulation of transcription by activators is assisted by another group of proteins called co-activators, which act through protein-protein interactions. This model depicts that transcription is cyclic phenomenon where after completion of first cycle of RNA synthesis, RNA Pol II, IIB and IIF return back to core promoter to join in a scaffolding complex comprising the rest of the members of PIC and to reinitiate the transcription (Maston *et al.* 2006; Hahn 2004; Alberts *et al.* 2002). (DBD, DNA binding domain; AD, activator domain; PIC, Preinitiation complex and MD, mediator).

In vitro study demonstrated that the binding of PIC at core promoter stimulates properly initiated transcription at low level, also known as basal transcription. However, high level of transcription can be stimulated by activators also called second class factors. An activator is DNA specific sequence binding protein whose recognition site is located upstream of the core promoter. Activators bring about gene activation by recruiting RNA Pol II through directly contacting different binding site of the enzyme (Ptashne & Gann 1997). There are many activators which have their own known specific DNA binding sequences such as homeobox, helix-loop-helix (HLH), basic leucine zipper (bZIP), fork head, ETS, or Pit-oct-UNC (POU) DNA binding domain (Pabo & Sauer 1992). The mechanism by which the activators bring about increased PIC formation is diverse including direct interaction with one or more parts of PIC (Ptashne & Gann 1997), by activating one or more processes during initiation, elongation or reinitiation(Lee & Young 2000) and or through chromatin modification (Lemon & Tjian 2000).

5.2.2. Transcriptional regulatory elements in eukaryotes

The regulation of expression of eukaryotic protein coding genes occurs at many steps such as transcription initiation, elongation and mRNA processing, transport, translation and stability, however, the major regulation is at transcriptional level (Wray *et al.* 2003). Gene control region is a general term that describes sets of regions of DNA including the promoter where RNA Pol II and GTFs bind to initiate transcription and regulatory sequences where gene regulatory proteins bind to regulate the process of assembly of different components of PIC. In eukaryotes and plants, it is very common to find a region of DNA called spacer which is flanked by promoter regions and regulatory sequences. A given gene that encodes a protein is flanked by regulatory sequences. With combined effort of regulatory proteins, the regulatory sequence controls the expression of a given gene and it determines the rate of gene expression, the cell or tissue specificity of expression and the level of expression (Alberts *et al.* 2002).

5.2.2.1. Promoter

Promoters are loci of a gene that play critical functions in the gene regulation. The principal function of a promoter is properly positioning of the transcription initiation complex. RNA Pol II

transcribed genes of eukaryotes vary with great extent both in temporal and spatial specificity of expression. Therefore this dictates the need for unique and vibrant regulation of gene expression. Conceivably, promoters confer the dynamic and special regulation of gene expression, which is attributed to their architectural diversity that determines the function of promoters (Lenhard *et al.* 2012). The presence of several regulatory elements in the promoter makes the eukaryotic promoter very complex. The complexity of the eukaryotic promoter is due to the presence of a large number of protein coding genes and each of them has very distinct and specific expression pattern that controls the rate, duration and place of the gene expression (Maston *et al.* 2006). However, the concept of gene expression is redefined recently as 75% of human genome is predicted to be transcribed (Djebali *et al.* 2012). Nevertheless, several gene expression patterns which exist in different tissues and cells with different rate and level are accomplished by multiple combinations of factors controlling gene regulation. The promoter consists of two regions called core promoter and proximal regulatory elements (Maston *et al.* 2006).

5.2.2.1.1. Regions of promoters

5.2.2.1.1.1. Core promoters

Core promoter is the region of PIC assembly and landing site for basic transcription machinery. The recognition of core promoter by transcription machinery underlines the correct position of transcription start site (TSS) and the directions of transcription (Maston *et al.* 2006; Hahn 2004). There are three main functional classes of core promoters such as type I, type II and III and one minor core promoter called TCT promoter. Type I core promoters are also called adult core promoters because genes with these types of promoters are specifically expressed in adult peripheral tissues. The common features of this class of promoters are TATA box enrichment, sharp TSS and disordered nuleosomes (Lenhard *et al.* 2012). The TATA box element binds TATA binding protein (TBP), which is a subunit of TFIID (Maston *et al.* 2006; Alberts *et al.* 2002). Type II promoters are also called ubiquitous promoters because the genes and genes with these types of promoters are ubiquitously expressed throughout the life of the organisms. The common feature of ubiquitous promoters is broad TSS, orderly arranged nucleosomes and in vertebrates they lack TATA and are known to have CpG islands. Type III promoters are also called developmentally regulated promoters and genes with this kind of promoters are differentially regulated. Most of them regulate multicellular development and differentiation (Engström *et al.* 2007). Minor functional classes of promoters are also called TCT promoters, which are known to regulate genes with upregulated expression feature. Promoters of this class are characterized by pyrimidine –stretch (TCT) usually rich in TATA box at its initiator sequence (Parry *et al.* 2010).

5.2.2.1.1.2. Proximal promoter element

A region of promoter few hundred genes upstream to the core promoter with binding site for activators is called proximal promoter element. CpG islands, small portion of DNA that range from 0.5kb to 2kb, are typically rich in C+G nucleotide content with high rate of CpG dinucleotides compared to the rest part of the genome. Normally the CpG islands in the promoter

regions are not methylated; however, CpG islands spread all over the genome are methylated (Bird 1987). DNA methylation at CpG island causes transcriptional silencing through blocking the recognition site of the promoters and recruiting chromatin repressive histone-modifying complexes such as a methylation specific protein, for example MeCP2, which in turn binds methylated CpG dinucleotides (Jones *et al.* 1998).

Table III. Summary of components and functions of general transcription factors in humans (adapted from (Thomas & Chiang 2006)).

GTFs	Protein compositions	Function
TFIIA	p35(α), p19(β) and p12(γ)	Anti-repressor; TBP-TATA complex stabilizer; co-activator
TFIIB	P33	Involves in the selection of TSS, TBP-TATA complex stabilizer, recruits Pol II /TFIIF
TFIID	TBP and TAFs (TAF1-TAF14)	Core promoter-binding factor, coactivator, phosphorylates RNA Pol II, activates ubiquitin and histone acetyltransferase
TFIIE	P56(α), and p34(β)	Recruits TFIIH, play role in promoter clearance, involves in the initiation competent RNA Pol II
TFIIF	RAP30 and RAP74	Facilitates the recruitment of RNA Pol II to the promoter through direct binding Involves in the recruitment of TFIIE and TFIIH Also involves in TSS selection Assist promoter escape of RNA Pol II Increases the efficiency of RNA pol II elongation
TFIIH	P89/XPB, p80/XPD, p62, p52 p44, p40/CDK7, p38/Cyclin H p34, p32/MAT1, and p8/TFB	ATPase activity for promoter clearance and initiation of transcription Involves in the promoter opening through its helicase activity Involves in nucleotide excision repair Phosophorylates RNA Pol II CTD Have E3 ubquitin ligase activity
Pol II		Major player in transcription limitation, elongation and termination mRNA capping enzyme recruitment CTD phosphorylation, glycosylation, and ubiquitination Facilitates the recruitment of splicing and 3'end processing factors during transcription

5.2.2.1.2. Distal regulatory elements

The distal promoter elements are consisted of enhancers, silencers, insulators and locus control regions (Maston *et al.* 2006). Enhancers are a group of DNA binding sites for transcriptional regulators, which have been demonstrated to regulate transcription both temporally and spatially regardless of their direction and the distance (as remote as 85, 000 bases) from TSS (Blackwood & Kadonaga 1998). SV40 tumor virus genome regions, most notably the first 72 bp at the beginning of the viral genome, have demonstrated tremendous transcriptional enhancement of heterologous human gene with promoter and are the first reported enhancers (Lee & Young 2000; Banerji *et al.* 1981) whereas immunoglobulin heavy chain locus is the first identified human enhancer (Banerji *et al.* 1983). The division between enhancer and proximal promoter element is not clear. For example, an activator that binds an enhancer element in one gene may bind proximal promoter element in other gene. However, enhancers can be located anywhere distally from the core promoter including upstream of hundreds of kilo base pairs from the core promoter or in the introns located downstream of the core promoter as well as 3' of the gene (Lettice *et al.* 2003). DNA looping model is a widely accepted model to enlighten how enhancers exert their influence on distantly located core promoter. The DNA looping model is based on thermodynamic concept first discovered in *E.*coli (Dunn *et al.* 1984) which explains how enhancers create physical contact with the core promoter by looping out the intervening DNA (Vilar & Saiz 2005).

5.3. RNA Biogenesis

Eukaryotic cells produce several types of RNA and these RNA are broadly divided into protein coding and none protein coding RNAs. Coding genes in a given cell denote amino acid sequence of proteins and are copied to single stranded nucleic acids also called messenger RNAs, which in turn are translated into proteins. Therefore mRNA mediated transfer of genetic message from nucleus to cytoplasm and subsequent translation of the message into functional proteins is a multi-step, critical and complex process that requires very strict regulation. In eukaryotes, RNA Pol II transcribes heterogeneous nuclear RNA which is meticulously processed by different mechanisms including 5'-methyl guanosine addition, splicing of introns, polyadenylation and cleavage of 3' end (Alberts *et al.* 2002). The process of posttranscriptional regulation of mRNA begins from the very early stages of transcription until mature mRNA is translated at ribosome into the functional protein (Jensen *et al.* 2003). CTD of the largest domain of RNA Pol II has been shown to facilitate each process of pre-mRNA processing suggesting that transcription process and regulation of mRNA biogenesis are coupled cellular processes (Fong & Bentley 2001).

The processing of transcript is attained through interaction between maturing mRNA transcript and various proteins also called mRNA binding protein (RBPs) which facilitate the process of mRNA maturation (Jensen *et al.* 2003). When processing of nascent mRNA is properly completed the transcript will be transported to cytoplasm through nuclear pores and the transportation of the transcript is assisted by complex formed by motor proteins and RBPs or through signal recognition particles (Jensen *et al.* 2003). Furthermore, the surveillance process known to eliminate pre-mRNAs and mRNA transcripts with aberrations or mutations acts

simultaneously with mRNA biogenesis and consequently allows the translation of only accurately transcribed mRNAs into functional proteins (Maniatis & Reed 2002).

5.3.1. Post-transcriptional gene regulation

5.3.1.1. RNA capping

Unlike in prokaryotes where mRNAs are transcribed exclusively by RNA polymerase enzyme, in eukaryotes generation of mRNA is accomplished by several processes among which transcription is the first essential step. However, among other posttranscriptional modifications, capping is the first post-transcriptional modification of eukaryotic pre-mRNAs. Immediately after synthesis of about 25 nucleotides of RNAs 5' end, enzymatic modification of guanine nucleotide at 5' end of the new transcript pre-miRNAs begins. These enzymatic modifications which are also called 5' RNA capping is carried out by three sequentially acting enzymes. The three enzymes are a phosphatase which takes away the phosphate group from 5' end of nascent mRNA, a guanyl transferase which adds guanine mono phosphate (GMP) to 5' end and a methyl transferase which adds methyl group to guanosine (Alberts *et al.* 2002; Cho *et al.* 1998). Phosphorylated RBP1 at serine-5 of CTD heptad repeats position by TFIIH has been demonstrated to bind the three enzymes suggesting that RNA Pol II mediated transcription and 5'capping are coupled from the very beginning of transcription initiation. The interaction between RNA pol II with phosphorylated CTD at RBPp1 subunit and capping enzymes is not stable; however, H8-a CTD-kinases inhibitor impedes this complex. The deletion of a part of CTD, which is known to have no effect on transcription, has significantly decreased in the capping level of nascent mRNA transcript (Cho *et al.* 1997). Furthermore, structural studies revealed that the exit groove of pre-mRNA from RNA Pol II is located next to CTD (Cramer *et al.* 2001).

Capping of pre-mRNA is carried out during transcription stages namely initiation and elongation. Phosphorylation of CTD at serine-5 of CTD heptad repeats elicits a sequence of events such as removal of general transcription factors from CTD, capping complex machinery recruitment and allosteric activation of capping enzymes. When capping is properly completed the capping machinery is removed through dephosphorylation of the phosphate group from serine 5 position. The removal of phosphate group from serine position is subsequently followed by phosphorylation of serine 2, which in turn facilitates the other mRNA processes such as mRNA maturation by recruiting specific factors required for the processing of mRNA. This suggests that the mRNA capping and processing are dynamic and coupled processes (Cho *et al.* 2001; Komarnitsky *et al.* 2000).

5'-Methyl cap that is attained by adding 7-methylguanosine cap on the first synthesized nucleotide (**Fig. 6**.) is a land mark for eukaryotic mRNAs, which allows the cells to identify mRNA from other types of RNAs existing in the cell. Besides, it is implicated to play a key role in many steps of mRNA processing and maturation including mRNA stabilization by preventing mRNA from exonucleases. Stabilization of mRNA is essential for most of mRNA translation. It also facilitates transcription, splicing, polyadenylation and export of mRNA from the nucleus to the cytoplasm (Cowling 2010). In mammalian cells, 2-CTD kinase is a known transcription elongation factor also known as Positive transcription elongation factor (P-PEFb). It

phosphorylates hsPT5, another transcription elongation factor, suggesting that P-PEFb is a major player in the coupling process of transcription and 5'capping of mRNA (Cho *et al.* 2001).

5.3.1.2. RNA splicing

In eukaryotic genes, the sequence that encodes the protein, also called exons, is intervened by non-protein coding sequences known as introns. During gene transcription both exons and introns are transcribed into mRNA, however, introns are precisely and efficiently excised and removed by the process known as RNA splicing. However, important question to be answered is why genes are intervened by non-coding introns and wasting their time by splicing introns? The proposed explanation for the existence of numerous introns is that they confer a special ability to DNA and space for genetic recombination of exons of different genes, which allows the generation of valuable and new proteins during the course evolution (Alberts *et al.* 2002). The process of removal of introns and production of mRNA is either constitutive or unique mRNAs each with ability to be translated into specific protein can also be generated by the process known as alternative splicing from a particular pre-mRNA by different grouping at 5' and 3' splice sites (SS) of various spliced exons (Shin & Manley 2004). Thirty-sixty percent of human mRNAs are originated from alternatively spliced transcripts and 70-90% of alternatively spliced transcripts are predicted to be translated into functional proteins (Modrek *et al.* 2001).

Both alternative and constitutive splicing is carried out by macromolecular machinery also called spliceosome, which is composed of U1, U2, U3, U4/ U6 and U5 small nuclear ribonucleoprotein (snRPs) and many other proteins (Wahl *et al.* 2009). The classical spliceosome assembly model describes the process of spliceosome formation as a sequential phenomenon that begins with binding of U1 snRNP at first 5' splice site (SS). Thereafter, base pairs are subsequently formed at 5' splice junction followed by binding of the branching site of introns by branch site binding proteins (BBP) and U2 snRNP binds the 3'SS that ultimately leads to the formation of complex A also called prespliceosome. Tri-snRNP [U4/U6.U5] addition to the complex A results in the formation of complex B, where U4 and U6 snRNPs are firmly held with base pair interaction. Subsequent conformational changes with alterations in composition of the complex including removal of U1 and U4 snRNPs and recruitment of other factors like CDC5L activate the spliceosome (Alberts *et al.* 2002; Makarov *et al.* 2002).

The recognition sites for spliceosome assembly on the introns of nascent pre-mRNA includes GU at 5' SS, branch point A, polypyrimidine tract and AG at 3' SS. The activated spliceosome excise introns first by cleaving at 5' SS then by forming lariat structure where the first nucleotide of the intron is bound through 2'-5' phosphodiester to adenosine at the branching point nearby 3'ss (Alberts *et al.* 2002; Matlin & Moore 2008). Thus spliceosome is very dynamic, cooperative and very complex machine composed of five stable snRNAs, large number (more than 300) of firmly and transiently interacting proteins (Alberts *et al.* 2002; Jurica & Moore 2002). These proteins are scaffolding proteins, RNA binding factors and proteins with domains rich in arginine and serine repeats. Crucial enzymatic activity for splicing includes ATPase, GTPase and cis-trans prolyl isomerase (Matlin & Moore 2008).

5.3.1.3. RNA polyadenylation

A poly (A) tail is an integral component almost in all fully processed mRNAs of eukaryotes. Poly (A) tail has been implicated in several functions almost in all stages of mRNA metabolism such as mRNA stability, promoting mRNA translation efficiency and facilitating the transportation of mRNA from nucleus to cytoplasm (Lewis *et al.* 1995). 10-30 bases upstream of polyadenylation or cleavage site each mRNA contains one of the most conserved sequences called polyadenylation signal sequence (AAUAAA). Another sequence element that is reported to be found in 70% of mammalian mRNA is GU, which is located 20-40 bases downstream of cleavage site (Lewis *et al.* 1995; Tabaska & Zhang 1999). These two genomic sequences which are transcribed by RNA pol II specify the cleavage site and can determine the strength of the signal of poly (A) (Alberts *et al.* 2002; Chen *et al.* 1995). Eventually these sequences are recognized by a group of RNA binding proteins and enzymes that process mRNA (Alberts *et al.* 2002).

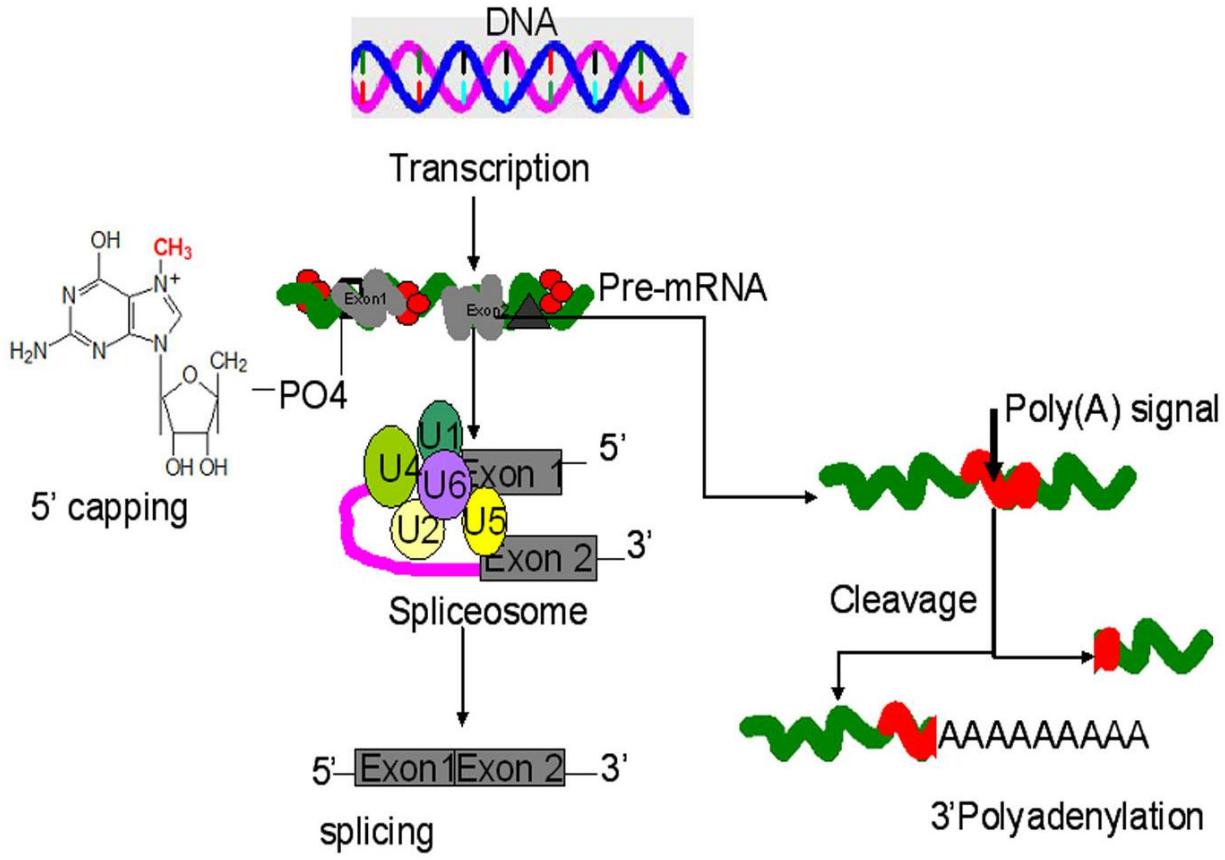


Figure 6. Post-transcriptional processing of mRNA. Post-transcriptional processing of mRNA such as 5' capping, splicing and polyadenylation during RNA biogenesis are schematically illustrated.

Two multisubunit proteins called cleavage stimulation factor (CstF) and cleavage and polyadenylation specificity factor (CPSF) travel with RNA pol II during transcription and upon recognition of AAUAAA sequence they transfer to and bind the 3'end mRNA precursor (Manley 1995). CPSF also links polyadenylation with transcription as it has been shown to exist in the stable complex with TFIID in PIC; however, as CTD of RNA pol II is phosphorylated during transcription elongation, CPSF dissociates from TFIID and joins RNA Pol II (Dantonel *et al.* 1997). CPSF through interaction with poly-A-polymerases (PAP) and CstF define polyadenylation site and the cleavage site (Murthy & Manley 1995). CstF and CPSF bind immediately the emerging RNA from RNA pol II (Alberts *et al.* 2002) and other proteins which is followed by subsequent recruitment of PAP, and thereafter PAP dictates the synthesis of poly(A) tail by adding one A at a time. The synthesis of poly (A) tail with PAP is carried out without any template which highlights the fact that poly (A) tail in eukaryotic mRNA is not encoded (Alberts *et al.* 2002). Furthermore, polyadenylation is also linked with splicing, as *in vitro* studies demonstrated enhanced splicing of 5'-terminal intron by polyadenylation signal, which further suggests the binding between last intron of mRNA and polyadenylation (Wassarman & Steitz 1993). After cleavage of pre-mRNA at 3' end, RNA pol II proceeds transcription, in some cases hundreds of nucleotides can be transcribed. Nevertheless, because of the lack of 5' capping, the transcript will be degraded by 5'→3' exonucleases. The degradation of the transcript transcribed after pre-mRNA cleavage is believed to be the cause for the release of RNA pol II from DNA (Alberts *et al.* 2002).

5.3.2. Transportation and localization of RNAs

In eukaryotic cells subsequent to transcription and post-transcriptional processing, transportation and localization of different species of RNAs from nucleus to cytoplasm is indispensable for gene expression. The transportation of RNAs and RNPs is facilitated by nucleocytoplasmic transport machinery and nuclear pore complexes (NPCs) (Görlich & Kutay 1999; Stewart 2007). A conserved protein family, also known as nuclear protein receptors or karyopherines or import-β family recognizes nucleotide motif on cargo RNA such as a nuclear localization signal (NLS) or nuclear export signals (NEs) and facilitates the nucleocytoplasmic transport of various species of RNAs through direct interaction with NPCs (Görlich & Kutay 1999). The nuclear receptor protein super families have a common RanGTP-binding N-terminal motif and are regulated by RanGTPase. In the nucleus, Ran stays in GTP bound state through regulatory action of Ran-GDP-exchange factor (RanGAP) whereas in cytoplasm it switches to GDP bound state through regulatory action of RanGEF (RanGTPase activating protein). The karyopherines are also known as exportins transport cargos of nuclear orgin bound to RanGTP only to the cytoplasm and the hydrolysis of RanGTP releases the cargo at desired location in cytoplasm. The cargo of cytoplasmic origin bound to RanGDP only is translocated into the nucleus (Görlich & Kutay 1999; Moore & Blobel 1993; Köhler 2007).

Transportation of different species of small RNAs such as tRNA, microRNAs (miRNAs), small nuclear RNAs (snRNAs) and ribosomal RNAs (rRNAs) can be executed with the relatively easy mechanism described for exportins. However, mRNA uses different receptors and does not need RanGTP-RanGDP gradient (Rodriguez *et al.* 2004; Conti & Izaurralde 2001). The different and complex mechanism of transportation of mRNA is required because mRNA differs in length, structure and sequence from other small RNAs species. These unique features of mRNA require

specific group of exporters, which have ability to channel mRNA to cytoplasm in a harmonized manner with posttranscriptional processing of mRNA and RNPs assembly. This further suggests that RNA transcription, processing, exporting and translation function in a coordinated fashion to bring about the gene expression (Köhler 2007). For example, export adapters bound to pre-mRNAs can serve as important factors to physically link the mRNA molecule after processing and export receptors responsible of mRNA translocation into cytoplasm (Hieronymus & Silver 2003). Tap-p15 complex also called NXF1-NXT1 serves as important general export receptors for mRNAs in higher organisms by facilitating transportation of mRNPs through NPCs (Grüter *et al.* 1998). The exporter can bind mRNA directly or using RNA binding proteins as adaptor and similar to karyopherines mRNA exporters interact physically with the group of proteins that lack secondary structure and are rich in Phel-Gly – repeats also known as FG nucleoporins (Cole & Scarcelli 2006). FGs form a mesh on NPC to create permeability barrier; however, by interacting with FG mRNA exporter allows the cargo to pass through NPC (Köhler 2007; Cole & Scarcelli 2006).

5.3.3. Small RNA biogenesis

Eukaryotic small RNAs belong to the non-coding group of RNAs comprised of 20-30 nucleotides with diverse function including targeting chromatin, mRNA destabilization and translation. Because of these diverse actions that target genome and transcriptome, small RNAs functionally participate in many biological processes such as cell differentiation, developmental process, apoptosis, regulation of metabolism, combating virus, silencing transposons and cell proliferation (Chu & Rana 2007; Filipowicz *et al.* 2008; Kawaoka *et al.* 2009; Kim *et al.* 2009). Their dysregulation has deleterious consequences during development and in the tumorigeneses (Leval *et al,* 2013). Furthermore, recently it has become apparent that they are crucial regulators of mammalian inflammatory response. For example, some specific miRNAs are expressed in activated T lymphocytes. During the course of inflammation these groups of miRNAs target and subsequently repress certain transcription factors, which affect the differentiation of specific inflammatory T lymphocytes (O'Connell, *et al,* 2012). The typical features of small RNAs are the short nucleotide sequence and their association with Argonaute (Ago)-family proteins. Based on the mechanism of biogenesis and the type of Ago proteins they are associated with, small RNAs are divided into three types such as microRNAs (miRNAS), endogenous interfering RNAs (endo-siRNAs or esiRNAs) and Piwi-interacting RNAs (piRNAs) (Kim *et al.* 2009). Two RNase III type proteins namely Drosha and Dicer act on hairpin structure of small mRNAs to generate miRNAs, and subsequently mature miRNAs bind Ago subfamily proteins whereas piRNAs which are also abundant in germ cell lines do not need dicer for biogenesis but they are associated with Piwi subfamily proteins (Vagin *et al.* 2006). esiRNAs like miRNAs are associated with Ago subfamily proteins but they do not require Drosha for biogenesis (Czech *et al.* 2008) and some of them have been shown to involve in post-transcriptional regulation of a gene through targeting RNAs (Kim *et al.* 2009).

5.3.3.1. microRNA biogenesis

miRNA gene is transcribed by RNA pol II with hosts of RNA Pol II transcription factors into pri-miRNAs transcripts usually with many kilobases long, a local hairpin structure and stem-loop structure. However, small numbers of miRNAs associated with Alu repeats are transcribed by

RNA pol III (Lee *et al.* 2002; Garzon *et al.* 2009). In the nucleus, RNase III Drosha cleaves the stem-loop structure from primary transcript and the cleavage product, small hairpin product, is called pre-miRNA or precursor-miRNA (Lee *et al.* 2003). The cleaved out remnant fragment is believed to be degraded in the nucleus; however, whether 5' and 3' fragments which surround stem-loop structure have functions is remained to be seen. Drosha is 160 kDa protein with two tandem RNase II domains and an essential domain for binding of double stranded DNA also called double stranded DNA binding domain (DBD) (Wu *et al.* 2000; Han *et al.* 2004).

Drosha is an integral component of a large complex called microprocessor that is 500 kDa in *D.melanogaster* and 650 kDa in human. Drosha interacts with a protein called DiGeorge syndrome critical region gene *8 (DGCR 8)* in humans and Pasha in *D.melanogaster* (Han *et al.* 2004; Landthaler *et al.* 2004). However, function of *DGCR8/Pasha* has been elusive though it is thought to serve as cofactor for Drosha's activity notably in substrate recognition (Han *et al.* 2004) by interacting with single stranded RNA (ssRNA) segment and the stem of Pri-miRNA(Han *et al.* 2006). Besides, the lack of miRNAs in *DGCR* 8 deficient mouse ES cells and ES cells with deleted *DGCR* 8 is shown to have defects both in proliferation and differentiation, which further highlight the crucial role played by *DGCR8* in the processing of miRNA and the role of miRNA in the important biological processes (Wang *et al.* 2007). In animal cells there are many types of pri-miRNAs which do not have any common sequence motif that may serve as recognition sequence for Drosha binding and trimming; however, the tertiary structure of pri-miRNA (both double stranded nearby cleavage site and the large terminal loop) specifies the cleavage site for Drosha (Lee *et al.* 2003; Zeng & Cullen 2003).

It has been shown that pri-mRNA processing is coupled with transcription. Previously it was believed that the splicing reaction occurs before Drosha mediated cleavage of intronic pri-miRNAs; however, it has been shown that the processing of intronic miRNAs can be achieved before the splicing reaction. Besides, the synthesis of mature mRNA has not been affected by Drosha mediated cleavage of intron suggesting that both mRNA transcript and miRNAs can be processed from a common transcript transcribed by RNA Pol II (Kim & Kim 2007). This goes along with the previously described "molecular tether" or "exon tethering" model that demonstrates co-transcriptional assembly of RNA Pol II transcribed exons with spliceosome (Dye *et al.* 2006). Therefore pri-miRNAs processing by Drosha is most likely taking place following binding of mRNA transcript to spliceosome complex, but before cleaving the intron. The localization of pri-miRNA and Drosha to splicing site of pre-mRNA transcript further strengthens the view that Drosha mediated trimming of pri-miRNA and splicing are strongly synchronized co-transcriptional processes (Kim *et al.* 2009; Pawlicki & Steitz 2008). However, the processing of exonic miRNA hairpin by Drosha has been shown to result in destabilization of mRNA transcript with hairpin loop with subsquent reduction in protein production and the same study demonstrates that Dorsha not only processes pri-miRNA but also cleaves mRNAs with long hairpin structure (Han *et al.* 2009).

Subsequent to nuclear processing by Drosha, pre-miRNAs are transported to the cytoplasm for further processing (Kim 2005) and the transportation of pre-miRNA is mediated by a nuclear transport family member protein known as Exportin 5 (EXP 5) whose main cargo is pre-miRNAs (Bohnsack *et al.* 2004; Lund *et al.* 2004). To transport the cargo, EXP5 like any nuclear family protein cooperates with a GTP bound cofactor known as Ran and it binds the cargo using 14 bp

dsRNA with 3' overhang of pri-miRNAs in the nucleus and the cargo is eventually released upon hydrolysis of GTP in the cytoplasm.

Pre-miRNA in cytoplams is further trimmed near the proximal loop by Dicer and 22 nt miRNA duplex is ultimately released. Dicer is a highly conserved protein in evolution and known to exist in all eukaryotic organisms. Following cleavage by Ddicer, an effector complex called RNA induced silencing complex (RISC) is generated by loading 22 nt miRNA duplex onto an argonate protein (Ago). The Ago binding leads to formation of mature miRNA also called the guide strand or miRNA; however, another strand also called passenger strand or miRNA* is subjected to degradation. Which strand is chosen for degradation or for generation of mature miRNA is determined by thermodynamic stability where the strand containing high number of unstable base pairs at 5' end such as GU pair survives to become mature miRNA whereas the strand with GC is prone to degradation (Kim *et al.* 2009; Khvorova *et al.* 2003).

5.3.3.2. Piwi-interacting RNAs biogenesis

piRNAs are a unique group of small non-coding RNAs (22-29 nt) that generates piRNA induced silencing complex (piRISC) particularly in the germ line of many animal species. piRISC ultimately silences the transposable elements so as to safeguard the genomic integrity of the cells of germ line (Siomi *et al.* 2011). Based on extensive investigation of PIWI protein associated piRNAs in flies and mice, two mechanistic models of piRNA biogenesis have been proposed including primary piRNA biogenesis pathway and the ping-pong amplification loop. The primary piRNA biogenesis pathway model describes a mechanism of biogenesis of piRNA that targets multiple transposable elements with initial pool of piRNAs. The ping-pong amplification loop model proposes that class of piRNAs with amplified sequence targets the active transposon. Both proposed models are evolutionary conserved in many species such rats, zebra, fish, frogs and silkworms (Kawaoka *et al.* 2009; Lau *et al.* 2006; Houwing *et al.* 2008; Robine *et al.* 2009).

5.3.3.3. Endo siRNA biogenesis

The biogenesis of endo-siRNA is similar with exo-siRNAs but it is different from miRNAs in which the processing of endo miRNAs for example in flies does not require Dicer2 but Dicer1. Besides, as opposed to exo-siRNAs, enod-siRNAs do not need R2D2; however, particularly endo-siRNAs whose origin is long-stem loops instead of R2D2 require association with LOQs that plays an important role in miRNAs pathways (Siomi *et al.* 2011; Okamura *et al.* 2008). Transposones derived from sense-antisense pairs are the principal precursors of endo-siRNAs. Unannotated regions of genome as well as convergent transcription coding genes can also serve as origin of endo-siRNAs precursors (Chung *et al.* 2008; Ghildiyal *et al.* 2008).

The other type siRNA precursors are single stranded RNAs with ability to form a long stem-loop structure through process of self hybridization and they differ from miRNAs by the presence of longer stems compared to that of miRNAs (Chung *et al.* 2008; Ghildiyal *et al.* 2008). Twenty % of siRNAs bound to Ago2 at least in S2 cells show mutations in their sequence and ADAR mediated RNA editing is thought be the probable cause behind these mutations. This suggests that formation of dsRNA most likely occurs in the nucleus as activity of ADAR is limited only to the nucleus and its lone substrate is dsRNA. The nucleotide modifications at posttranscriptional

level may be the cause for expanding little bulges in the endo-siRNAs precursors. Dicer 2 and LOQs selectively recognize the long dsRNA and the bulge structure. Dicer 2 is believed to play major role in the processing the long dsRNA and LOQs facilitates Dicer 2 binding to dsRNA mismatches as well as it guides dsRNAs to the RISC (Siomi *et al.* 2011; Li *et al.* 2009; Li *et al.* 2009; Nishikura 2006).

5.4. Epigenetic regulation of gene expression

Epigenetics is covalent chemical modification of the DNA or regions around the DNA, which occurs without altering the genetic code or DNA sequence and is triggered by environment or during development to regulate the gene expression in a tissue and context-specific manner (Natoli 2010). These modifications occur from early development to late in adult stage of life in response to random change or environmental pressure. Epigenetic changes confer a unique mechanism through which the genome acclimatizes a new pressure during development and or by environment and these modifications are indispensable for development and differentiation. Post-translational modifications (such as methylation, acetylation and phosphorylation) of histone, methylation and hydroxy-methylations cytosines of dinucleotide sequence CpG DNA motifs and non-coding RNA are the principal mediators of the mechanism of epigenetic modifications (Jaenisch & Bird 2003). Epigenetic modifications can be transient alterations in chromatin and or DNA methylation and they can also be stable alterations that could be inherited to the progeny. Therefore epigenetic modifications give an insight to both temporal or spatial differential gene expression or differentiation pattern in the cells and tissues of an organism irrespective of having an identical DNA (Natoli 2010; Jaenisch & Bird 2003; Jaenisch & Bird 2003).

DNA methylation, chromatin modification as well as promoter and enhancer binding proteins facilitate the localization and binding of the signaling transcription factors such as STATs or NF-kB to genome in response to environmental stimuli (Buecker & Wysocka 2012). The 'epigenetic land scape' (such as the sum total and configuration of DNA methylation, chromatin modification as well as promoter and enhancer binding proteins) is shaped by previous developmental history and environmental pressure in response to a given environmental stimuli. The acute stimuli activate remodeling of the epigenetic land scape and this facilitates the reprogramming of cells by integrating signals in response to stimuli through changing the expression of genes (Jaenisch & Bird 2003; Gordon & Martinez 2010).

5.4.1. DNA methylation

There are several mechanisms by which gene expression is regulated. DNA methylation is a common and well-studied epigenetic mechanism of gene regulation. DNA methylation refers to an enzymatic modification of cytosines where methyl group is added on cytosines at position of 5 exclusively in CpG dinucleotides and provides very crucial epigenetic signaling to regulate genes in transcriptionally repressed state through repressing gene promoters. In animals, the addition of methyl group to cytosine is catalyzed by DNA (cytosine 5) methyl transferases (DNMTs). DNMTs serve as *de novo* DNMTs by adding initial methyl group in DNA sequence or maintenance DNMTs by copying methylation from the sequence of DNA after replication (Kass *et al.* 1997; Hsieh 1994; Boyes & Bird 1992). In vertebrates, DNA methylation confers genomic stability (Maloisel & Rossignol 1998), regulates imprinted genes (Bell & Felsenfeld 2000) and X-chromosome inactivation (Csankovszki *et al.* 2001). Alterations or defects in the pattern of

DNA methylation have a harmful outcome such as prenatal mortality and tumorigenesis. As any epigenetic modifications, DNA methylation can also be inherited and if defects and alterations of DNA methylation triggered by environmental changes are inherited, it can have a deleterious consequence for many subsequent generations (Li *et al.* 1992; Robertson 2005).

A genetic region that contains high frequency of CpG sites is known as CpG islands. In vertebrates, 70-80% of cytosines in CpG islands are methylated. About 70% of promoters in humans are reported to have high CpG content and CpG islands characteristically occure near or at TSS. In mammals methylation is global but very rare that is dispersed in the entire genome except in CpG islands (Bird & Taggart 1980). DNA methylation regulates gene expression by blocking the site where activating transcription factors bind promoters. The mechanism by which DNA methylation regulates gene expression is still poorly understood, nonetheless, excess methylation near to the promoter is associated with low or no transcription. Proper methylation is indispensable for prenatal development and differentiation. Furthermore, diseases such as cancer, lupus, muscular dystrophy and many birth defects are associated with methylation defects (El-Maarri 2004). Tumor suppressor genes are usually repressed during cancer because of hypermethylation; however, with the exception of cell cycle regulatory genes, hypomethylation is a common observation in the whole genome of cancer cells compared to normal cells (Suzuki & Bird 2008).

5.4.2. Histone modification

In the cells of eukaryotes, histones in combination with some other proteins wrap genomic DNA to form a dynamic protein polymer known as chromatin and the repeating unit of chromatin is nucleosome. Chromatin exists in two forms i) euchromatin which is less condensed chromatin known to exist in the region of promoter and enhancer elements and facilitates the transcription by permitting the transcription factors to access the promoter of transcriptionally active genes ii) heterochromatin which is more condensed form of chromatin and known to present in transcriptionally suppressed regions of the genome (Suganuma & Workman 2011). Histone protein is comprised of dimers of each histone (H2A, H2B, H3 and H4). Post translational modifications of N-terminal region of each core histone determine the structural organization of chromatin (Luger *et al.* 1997). There are several types of histone modifications including acetylation, methylation, phosphorylation, ubiquitination, biotinylation, SUMOylation, and poly-ADP-ribosylation (Heemers & Tindall 2007). The modification of histone alters the physical feature of histones, which in turn has influence over nucleosomes and chromatin. The modifications of histones regulate genome accessibility for different transcription activators of gene and, therefore, histone modification is an important and integral component of epigenetic gene regulation. Histone modification can serve as a spot for recruitment and activity of chromatin or nucleosome remodeling protein complexes (Felsenfeld & Groudine 2003; Cosgrove & Wolberger 2005).

Acetylation of histones is the most investigated histone modification. Most notably, acetylation of H3 and H4 has been reported to play a critical role in the organization of chromatin and gene regulation (Eberharter & Becker 2002). The hyperacetylation of histones generally promotes the activation of the gene expression whereas hypoacetylation of histones is often linked with the genes that are not expressed (Munshi *et al.* 2009). The acetylation of Lys (K) residues of H3 and

H4 by an enzyme known as histone acetyl transferase (HATs) enable the transcriptionally repressive heterochromatin to switch to euchromatin that permits transcription. However, the deacetylation of histones by histone deacetylases (HDACs) switches the chromatin to the status of transcription repression. Therefore, acetylation of histones by HATs and deacetylation by HDACs confer histones a dynamic regulatory ability on chromatin and facilitates the gene regulation by opening transcriptionally active chromatin when histone is acetylated and by repressing transcriptionally inactive chromatin (Munshi *et al.* 2009; Allfrey *et al.* 1964; Struhl 1998).

5.5. Hematopoiesis

Hematopoiesis is the process of formation of the cellular components of the blood. It is a very complex process in mammals due to both spatial and temporal differences in the sites of formation of the blood cells. Besides, cells of the hematopoietic system are not stationary as opposed to several other tissues. Nevertheless, all blood cells of the hematopoietic system are derived from hematopoietic stem cells (HSCs) (Dzierzak & Speck 2008). The life span of mature blood cell is short, however; throughout life HSCs differentiate and give rise to billions of blood cells. In adult mammals, HSCs reside in the bone marrow as a rare population of cells and differentiate to progenitor cells, which in turn differentiate into several blood cell precursors and through further differentiation, the precursors yield mature blood cells such as red blood cells, megakaryocytes, myeloid cells (monocytes/macrophages, neutrophils, eoisinophils and basophils) and lymphocytes. Furthermore, like any stem cells, HSCs conserve the ability of self-renewal and differentiation (Orkin & Zon 2008).

The origin of the blood cells has been shown to be extra-embryonic yolk sac during embryogenesis of vertebrates and the formation of blood cells occurs in association with the development of blood vasculature (**Fig. 7**). In mammals, the HSCs migrate from yolk sac to aorta-gonad mesonephros (AGM) region, and fetal liver and finally to bone marrow as their final destination and reside in bone marrow throughout the life of an organism. Recently placenta is also described as residential site of HSCs (Dzierzak & Speck 2008; Orkin & Zon 2008). The features of HSCs differ in each distinct residential site and the differences are attributed to diverse niches in each separate residential site that may promote expansion and/or differentiation in a site-dependent manner, and HSCs have distinct characteristics during each stage of migration. For example, HSCs in fetal liver undergo cell cycle; however, HSCs in adult bone marrow remain quiescent (Dzierzak & Speck 2008; Orkin & Zon 2008; Orkin 2000).

In mammals, the AGM region is the next site of hematopoiesis (Orkin & Zon 2008). In mouse umbilical artery and allantois, hematopoietic and endothelial cells are co- localized (Inman & Downs 2007). Large numbers of HSCs have been shown to reside in mouse placenta (Gekas *et al.* 2005). Placental accumulation of HSCs cells is attributed to both *de novo* generation and colonization of HSCs. However, the contribution of these sites for the final pool of HSCs in adult mammals has not been well understood (Orkin & Zon 2008). Definitive hematopoiesis commences with colonization of fetal liver, thymus, spleen and finally bone marrow. Nevertheless, none of these definitive hematopoietic sites are involved in *de nevo* generation of HSCs (Orkin & Zon 2008). However, in vertebrates it seems that the sites of adult hematopoiesis are not conserved, as different organisms have different sites of adult hematopoiesis. For example,

adult hematopoiesis takes place in the kidney in fish, liver in frog, and bone marrow in mammals and birds (Orkin & Zon 2008).

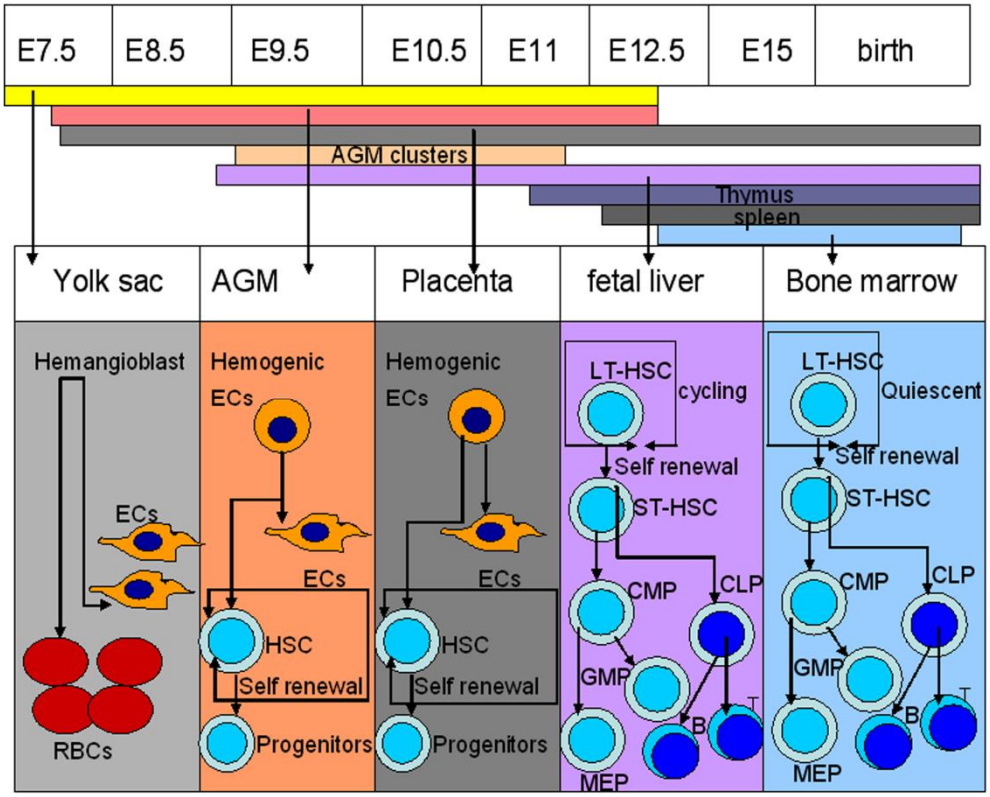


Figure 7. Hematopoiesis during development of mouse. Hematopoiesis first begins in the blood islands of the yolk sac where hemangioblast cells differentiate into red blood cells (RBCs) and endothelial cells (ECs). Later hematopoiesis continues at aorta–gonad meso-nepheros (AGM) region, placenta and fetal liver. Finally the blood cells reside in bone marrow and continue to differentiate into progenitors and precursors that eventually differentiae into mature blood cells throughout adult life. LT-HSCs, long term hematopoietic stem cells; ST-HSCs, short term term hematopoietic stem cells, CMP, common myeloid progenitor; MEP, megakaryocyte eythroid progenitor; GMP, granulocyte macrophage progenitor and CLP, common lymphoid progenitor (Adapted from (Dzierzak & Speck 2008; Orkin & Zon 2008).

5.5.1. Lineage commitment of hematopoietic stem cells

Traditionally, the mature blood cells are divided into two distinct categories including lymphoid and myeloid lineages. T, B and natural killer (NK) cells belong to the lymphoid lineage whereas granulocytes (neutrophils, eosinophils, and basophils), monocytes, macrophages, erythrocytes, megakaryocytes and mast cells belong to the myeloid lineage. These cells which comprise myeloid lineage are diverse both morphologically and functionally. Dendritic cells are unique in

their development as they can be activated from both myeloid and lymphoid lineages (Manz *et al.* 2001; Traver *et al.* 2000). Maintaining the continuous but regulated production of these cells throughout mammalian life is carried out by rare HSCs of bone marrow which undergo a process of self renewal and differentiation that eventually yields different progenitor and precursor cells which in turn give rise to mature blood cells upon further differentiation. Therefore, hematopoiesis is a hierarchical process where HSCs are at the top of hierarchy (Orkin 2000).

Hematopoietic progenitors committed to common lymphoid (CLP) and myeloid (CMP) categories have been identified on the bases of surface markers and bioassays. For example in mouse, HSCs are c-Kit^{+} (a tyrosine kinase receptor for stem cell factors), Sca-1^{+}, also called Ly-6A/E^{+} (a phosphotidylinistol-anchored protein), and CD34$^{+/-}$(a glycoprotein expressed on endothelial and some of hematopoietic cells) but Lin^{-} (a cocktail of monoclonal antibody that recognizes mature hematopoietic cells) (Orkin 2000; Spangrude *et al.* 1988; Iwasaki & Akashi 2007). With the aid of the multicolor flow cytometry, Weissman's laboratory was able to characterize HSCs and intermediate precursors. The proposed model from Weissman's laboratory divides HSCs in two categories such as long-term and short-term HSCs. Long-term HSCs are HSCs characterized by c-Kit^{+} IL-7Rα^{-} Sca-1^{+} Lin^{-} CD34^{-}Thyl^{low}FLT3^{-} and form a rare subpopulation of bone marrow cells, which have been shown to posses a distinct ability to undergo self-renewal and multilineage differentiation potential (Weissman *et al.* 2001; Weissman *et al.* 2001; Adolfsson *et al.* 2001) whereas short-term HSCs are derived from long term HSCs which preserve multilineage differentiation potential and low self renewal feature. Short term HSCs are characterized by c-Kit^{+} IL-7Rα^{-} Sca-1^{+} Lin^{-} CD34^{+} Thyl^{low} FLT3^{low} (Morrison *et al.* 1995; Christensen & Weissman 2001). Multipotent progenitors (MPPs) have lost the ability of self renewal but the ability to differentiate into blood cells is retained and MPPs are characterized as Lin^{-} IL-7Rα^{-} Sca-1^{+} c-Kit^{+} CD34^{+}Thyl^{-} FLT3$^{low\text{-}high}$ (Weissman *et al.* 2001).

Both common lymphoid precursors (CLPs; Lin^{-} IL-7Rα^{+} Sca-1^{low} c-Kit^{low})(Kondo *et al.* 1997) and common myeloid precursor (CMPs; Lin^{-} Sca-1^{-} c-Kit^{+} CD34^{+}FCγRII^{-}FCγRIII^{-}) are derived from MPPs (Akashi *et al.* 2000). CLPs do not retain any feature of myeloid cells but have preserved the ability to differentiate into cells of the lymphoid lineage whereas myeloid precursors including erythroid progenitors are derived from CMPs (Akashi *et al.* 2000). CLPs and CMPs further differentiate into precursors of several lineages which are also characterized by lineage-specific surface markers (Phillips *et al.* 2000). CMPs further differentiate into more specified progenitors namely granulocyte/monocyte progenitors (GMP; Lin^{-} Sca-1^{-} c-Kit^{+} CD34^{+} FCγRII^{+} FCγRIII^{+}), megakaryocyte/erythroid progenitors (MEPs; Lin^{-} Sca-1^{-} c-Kit^{+} CD3-$^{+}$ FCγRII^{-} FCγRIII^{-}), basophil, macrophage and dendritic cell progenitors (Arinobu *et al.* 2005; Fogg *et al.* 2006).

5. 5.1.1. Transcription factors regulating hematopoietic lineage commitment

The lineage-specific differentiation is regulated by specific nuclear transcription regulatory factors which are specifically expressed in specific lineage and their expression leads to intrinsic diversification of cells (Porcher *et al.* 1996). The functions of transcription factors that regulate hematopoiesis are principally obtained from knockout mice and forced expression studies (Orkin & Zon 2008). The major hematopoietic transcription regulators carry out their task in a context dependent manner where a given crucial lineage specific transcription factors perform functions

that support a given a lineage differentiation and concomitantly hamper the activity of other factors that support another lineage differentiation (Orkin 2000). For example, *GATA-1 zinc finger factor* supports erythroid/megakaryocytic/eosinophil differentiation whereas PU.1 supports myeloid differentiation. However, both *PU.1* and *GATA-1* proteins functionally oppose each other through direct physical interaction. For example, over-expression of *GATA1* in multi-potent chicken hematopoietic cells transformed by retrovirus-containing oncogenes such as *myb* and *Ets* has been shown to reprogram these progenitor cells into three different lineages including erythroid cells, eosinophils and thromboblasts (Graf *et al.* 1992). On the contrary, the expression of *Ets* family transcription factor *PU.1* in transformed multi-potent chicken hematopoietic progenitors has guided them to differentiate along myeloid lineage, which is characterized by up-regulated expression of myeloid-specific surface antigens and building up of a trait dependent on myeloid growth factor as well as down regulation of *GATA-1* and thrombocyte-specific cell surface antigens. Short term activation of these factors induces formation of juvenile eosinophils whereas prolonged activation of these factors has led to formation of myeloid lineage (Nerlov & Graf 1998). Furthermore, the expression of BZip protein mafB in transformed chicken hematopoietic precursors has been shown to instruct the differentiation into cells with typical features of macrophages including competence in phygocytotic activity, nitric oxide formation in response to lipopolysaccharide challenge, morphology and macrophage-specific surface markers (Kelly *et al.* 2000).

5.5.1.1.1. Transcription factors regulating generation of HSC from mesoderm

Transcription factors function as natural determinants to confer a capacity for HSCs to expand and differentiate in a lineage-specific fashion during embryogenesis and throughout adult life. However, the “master gene” that serves as the transcription factor to induce mesodermal cell development into HSCs has been poorly understood (Orkin 2000). Nevertheless, Vascular growth factor (*VGEF*) in association with basic fibroblast growth factor (*bFGF*), Tumor growth factor-β (*TGFβ*) and bone morphogenic factor 4 (*BMP4*) also known as ventralizing factors have been reported to regulate hematopoiesis in mouse(Fehling *et al.* 2003). For example, *BMP4-/-* mice die at gastrulation stage but few surviving mice have very small yolk sac mesoderm (Winnier *et al.* 1995). BMP4 has been shown to increase HSCs at AGM explants (Winnier *et al.* 1995), and in both human and mouse (Winnier *et al.* 1995), *BMP4* has been shown to be located in mesenchyme of aortic clusters (Marshall *et al.* 2000). Besides, both runt-related transcription factor 1 (*RUNX*1; also known as *AML1*) and stem cell leukemia factor (*SCL*; also known as *TAL1*) are also considered to be among the early transcription factors that devise the development of HSCs from early yolk sac mesodermal cell. Mice null for both SCL and RNUX1 die prenatally without any evident hematopoiesis like that of BMP4 (Shivdasani *et al.* 1995; Okuda *et al.* 1996).

5.5.1.1.2. Transcription factors regulating myeloid progenitors

PU.1 which is a member of Ets transcription factors containing glutamate-rich trans-activating N-terminal domain and its DNA binding C-terminal domain is an essential transcription factor for the formation of early myeloid progenitor (Ward *et al.* 2000). Expression of PU.1 is limited only to blood cells (HSCs, CMPs, CLPs, GMPs, monocytes, granulocytes and B cells) (Rosenbauer & Tenen 2007). However, its expression varies in different cells. For example, its expression level is high in immature myeloid cells and detectable but similar level of PU.1 expression is reported

in HSCs, CMPs, CLPs and B cells (Rosenbauer *et al.* 2005; Back *et al.* 2005). The low level of PU.1 has been shown to support macrophage formation whereas high level of PU.1 supports B-cell formation (DeKoter & Singh 2000; Anderson et al. 2002). In T-cells and early erythroids, down regulated expression of PU.1 has been observed, and in the absence of PU.1 down-regulation, these cells fail to differentiate and transform into malignant cells (Rosenbauer *et al.* 2005; Anderson *et al.* 2002).

In PU.1-null mice, neutrophils develop normally and express neutrophil-specific markers Gr-1 and cloroacetate esterase; however, PU.1-deficient neutrophils have lost the ability to differentiate terminally. Neutrophils devoid of PU.1 lack the ability to properly respond for some chemokines, they are not capable to produce superoxide ions and they have low bacterial engulfing and killing efficiency (Anderson *et al.* 1998). PU.1 is encoded by an oncogene known as *SPI1* and defects in prenatal and neonatal hematopoiesis are observed in SPI1-null mice. Granulocytes from *SPI1* null mice fail to completely mature, suggesting the fact that PU.1 is very critical in cell fate decision for both lymphoid and myeloid lineages (Rosenbauer & Tenen 2007; Scott *et al.* 1994; McKercher *et al.* 1996). However, in PU.1 null mice, yolk sac -derived phagocytes have been shown to develop without any defect (Lichanska *et al.* 1999).

5.5.1.1.3. Transcription factors regulating GMP

PU.1 is an essential transcription factor to convert HSCs into CMPs whereas C/EBPα is a family member of bZip transcription factors, such as C/EBPβ, C/EBPγ, C/EBPδ and C/EBPε, and is necessary for the formation of GMPs from CMPs. C/EBPα is expressed in HSCs, myeloid progenitors and granulocytes but not in macrophages (Akashi *et al.* 2000; Ward *et al.* 2000). The C/EBPα-null mice develop normal number of CMPs but GMPs and all successive granulocyte stages fail to develop in these mice, suggesting that C/EBPα is a "master" regulator of granulopoiesis (Zhang *et al.* 1997; Zhang *et al.* 2004b). However, normal granulopoiesis after GMP stage has been observed in mice where C/EBPα is conditionally deleted and this suggests that C/EBPα is not needed for differentiation of neutrophils after GMP stage (Zhang *et al.* 2004b). C/EBPα, like PU.1, has been shown to regulate the expression of many myeloid -specific genes (Tenen *et al.* 1997). C/EBPα facilitates the exit from cell cycle by supporting cell-cycle arrest through different mechanisms such as stabilization of wild type P53; it recruits retinoblastoma protein to CEBP responsive promoters, inhibits E2F activities and represses the activity of cyclin dependent kinase 2(CDK2)(CDK4)(Nerlov 2004).

5.5.1.1.4. Terminal differentiation of myelocytes

GFI1 and C/EBPε are the essential transcription factors that determine the remaining hematopoietic differentiation after GMP stage to generate myeloid cells. GFI1 represses transcription and, with its closely related protein, GFI1 shares identical zinc finger and transcription repressor domain (Zweidler-McKay *et al.* 1996). GFI1 is expressed in HSCs, nuetrophils, and early B and T cells (Rosenbauer & Tenen 2007). Severe neutropenia and accumulation of immature mononuclear cells in blood and bone marrow have been reported as the phenotype of GFI1 deficient mice. In these mice stimulation of myeloid precursors with granulocyte colony stimulating factor (G-CSF) is able to induce differentiation into mature macrophages but it has failed to induce granulocyte differentiation beyond promyelocyte stage

(Karsunky *et al.* 2002). Similar phenotype has been observed in C/EBPε-null mice where granulopoiesis failed to advance past promyelocyte stage that led to early death of mice by opportunistic infections and tissue destruction (Yamanaka *et al.* 1997). Similarly to PU.1 and C/EBPα deficient mice, HSCs from GFI1 deficient mice are functionally impaired and they lack the ability for self-renewal that is due to GFI1 role on cell cycle (Hock *et al.* 2004).

5.6. Tudor-SN

Tudor-SN (SND1, P100) exists as a single gene without any close homology and it maps to chromosomes 7q31.3 in human, 6 in mouse and 4q23 in rat. Tudor-SN is composed of four complete tandem repeats (SN1-4) and Tudor domain flanked by two incomplete SN domains (Ponting 1997; Selenko *et al.* 2001). The protein has been shown to be localized both in cytoplasm and nucleus (Broadhurst & Wheeler 2001; Zhao *et al.* 2003; Caudy *et al.* 2003; Saarikettu *et al.* 2010). Tudor-SN is conserved along evolution in mammals, fish, drosophila, *C. elegans*, ciliates and fission yeast and the conservation along evolution underlines the importance of the protein. In mammals Tudor-SN is expressed in many organs including mammary gland, pancreatic cells, parotid, anterior pituitary, corpus lutetium, ovarian follicular cells, placenta and small intestine (Broadhurst & Wheeler 2001; Broadhurst *et al.* 2005). Tudor-SN protein is implicated in several functions including transcriptional co-activation (Tong *et al.* 1995a; Leverson *et al.* 1998; Paukku *et al.* 2003; Yang *et al.* 2002; Välineva *et al.* 2006; Välineva *et al.* 2005). Tudor-SN also facilitates spliceosome assembly and increases the activity of splicing of pre-miRNAs (Yang *et al.* 2007), component of RISC (Caudy *et al.* 2003; Paukku *et al.* 2008), is involved in the degradation of hyper-edited dsRNA (Levanon *et al.* 2004; Li *et al.* 2008), plays an important role in tumor angiogenesis(Santhekadur *et al.* 2012), increases stability of mRNA intended to secretory pathways (dit Frey *et al.* 2010) and have a role in apoptosis (Sundström *et al.* 2009) and is localized in the stress granules (Caudy *et al.* 2003; Scadden 2007; Gao *et al.* 2010).

Tudor-SN is also implicated in tumorigenesis. The expression of Tudor-SN is up-regulated in prostate cancer, most notably in castration-resistant cancer, hepatocellular carcinoma, colon cancers and metastatic breast cancer (Tong *et al.* 1995a; Santhekadur *et al.* 2012; Kuruma *et al.* 2009; Tsuchiya *et al.* 2007; Blanco *et al.* 2011). Furthermore, non-cleavable Tudor-SN mutant has been shown to increase cell survival and ectopic over-expression of Tudor-SN in the intestinal epithelial cells has been shown to enhance the cell proliferation through Wnt signaling mediated pathway (Tsuchiya *et al.* 2007). Thus Tudor-SN is implicated in a multitude of functions; however, the exact mechanism that explains the role of Tudor-SN is poorly understood. Nevertheless, recent cellular studies suggested that Tudor-SN is promoting tumorigenesis by activating NF-κB that in turn activates miR-221(Santhekadur *et al.* 2012). On the contrary, another recent study suggested that Tudor-SN is a NF- κB -responsive gene and the promoter of Tudor-SN has been shown to bind NF- κB, Sp1 and NF-Y. Generally, Tudor domain containing proteins are conserved, and serve as molecular adaptors (Armengol *et al.* 2012). Besides, Tudor domain containing proteins are involved in the regulation of development particularly through piwi RNA pathway, RNA biogenesis and DNA damage response (Pek *et al.* 2012).

5.6.1. Biochemical and cellular functions of Tudor-SN

5.6.1.1. Tudor-SN as transcriptional co-activator

Tudor-SN is first reported as co-activator of transcriptional activator (Tong *et al.* 1995a). EBNA 2 activates specific viral genes which encode two integral membrane proteins (IMPs) in latently infected B-lymphocytes. EBNA is indispensable for B cell transformation and it is one of the first genes to be expressed in Epstein-Barr virus (EBV) infected B lymphocytes (Alfieri *et al.* 1991; Rooney *et al.* 1989). The acidic domain of EBNA 2 has been shown to interact with general transcription factors such as TFIIB, TFIIH and TAF40 (Tong *et al.* 1995a; Tong *et al.* 1995b). Tong et al. (1995) demonstrated specific and stable association between Tudor-SN and the acidic domain of EBNA2. Moderate and low level expression of Tudor-SN has been shown to increase acidic domain of EBNA2 -mediated activation of gene. Indeed, direct interaction between Tudor-SN and EBNA2 suggests that the two proteins exist as complex in transformed B lymphocytes by EBV. Furthermore, the co-activation effect of Tudor-SN is attributed to its interaction with TFIIE, as its subunits P56 and P34 have been shown to independently associate with Tudor-SN (Tong *et al.* 1995a).

Another independent study also demonstrated Tudor-SN protein as a transcriptional co-activator of transcriptional activator c-*myb* by cooperating with Pim-kinase (Leverson *et al.* 1998). *C-myb* is a protooncogene that plays a key role in several cellular functions such as proliferation, differentiation, and apoptosis. Expression of *C-myb* is crucial for fetal hematopoiesis and has been shown to play a key role in tumorigenesis (Ness 2003). Pim-1 kinase is a protooncogene itself and has been shown as important molecule in lymphoid cell transformation as it cooperates with *c-myb* (Jonkers & Berns 1996). Pim-1 has been shown to interact with Tudor-SN: it forms a complex with Tudor-SN, phosphorylates Tudor-SN and activates C-myb in Tudor-SN -dependent fashion (Leverson *et al.* 1998).

Tudor-SN has also been shown to interact and co-activate signal transducer and activator of transcription (STAT) 5 (Paukku *et al.* 2003) and a STAT 6 (Yang *et al.* 2002; Välineva *et al.* 2005). STATs are generally cytokine activated cytoplasmic transcription factors, which have been shown to be involved in many cellular functions such as growth and differentiation (Takeda & Akira 2000). STAT 5 is crucial in the induction of several genes that activate the expression of the milk protein (Boutinaud & Jammes 2004). Interestingly, the expression level of Tudor-SN is up-regulated in the mammary glands during lactation (Broadhurst & Wheeler 2001). However, mRNA level of Tudor-SN is not changed during lactation suggesting that up-regulated expression of Tudor-SN protein is regulated post-transcriptionally (Broadhurst & Wheeler 2001). The functional association between Tudor-SN and prolactin has not been studied. Nevertheless, the regulatory loop is speculated to exist and confer the ability to prolactin to stabilize Tudor-SN protein. The stabilized Tudor-SN in turn physically interacts and co-activates STAT 5 (Paukku *et al.* 2003).

Tudor-SN has also been shown to physically interact with and co-activate STAT6 (Yang *et al.* 2002). IL-4 responsive genes are principally mediated by STAT6 (Kaplan *et al.* 1996) and IL-4 is a cytokine which has variable effects in different cells including lymphoid, myeloid, stromal and

epithelial cells (Nelms *et al.* 1999). Yang et al. (Yang *et al.* 2002) demonstrated the physical interaction of STAT6 and SN like domains of Tudor-SN. They also demonstrated that in IL-4 stimulated cells that over-express Tudor-SN STAT 6 responsive gene reporter activity is increased. This suggests that Tudor-SN is a STAT6 co-activator (Yang *et al.* 2002). In an attempt to explain the underlining mechanism of co-activation of STAT 6 by Tudor-SN, Yang et al. (Yang *et al.* 2002) described the physical interaction between Tudor-SN and the large subunits of RNA Pol II. The physical interaction between Tudor-SN and RNA Pol II is believed to link the co-activation role played between STAT 6 and the basal transcription machinery (Yang *et al.* 2002). Furthermore, chromatin immuno-precipitation studies revealed that Tudor-SN enhances the formation of complex containing STAT6-p100-CBP as well as acetylated histone H4 in the Igε promoter (Välineva *et al.* 2005). Based on these findings, Välineva et al. hypothesized that the recruitment of CBP to STAT 6 response elements is carried out by Tudor-SN, which subsequently open up the nucleosomes that permits an access to STAT 6-Tudor-SN protein complex to join the basal transcription machinery (Välineva *et al.* 2006; Välineva *et al.* 2005).

5.6.1.2. Tudor-SN in RNA interference and hyper edited dsRNA

Gene expression is also regulated post-transcriptionally by RNA interference through cleaving mRNA that eventually blocks the synthesis of protein. RNA interference is mediated by RNA induced silencing complex (RISC) (Fire *et al.* 1998). In *Drosophila*, the two dicer proteins Dcr-1 and Dcr-2 have been shown to play an independent function. Dcr-1 functions in the maturation of miRNA whereas Dcr-2 plays a role in the production of siRNA and loading one strand of siRNAs to the RISC (Lee *et al.* 2004; Rand *et al.* 2005). Human Dicer has also been implicated in the loading of one strand of siRNAs to RISC as cells from which dicer is knocked down failed to trigger efficient gene silencing following siRNA transfection (Meister & Tuschl 2004). During RISC assembly, siRNA is bound with another *Drosophila* protein called R2D2 which has a notable specificity to bind stable end of siRNAs and Dcr-2 to form RISC loading complex. R2D2/Dcr-2 complex is suggested to recruit the remaining components of RISC such as Dcr-1, Tudor-SN, and Ago2 (Caudy *et al.* 2003; Rand *et al.* 2005; Scadden 2005). The association of R2D2/Dcr-2/siRNA complex with PIWI domain of Ago2 unwinds siRNA that ultimately through unknown protein leads to the degradation of passenger strand of siRNA. The degradation of passenger strand of siRNA ensures the formation of mature RISC with only single stranded RNAs (Rand *et al.* 2005).

However, the functional role of Tudor-SN in the RISC is not clear and controversial. In the first study that demonstrates that Tudor-SN is a component of RISC, authors reported that Tudor-SN has nuclease activity and it may serve as catalytic engine in RNAi but they also noted some inconsistencies. These inconsistencies include i) unlike the RISC, Tudor-SN lacks specificity in its sequences for target mRNA, ii) Tudor-SN is expected to cleave both RNA and DNA but there is no assessable DNase activity in the RISC iii) Tudor-SN and related enzymes can not be matched with any feature of specific cleavage siRNA-mRNA hybrid. Therefore, it was speculated that Tudor-SN may degrade the remaining fragments after RISC or it may not have any catalytic role in the RISC (Caudy *et al.* 2003). Indeed, another study described RISC as endonuclease that is dependent on Mg^{2+} but not Ca^{2+}. This eventually eliminates the idea that Tudor-SN is the protein responsible for endonuclease activity in RISC because as a member of Staphylococcal nuclease family, Tudor-SN is expected to require Ca^{2+} but not Mg^{2+} and therefore

is not expected to produce 3' hydroxyl, 5' phosphate termini that are obtained from RISC mediated cleavage (Schwarz *et al.* 2004). However, a recent study highlighted that Tudor-SN has nuclease activity in plants (Sundström *et al.* 2009).

Further investigation carried out to understand the functional role of Tudor-SN in the RISC suggested that Tudor-SN is required for degradation of hyperedited dsRNA. These findings led to the hypothesis that Tudor-SN links to miRNA and RNAi pathway (Scadden 2005). The presence of dsRNA is strange in cells and may reflect the production of antisense RNA for gene regulation, for example as defense to viral infection or invading nucleic acid molecules (Maquat & Carmichael 2001). Inside cells, dsRNA follows different fates including activation of antiviral system (Zhou *et al.* 1993) or go through adenosine deaminase (ADAR) -mediated covalent modification also known as editing (Bass 2002). They can also be utilized by RNAi pathway to inactivate the target gene (Novina & Sharp 2004). Adenosine is converted to inosine in dsRNA by ADARs and this modification specifically targets adenosines at very critical positions, for example at glutamine-arginine site, resulting in hydrolytic deamination that converts adenosine to inosine. Inosine is similar to guanosine except it lacks exocyclic amino group. Therefore it favors pairing with cytosine and is subsequently translated as guanosine resulting in the inclusion of a different amono acid (Bass 2002). Tudor-SN, an integral component of RISC, has been shown to interact with dsRNA with multiple I.U and U.I. pairs but no interaction has been observed between Tudor-SN and dsRNA G.U and U.G. pairs (Scadden 2005). This suggests Tudor-SN selectively binds hyperedited dsRNAs and binding of Tudor-SN to the RNA is not surprising as Tudor domain proteins are predicted to bind RNAs (Pek *et al.* 2012).

The binding of Tudor-SN to hyperedited IIUI dsRNA resulted into specific cleavage of IIUI dsRNA and the addition of staphylococcal nucleases inhibitor and EGTA blocked the IIUI dsRNA cleavage but addition of Tudor-SN alone failed to trigger cleavage of the IIUI dsRNA suggesting that additional factors that confer stability to Tudor-SN is required or alternatively Tudor-SN may be activating nucleases that target hyperedited dsRNA (Scadden 2005). These finding suggests that the fate of dsRNA in the cell is determined by whether they first encounter ADARs or dicer in the cytoplasm. If dsRNA first encounters ADARs they undergo the process of hyperediting and they become unsuitable substrates for dicer-mediated cleavage. The hyper-edited dsRNA undergoes Tudor-SN -mediated degradation. Alternatively, if dsRNAs are first subjected to dicer they are processed to siRNA and become an integral part of RISC (Scadden 2005; Knight & Bass 2002). Pri-miRNA precursors have also been shown to be edited by ADARS (Luciano *et al.* 2004) suggesting that improperly processed pre-miRNA may also be subjected to ADAR-mediated covalent modifications and subsequent Tudor-SN-mediated degradation (Tonkin & Bass 2003). However, recent studies suggested that by activating NF-κB, Tudor-SN induced miR-221 that in turn promoted angiogenesis during tumorigenesis (Santhekadur *et al.* 2012).

5.6.1.3. Tudor-SN in spliceosome assembly and apoptosis

Tudor-SN has also been shown to interact with many snRNAs such as U1, U2 U3, U4, U5, U6 and U7, and subsequently speed up the formation of spliceosome; however, it does not change the total level of splicesome (Yang *et al.* 2002). Nevertheless, Tudor domain of SMN proteins has been shown to bind spliceosomal Sm proteins and therefore plays crucial role in the

formation of spliceosome (Bühler *et al.* 1999). A recent study demonstrated that the interaction between Tudor-SN and snRNP brings about efficient association of Sm proteins. Specifically the interaction between Tudor-SN and symmetrically dimethylated SmB/B', SmD1 and SmD3 proteins have been shown to recruit Sm proteins to the U snRNAs to subsequently form spliceosome complex A. Furthermore, the knocking down of Tudor-SN resulted in the reduction of the level of U snRNAs bound Sm proteins (Yang *et al.* 2007).

Tudor-SN has also been shown to play a role during transition from complex A to complex B as it forms stable complex even in harsh conditions with U5-116 through interacting with different domains of Prp8 protein (Gao *et al.* 2012). The interaction between Tudor-SN and components of spliceosome is mediated by carboxyl-terminal region of Tudor-SN protein. Carboxyl-terminal region of Tudor-SN is consisted of hook like structure also called Tudor and SN5 domains (Shaw *et al.* 2007). Caspase-mediated cleavage between Tudor and SN5 domains has been shown to hinder the stimulatory effect in pre-mRNA splicing. This inhibitory effect on pre-mRNA splicing is most likely due to aggregation of proteins on the hydrophobic area on the exposed surface as consequence of cleavage by caspase as it has been predicted by molecular modeling. N-terminal domains of Tudor-SN have been shown to have mild stimulatory effect during spliceosome assembly (Sundström *et al.* 2009).

Most of the proteins which interact with Tudor-SN and are involved in the regulation of transcription, such as RNa Pol II, RNA helicase A (RHA), CBP/p300, and splicing, such as snRNP70, nuclear protein (SKIIP), splicing factor 3 B subunit 2 (SF3b150), splicing factor U2AF subunit (U2AF65) and U6 snRNA-associated Sm like protein LSM3 (LSM3), are substrate for caspase (Välineva *et al.* 2006; Yang *et al.* 2007). The multitude of caspase substrates comprises the principal component of programmed cell death (PCD) degradome in animals (López-Otín & Overall 2002). Metacaspase, ancestral family of cysteine proteases, has been shown to cleave Tudor-SN during development as well as during stress induced apoptosis in plants. Furthermore, human Tudor-SN has been shown to be cleaved by caspase 3 during apoptosis (Sundström *et al.* 2009). PCD is indispensable during early development of plant and metacaspase mcII-pa an effecter metacaspase that execute PCD (Bozhkov *et al.* 2005). PCD activation is reported to be triggered as a consequence of reverse genetic effect of the two Tudor-SN homolog knock-down in *Arabidopsis*. The activation of PCD is reported as cytological hall mark of apoptosis in plant and 30% nuclear DNA fragmentation with subsequent degeneration is also observed in pollen grain of Tudor-SN knockdown *Arabidopsis.* Besides, inhibition of nuclease activity of Tudor-SN by pdTP (3', 5'-deoxythymidine bisphosphate) has been shown to increase the mass of embryos containing fragmented DNA by four times (Sundström *et al.* 2009). This suggests the fact that nuclease activity of Tudor-SN is important for survival of cells. Both plant metacaspase and animal caspase have been shown to act on the same Tudor-SN substrate to elicit apoptosis despite their long divergences (Sundström *et al.* 2009).

5.6.1.4. Tudor-SN in stress granules

Stress granules (SGs) are dynamic dense cytoplasmic structure that form quickly and transiently to sequester mRNAs in response to external environmental stress. Sequestration of a group of translationally apprehended mRNAs during stress permits selective translation of important mRNAs for the survival of cells and, therefore, the formation of SGs is suggested to be one of the

important means of survival for eukaryotic cells during stress (Anderson & Kedersha 2008). The formation of SGs is associated with general dynamic features of cells in response to stimuli of stress most notably the process of translation and transcription in which the expression level of certain genes are up-regulated whereas the expression level of other genes are down-regulated (Kedersha & Anderson 2002). However, it has been proposed that the formation of SGs can be the cause for the modification of mRNA translation during stress or its formation is triggered as a consequence of modified mRNA translation (Anderson & Kedersha 2009). Although several constituents of SGs are identified, their total lay-out has been partly dissected. Translational initiation factors such as eIF2, eIF2B, eIF24E, 40s subunit of ribosome, mRNAs and RNA binding proteins like TA1-1 and G3BP are known components of SGs (Gilks *et al.* 2004; Tourrière *et al.* 2003).

IU-dsRNA has been shown to interact with the protein complex of main component of cytoplasmic SGs such as TA1-1 and G3BP (Scadden 2007) and IU-dsRNA also specifically interacts with Tudor-SN (Scadden 2005). Indeed Tudor-SN is co-localized in the SGs with G3BP. Although knocking down of endogenous Tudor-SN has not affected the formation SGs, the assembly of large SGs from smaller SGs has been delayed in Tudor-SN knock-down cells, suggesting that Tudor-SN is not essential for the formation of SGs but it may have an important role in the aggregation of SGs (Gao *et al.* 2010). The interaction between Tudor-SN and hyper-edited dsRNA, which are rare species of RNA in cells but are generated in the stress conditions, and the interaction between Tudor-SN and the main components of SGs with ultimate co-localization in heat shock or arsenite-induced stress suggest that Tudor-SN has an important pathophysiological implication during stress (Scadden 2007; Scadden 2005).

SGs formed in tumors are believed to contribute to the formation of radiation resistance vasculature observed in the hypoxic area of tumors (Moeller *et al.* 2004). Besides, Tudor-SN is specifically co-purified with 3'end of transmissible gastroenteritis coronavirus geneome (TGEV) (Galán *et al.* 2009). Tudor-SN has also been shown to interact with the equine arteritis virus nsp 1, and nsp 1 is indispensable for mRNA synthesis (Tijms & Snijder 2003). dsRNA is produced during viral infection and it triggers antiviral response so as to increase cell survival from invading virus. Furthermore, interaction of Tudor-SN with hyperedited dsRNA and subsequent degradation of hyperedited dsRNA by turning off RNAi pathway may suggest that hyperedited dsRNA are not suitable for RNAi pathway (Scadden 2007; Scadden 2005; Weissbach & Scadden 2012). This can negatively affect the cell survival and their degradation by Tudor-SN additionally contributes to cell survival during virus infection-triggered stress. In *Arabidopsis,* double Tudor-SN homozygous mutant showed hypersensitivity to salt-induced stress conditions and yet by unknown mechanism Tudor-SN has been shown to stabilize a subset of stress responsive mRNAs that are intended to secretory pathway (dit Frey *et al.* 2010). Furthermore, another study demonstrated that Tudor-SN interaction with AT1R 3'-UTR through its SN domain has resulted in mRNA stability, which eventually boosts its expression by enhancing the translation of AT1R 3'-UTR through Ago2 dependent manner (Paukku *et al.* 2008).

5.6.1.5. Tudor-SN in cancer

Tudor-SN has been shown to increase cell viability, is antiapoptotic and up-regulated in various tumors (Santhekadur *et al.* 2012; Kuruma *et al.* 2009; Kuruma *et al.* 2009; Tsuchiya *et al.* 2007;

Blanco *et al.* 2011); however, its functional role in these cellular process has been elusive. Nevertheless, several studies have obtained compelling results that highlight Tudor-SN by an unknown mechanism to play a crucial role in the tumorigenesis. For example, antisense inhibition of Tudor-SN in EBV transformed B-lymphoblast has been reported to trigger cell death. Furthermore, the ability of Tudor-SN to co-activate EBNA 2, an indispensable gene for EBV mediated transformation of B-lymphocytes, suggests that Tudor-SN has important role in the transformation of cells and tumorigenesis (Tong *et al.* 1995a). In human colon cancer tissue, the expression level of mRNA of Tudor-SN has been shown to be remarkably upregulated. The upregulated level of mRNA of Tudor-SN is reported even in early stage of lesions. The up-regulated expression of mRNA of Tudor-SN is also observed in colon cell lines. The stable over-expression of mouse Tudor-SN in IEC6 rat intestinal epithelial cells induced transformation of IEC6 cells into tumor cell phenotype where the cells lost contact inhibition, enhanced cell growth that continues after confluence, perturbed E-cadherin distribution from cell membrane to cytoplasm, and down-regulation of adenomatous polyposis coli (APC) protein without noticeable alterations in the expression level of mRNA. This study suggested that Tudor-SN is up-regulated in early stage of colon cancer development through an unknown post-transcriptional mechanism that regulates both B-catenin and APC (Tsuchiya *et al.* 2007).

Up-regulated expression of Tudor-SN is also reported in prostate cancer, most notably in castration-resistant cancer (Kuruma *et al.* 2009). The expression level of Tudor-SN is observed in 97% of specimens from prostate cancer patients; however, weak or no expression is observed in normal and hyperplasia specimens. In Tudor-SN knockdown prostate cancer cell lines, cell growth is significantly decreased. This study suggested that Tudor-SN can serve as a marker and a drug target for prostate cancer (Kuruma *et al.* 2009). Up-regulated expression of Tudor-SN is reported in hetaptocellular carcinoma (HCC). In 74% HCC patient specimens Tudor-SN is upregulated compared to normal liver. Tudor-SN has also been shown to interact with Astrocyte elevated gene-1 (AEG-1), which is one of the known genes playing a crucial role in hepatocarcinogenesis. In HCC, high RISC activity is observed and consequently high RISC activity most likely conferred by Tudor-SN or AEG-1 degraded mRNA of tumor supperessor genes. Blocking of enzymatic activity of Tudor-SN in HCC has led to cessation of cellular proliferation. This independent study also suggests that Tudor-SN most likely, by increasing RISC activity and with subsequent degradation of mRNA of target tumor suppressor gene by oncogenic miRNAs such as miR-221, plays a crucial role in the tumorigenesis of HCC and can be an ideal drug target for HCC (Yoo *et al.* 2011).

Tudor-SN has also been shown to play a crucial role during metastasis of breast cancer. Tudor-SN has been shown as a metastasis promoting novel gene enhancing metastasis by interacting with Metadherin (MTDH), which is also known as Lyric and AEG1(Blanco *et al.* 2011). MTDH serves as a functional target at 8q22 regional genomic gain, which is very common in poor prognosis breast cancer patients. MTDH is over expressed in 40% of breast cancers with 8q22 regional genomic gain. The functional study further suggests that MTDH plays a crucial role by facilitating metastatic seeding and boosting chemoresistance (Hu *et al.* 2009). Knocking down of Tudor-SN in SCP28 and LM2 cells promoted apoptosis to 2-3 fold; however, no significant changes in invasion and proliferation has been observed between Tudor-SN knockdown and control SCP28 and LM2 cells (Blanco *et al.* 2011). The microarray data from breast cancer patients with the history of recurrent relapse of metastasis compared to disease free subjects

suggests that Tudor-SN is significantly up-regulated in patients with metastasis (Blanco *et al.* 2011).

Tudor-SN has also been shown to promote tumor angiogenesis in HCC (Santhekadur *et al.* 2012). HCC is a solid cancer which is very rich in blood vessels and the generation of new of blood vessels is seminal for growth of HCC (Zhu *et al.* 2011). Tudor-SN enhances blood vessel formation by increasing the production of agiogenin and CXCL16 (Santhekadur *et al.* 2012). CXCL16 has been shown to enhance tumor angiogenesis, invasion and metastasis (Deng *et al.* 2010). It has been shown that Tudor-SN activates NF-κB and the activation NF-κB consequently elevates the expression of oncogenic miRNA, also known as mir-221, which in turn regulates enhanced expression of agiogenin and CXCL16(Santhekadur *et al.* 2012).

5.7. Polyamines

5.7.1. Biochemical and cellular functions of polyamines

The natural polyamines spermidine, spermine and their cellular precursor putrescine are evolutionary conserved cationic molecules known to exist in all living organisms except in few species of bacteria. Their conservation along evolution suggests that they are crucial molecules for the survival of organisms (Wallace *et al.* 2003). Leuwenhoek discovered spermine in 1678; however, the initial empirical formula of spermine was understood hundreds of years later (Dudley *et al.* 1924). However, the exact physiological function of polyamines despite their early discovery and intensive investigation has been enigmatic. Polyamines are implicated in several cellular functions such as supporting cell growth, maintaining chromatin conformation, regulation of gene expression, transcription, translation, post translational protein modification and membrane stability (Wang & Casero 2006; Matthews 1993).

Polyamines are positively charged molecules and therefore ionically bind with several cellular negatively charged macromolecules such as DNA, RNA, proteins and phospholipids. Unlike cellular bivalent compounds, such as magnesium and calcium, the charges of polyamines are distributed along the entire length of the molecule. For example, spermidine and spermine have been reported to stabilize nucleic acid structure of DNA by bridging the minor and major grooves of DNA (Matthews 1993). Furthermore, the depletion of polyamines has been shown to partially unwind the DNA nucleosome and subsequently expose the potential sequences to transcription regulation (Morgan *et al.* 1987). The prolonged incubation of calf thymus DNA with spermine has led to concentration dependent condensation of DNA, suggesting that polyamines are key players in the regulation of gene transcription (Morgan *et al.* 1987). Polyamines are also shown to play a crucial role in signal transduction pathways. For example, polyamines increased the activity of casein kinase II and were implicated in the regulation of membrane associated enzymes such as adenylate cyclase and tissue transglutaminase (Wright *et al.* 1978; Bauer *et al.* 2001).

5.7.2. Polyamine metabolism

5.7.2.1. Polyamine interconversion

Mammalian cells use L-ornithine and L-methionine as the main amino acid precursors to synthesize polyamines. Polyamine metabolism is a very complex process that is carried out by 6 enzymes **(Fig. 8).** Two cytoplasmic enzymes, ornithine decarboxylase (ODC) and S-adenosyl methionine decarboxylase (AdoMetDC) catalyse decarboxylation of ornithine and AdoMet, respectively. The production of putrescine from ornithine is the first rate-controlling step in polyamine biosythesis whereas decarboxylation of AdoMet produces decarboxylated AdoMet (dcAdoMet). Spermidine and spermine synthases catalyse the transfer of aminopropyl group from dcAdoMet to putrescine and spermidine to synthesize spermidine and spermine, respectively. The intracellular polyamine level is also regulated by catabolic reactions that convert spermine back to putrescine. To be converted back to putrescine, spermidine and spermine are first acetylated by spermidine/spermine N^1-acetyl transferase (SSAT). Flavin adenine dinucleotide (FAD) -dependent polyamine oxidase (PAO) then catalyses the oxidation of acetylated spermidine to putrescine, monoacetylated spermine to spermidine and, supposedly, diacetylated spermine to putrescine. However, spermine oxidase has been shown to oxidase non-acetylated spermine directly to spermidine (Vujcic *et al.* 2002; Jänne *et al.* 2004).

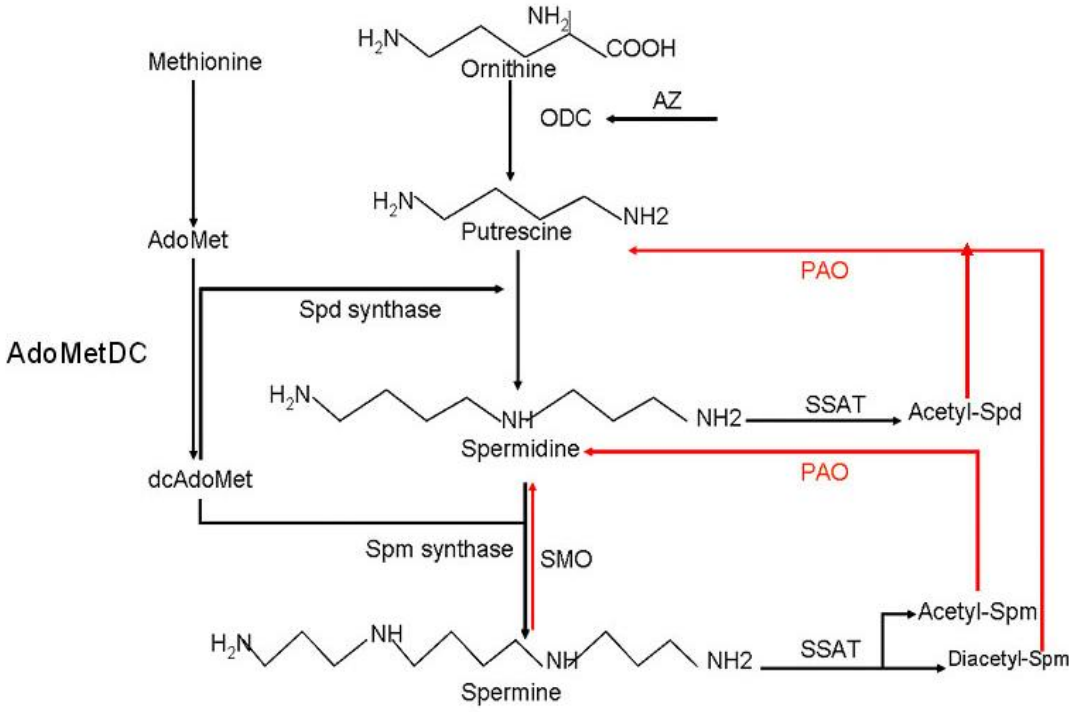


Figure 8. Polyamine Metabolism: ODC, Ornithine decarboxylase; AZ, antzyme; Spd, spermidine; SPM, spermine; PAO, polyamine oxidase; SMO, spermine oxidase; SSAT, spermidine/spermine N^1-acetyl transferase; AdoMetDC, S-adenosylmethionine decarboxylase; dcAdoMet, decarboxylated AdoMet. Black lines depict polyamine biosynthesis pathways and red lines depics polyamine catabolic pathways.

5.7.2.2. Ornithine decarboxylase

ODC (L-ornithine carboxylase, EC 4.1.1.17) is the first and rate controlling cytoplasmic enzyme in polyamine biosynthesis. Active ODC uses pyridoxal phosphate as cofactor and thiol group as reducing agent. ODC is extremely inducible in response to many trophic stimuli. The ODC monomer consists of two domains including a cofactor binding NH2-terminal domain which forms α/β-barrel and a COOH terminal. The activity of ODC is dependent on the formation the dimer, which leads to the formation of the active site at the interface between the two subunits. ODC contains two PEST regions that are rich in proline, glutamic acid, aspartic acid, serine and threonine. The growth stimulation of cells has been shown to increase the activity of ODC and, based on this observation; ODC is described as an immediate early response gene (Wallace *et al.* 2003; Coleman *et al.* 1994; Pegg 2006). ODC forms a non-covalent association with a protein called antizyme (AZ). The formation AZ-ODC heterodimer triggers a conformational change of ODC by exposing the C-terminal degradation signal (PEST), which subsequently leads to the ATP dependent proteosomal degradation of the enzyme (Zhang *et al.* 2004a). Besides, AZ can alter homeostasis of polyamines by decreasing polyamine uptake through its ODC-independent effect (Pegg 2006).

5.7.2.3. S-adenosylmethionine decarboxylase

The synthesis of AdoMet is the next important step in the biosynthesis of higher polyamines, and AdoMetDC is an enzyme that decarboxylates S-adenosylmethionine to generate dcAdoMet. AdoMetDC (EC 4.1.1.50) is expressed as a proenzyme with 334 AAs and have been shown to undergo putrescine –activated autolytic reaction to form two subunits, α with 266 AAs and β with 67 AAs, and a subsequent generation of a pyruvate group from an internal serine residue (Ser68) at N-terminus of α-subunit. In mammals, the active site of AdoMetDC consists of $(\alpha{:}\beta)_2$ dimer, where the two active sites consisting α:β are not interacting. Besides, AdoMetDC is strictly regulated at the translation, transcription, and proenzyme processing level (Pegg *et al.* 1998). It has also been shown that putrescine enhances the activation of AdoMetDC from the state of proenzyme to the mature form as well as it stimulates the catalytic activity of AdoMetDC; however, spermidine has been shown to repress both transcription and translation of AdoMetDC (Pegg *et al.* 1998; Stanley *et al.* 1994).

5.7.2.4. Spermidine and spermine synthases

Spermidine synthase (SPDsy, EC 2.5.1.16) is an enzyme that catalyzes a reaction of transfer of aminopropyl group from dcAdoMet to putrescine to yield spermidine. Similarly, spermine synthase (SPMsy, EC 2.5.1.22) catalyses the transfer of aminopropyl group from dcAdoMet to spermidine to yield spermine. Although these two enzymes perform a similar function they are independent enzymes and they have only one homologous domain which is predicted to be the binding site for dcAdoMet (Korhonen *et al.* 1995). SPDsy is a constitutively expressed and conserved in all organisms; however, SPMsy is expressed only in eukaryotes. Both SPDsy and SPMsy have stable half life and they are regulated by the availability of their substrates (Janne *et al.* 1978).

5.7.2.5. Enzymes of polyamine catabolism

The acetylation of higher polyamines, spermidine and spermine, is an indispensable part of their backconversion to putrescine. Subsequent reactions catalyzed by PAO convert the acetylated spermidine to putrescine and spermine to spermidine. The intracellular negatively charged macromolecules such as nucleic acids, phosphoproteins, and phospholipids are not tightly bound by acetylpolyamines and diamines and therefore the acetylation of polyamines is suggested to destabilize negatively charged macromolecules (Jänne *et al.* 2004; Jänne *et al.* 2006). SSAT is a rate limiting enzyme that acetylates higher polyamines in the reaction that transfers the acetylmoiety of acetyl-CoA to the aminopropyl groups of spermidine and spermine. However, SSAT does not acetylate the secondary amino group. SSAT contains a conserved acetyl-CoA binding region comprised of 20 amino acids, which starts from Arg101 with sequences of RGFGIGS. Acetylated spermidine and spermine are readily oxidized by PAO to yield putrescine and spermidine, respectively (Persson & Pegg 1984). Now, PAO is also called acetylpolyamine oxidase (APAO) to emphasize the fact it prefers an acetylated substrate over an unacetylated one. This feature makes PAO different from SMO that uses only unacetylated spermine as its substrate.

5.8. Polyamine analogues

Polyamine analogues are synthetically generated compounds. Historically, the inability to producte a single enzyme inhibitor of polyamine metabolic pathway created the need for analogue development (Porter & Bergeron 1983). The intracellular natural polyamine pool has been shown to be depleted or displaced by most of polyamine analogues and many polyamine analogues failed to substitute the function of their natural counterpart. Although, a reasonable concentration of intracellular natural polyamines is maintained, the distorted polyamine function owing to presenace of polyamine analogues eventually leads to apoptosis and cell death. This property of polyamine analogues provided the bases for utilizing them as chemotherapeutic drugs for cancer as cancer tissue is known to have elevated polyamine concentration (Thomas & Thomas 2003). The mechanism of action of polyamine analogues have not been yet fully comprehended; however, mimicking their natural counterparts is believed to be a possible mechanism of their action. Furthermore, direct interaction of polyamine analogues with DNA is thought be one of the major actions of polyamine analogues (Faaland *et al.* 2000). It is suggested that polyamine analogues can hinder DNA condensation, packaging and unraveling during transcription and replication and can alter cell cycle regulation (Thomas & Thomas 2003).

In contrast to growth-inhibitory polyamine analogues, C-methylated polyamines are able to fulfill the biological roles of the natural polyamines. As their natural counterpart, α-methylspermidine, α -methylspermine and α, ω-bismethylspermine have shown competence to convert right handed B-DNA to the left-handed DNA (Varnado *et al.* 2000).They are metabolically stable and well tolerated in vivo, and both α-methylspermidine and α –methylspermine are reported to induce SSAT both in wild type and SSAT transgenic mice (Järvinen *et al.* 2006; Järvinen *et al.* 2005). For example, α –methylspermidine has been used to restore liver regeneration (Räsänen *et al.* 2002), reverse cytostasis in fetal fibroblast (Järvinen *et al.* 2005) and prevent acute pancreatitis triggered by polyamine depletion (Hyvönen *et al.* 2006).

5.9. Hair cycle

A hair follicle is a mini organ of the skin and the principal unit for the production of a distinct hair shaft. Hair follicle formation mainly occurs during fetal and pre-natal skin development. The coordinated and highly regulated mesodermal-ectodermal interaction is believed to drive the development of hair follicle. Hair has several functions such as regulation of the body temperature, physical protection, transmitting sensory and tactile input and providing attractive feature for social interaction. To maintain these multiple functions, a stable and long-lasting supply of new hairs is required. Therefore in mammals, hair follicle undergoes life long cyclic transformation that is characterized by a rapid phase of growth (anagen), a short regression period (catagen) and a relatively long resting phase (telogen)(**Fig. 9)** (Müller-Röver *et al.* 2001).

5.9.1. Anagen

The whole shaft of hair from tip to root grows during the anagen phase. The histological appearance of hair follicle during anagen is straight and the cells of anagen follicles, notably proliferating matrix cells, are among the most rapidly dividing cells of animals with an estimated cycle length of 18hrs (Lavker *et al.* 2003). The daughter cells of proliferating matrix cells move upward to form the six lineages of the cell layer of the hair follicle from outermost to inner most layers. The layers are named as Henly, Huxley, cuticle layers of inner root sheath, cortex and medulla layers of hair shaft. The cells of hair shaft undergo terminal differentiation that is marked by removal of their organelles and by development of tightly assembled filaments of keratins that are physically cross-linked to confer strength and elasticity to hair shaft. The continued proliferation and differentiation of matrix cells determines the duration of anagen phase of hair cycle (Alonso & Fuchs 2006).

5.9.2. Catagen

During the late period of anagen, the matrix cell supply diminishes and inner root sheath and differentiation of HS cells become sluggish. Subsequently, the hair follicle undergoes an apoptosis-driven destructive phase known as catagen. The duration of the first catagen slightly differs from one strain of mice to another and varies considerably in different regions of skin. In black, mice for example, the skin color changes from dark anagen to gray catagen and pale to pink telogen skin. However, the molecular mechanism that explains the anagen to catagen phase of hair cycle has not been well understood (Alonso & Fuchs 2006). Nevertheless, molecular regulators, such as growth factors including epidermal growth factor (EGF), fibroblast growth factor 5 (FGF5), neurotrophins such as brain-derived neurotrophic factors (BDNF), P53 and TGFβ, have been reported to be involved in the regulation of anagen to catagen transition (Andl *et al.* 2004; Schmidt-Ullrich & Paus 2005; Foitzik *et al.* 2000).

5.9.3. Telogen

After the catagen phase, the hair follicle becomes dormant and quiescent also known as telogen or resting phase of hair cycle. In mice, the first telogen is very short: only about one or two days; however, the second telogen lasts for more than two weeks from postnatal day 42 (Alonso & Fuchs 2006). During telogen-anagen transition, multi-potent epithelial stem cells residing at hair

bulb or near to dermal papila are activated to trigger the new cycle of hair follicle growth (Taylor *et al.* 2000). The activation of follicular stem cells leads to very rapid proliferation of follicular stem cells which eventually leads to the formation of new hair shaft (Tumbar *et al.* 2004). Wnt siganling and BMPs are reported to play a crucial role in the transition from telogen to anagen (Lowry *et al.* 2005; Kulessa *et al.* 2000).

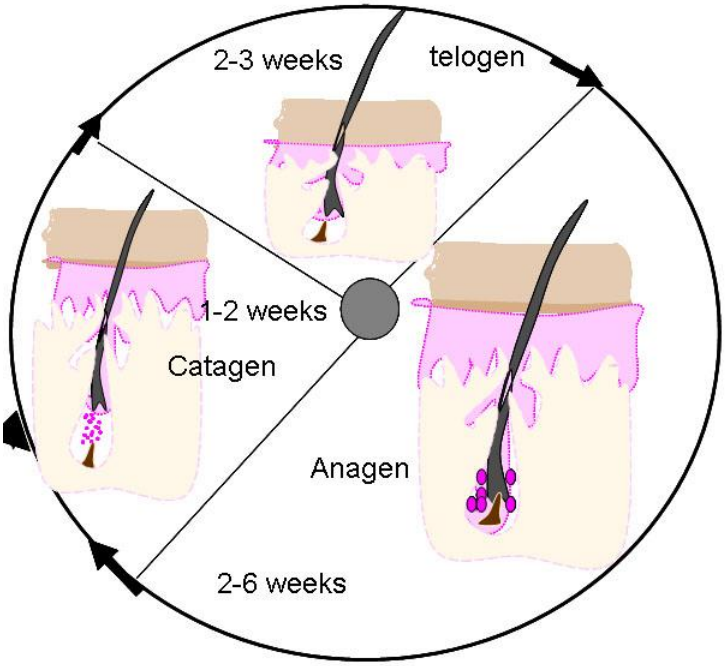


Figure 9. The hair cycle

6. AIMS OF STUDY

Tudor-SN is a multifunctional protein that is suggested to play multifunctional role in spliceosome assembly, as transcriptional co-activator, component of RISC, apoptosis, in the degradation of hyperedited dsRNA, tumorigenesis, tumor angiogenesis, stress granules and mRNA stability. Furtheremore, Tudor-SN is expressed ubiquitously almost in all organs. However, the exact function of Tudor-SN as well as comprehensive expression analysis of the protein has not been fully understood. The aim of this study was partly to dissect the expression of Tudor-SN in mice organs and tissues as well as to elucidate the physiological role of Tudor-SN using animal and cell culture models. Therefore, it was prosed that the generation of Tudor-SN knockout mouse unravel the physiological role of Tudo-SN in different biological processes during development and later in adult stage such as hematopoitetic differentiation, growth and metabolism in different organ system.

The hair cycle in C57/BL/6 mice undergo series of three cyclic growth phases also known as anagene (rapid growth phase), catagen (apoptosis driven regression phase) and telogen (relatively quiescent phase). In the study III, it was hypothesized that hair follicle growth can be activated by increasing intracellular pool of spermidine. Therefore, the aim of this study was to increase the intracellular pool of spermidine with the aid of metabolically stable α-methylspermidine that has been proven to serve as substitute for spermidine in telogen mice and analyze the expression of polyamine regulated proteins.

7. MATERIALS AND METHODS

7.1. Experimental animals

C57/BL/6 mouse has been used in all three studies (I, II, and II). In study I, major organs of mice were collected from three male and three female mice. In study II Tudor-SN knockout mice were generated in the C57/BL/6 background. Major organs were collected for histological and molecular analysis and observations were compared in the respective organs of Tudor-SN wild type litter mates. In study III, the hair of C57/BL/6 mice at the second telogen phase (7^{th} week) was shaved at caudodorsal region of animals and the shaved region of the skin was treated by 5% (w/v) α-MeSpd (n=5), 5% (w/v) spermidine (n=5) or vehicle (60% ethanol, 5% methyl cellulose and 20% propylene glycol) (n=5) to activate hair growth by α-MeSpd. Specimens from treated regions of skin and liver from each experimental animal were collected for histology, immunohistochemistry, molecular and biochemical assays. The animal experiments were approved by responsible regional authorities.

7. 2. Cell culture

Hek 293 cells, PC3, VCap, and 22RV1 cells were used to study the functional role of Tudor-SN. Hek 293 and PC3cells were grown in Dulbecco's Modified Eagle Medium (DMEM) whereas VCap, and 22RV1 were cultured in RPMI growth medium.

7. 3. Antibodies

Table IV. List of antibodies used in different studies

Name of the antibody	Host animals	Manufacturing company	Used in
Anti-androgen receptor	Rabbit polyclonal	Santa Cruz Biotechnology	**Fig 11**
Anti-drosha	Rabbit polyclonal	Santa Cruz Biotechnology	**Fig 11**
Anti-CD68	Mouse monoclonal	DAKO	**I**
Anti-CD3	Mouse monoclonal	DAKO	**I**
Anti-Tudor-SN	Mouse monoclonal	Home made	**I, II and Fig 10**
Anti-keratin 6	Mouse monoclonal	Santa Cruz Biotechnology	**III**
Anti-PCNA	Rabbit polyclonal	Santa Cruz Biotechnology	**III**
Anit-β-catenin	Rabbit polyclonal	Santa Cruz Biotechnology	**III**
Anti- β-actin	Mouse monoclonal	Santa Cruz Biotechnology	**III**
Anti-tubulin	Rabbit polyclonal	Santa Cruz Biotechnology	**I**

7.4. Histology and immunohistochemistry

For histology and immunohistochemistry, specimens collected from different organs of mice (study I, II), and treated area of skin for PCNA staining (study III) were fixed in 10% buffered

formalin solution) and skin samples from treated area for other than PCNA staining (study III) were fixed with histochoice for 24 hrs. After fixation, samples were washed with 0.1 M sodium phosphate buffer pH 7.4, dehydrated in graded ethanol, embedded in paraffin film and sectioned into 5 μM thick sections. The histological sections were stained with Hematoxylin-Eoisin (H-E) in all studies I, II and III. For immunohistochemistry, histological sections from mouse as well as arrays of prostate cancer patients were incubated in 3% H_2O_2 for 10 minutes and thereafter specimens were washed in Tris buffered saline buffer (TBS). To retrieve antigen, samples were boiled in pH 9.0 Tris –HCL buffer (study I, III) but for PCNA staining in specimens were boiled in 0.01 M citric acid buffer pH 6.0 (study III). Specimens were washed by TBS and blocked by pre-blocking solution for 30 minutes. Thereafter, specimens were incubated overnight with Tudor-SN monoclonal antibody (1:100)(Saarikettu *et al.* 2010) (study I), rabbit polyclonal anti-PCNA (1:750) (PC-10, Santa Cruz Biotechnology, Santa Cruz, CA) (study III), and anti-K6 (Covance, Princeton, NJ) and anti-BrdU-POD (Roche, Basel, Switzerland) (study III). For visualization Power Vision, Poly-HRP Rabbit IgG IHC kit (Immunovision technologies, Leica Microsystems, Wetzlar, Germany) was used. Samples were examined with the aid of light microscope and pictures were processed with Adobe Photoshop software.

7.5. Fluorescence and confocal microscopy

For fluorescence microscope, frozen sections of mouse tissues (study I), cytospinned bone marrow cells and HEK 293 cells cultured on the cover plates were used. Frozen tissue sections of 4-8 μm thickness were cut by cryostat at -20^{o}C. HEK 293 cells (200, 000 cells/ml) were cultured on sterilized cover slip for 24 hrs and thereafter rinsed by PBS. Air dried frozen sections, cytospinned bone marrow cells and HEK 293 cells were fixed by 4% paraformaldehyde (pH 7.4) (Merck KGaA, Darmstadt, Germany) for 15 min and following fixations specimens were rinsed by PBS and blocked by 5% bovine serum albumin (BSA). Besides, deparafinized sections from prostate cancer patients were also blocked with 5% BSA and processed with similar procedures. Thereafter, overnight incubation of specimens was carried after adding mouse anti-Tudor-SN monoclonal antibody (1:100), monoclonal mouse anti-human-CD3 antibody (Dako, Glostrup, Denmark) (1:10) and monoclonal mouse anti-human CD68 (1:100) (Dako) antibodies. After washing with PBS, specimens were incubated with anti mouse IgG1-AF555 (Invitrogen, Oregon, USA), anti-mouse IgG2a-AF488 (Invitrogen) and IgG-3a AF488 (Invitrogen) secondary antibodies to detect Tudor-SN, CD3 and CD68 expression, respectively. Specimens were incubated for an hour in dark room. Thereafter, specimens were washed with PBS and incubated with DAPI for 15 min in dark room and samples were examined under fluorescent and confocal microscopes.

7.6. May-Grünwald-Giemsa staining

Bone marrow, peripheral blood and splenocytes were cytospinned and stained with May Grünwald-Giemsa staining. Briefly, 200,000 cells were counted and cytospinned for 6 minute and Grünwald stain (Sigma) was added over dried cytospinned cells for 5 minutes. Excess Grünwald stain was removed by washing slides with 40 mM Tris-HCL for 1-2 minutes. Thereafter, diluted Giemsa stain was added on specimens for 15 minutes. Thereafter, specimens were washed by water and air dried slides were examined under light microscope.

7.7. Western blotting

For Western blotting RIPA buffer (Tris 50 mM, glycerol 10%, sodium deoxycholate 0.1 mg/ml (Sigma), NaCl 150 mM, EDTA 1 mM, SDS 0.1% and 1 tbl/50 ml complete protease inhibitor cocktail III) (Roche) was used to homogenize tissue samples except skin. Skin specimens were homogenized. under keratin urea buffer (8 M urea, 50 mM Tris pH 7.6,100 mM dithiothreitol, 0.13 M 2-mercaptoethanol, and 1tbl/50 ml complete protease inhibitor) (Roche). Protein sample concentrations were measured using protein measurement kit (Bio-Rad Laboratories, Hercules, CA). Proteins were separated by SDS-PAGE and transferred onto Protran® nitrocellulose filters (Whatman, Dassel, Germany) or hydrophilic PVDF transfer membrane (Immobilon P, Millipore, Billerica, MA) using a semi-dry transfer system (Millipore). Membranes were blocked with 5% of non-fat dried milk powder in TBS or PBS containing 0.1% Tween 20. Thereafter, membranes were incubated in specific primary antibodies, followed by washing and incubation of biotinylated (DAKO) or horseradish conjugated secondary antibodies (Santa Cruz Biotechnology). The membranes incubated with biotinylated antibodies were washed and incubated with strepavidin –biotin horseradish peroxidase complex (GE-health care, Little Chalfont, UK). Immunodetection was carried out by an enhanced chemiluminescence (ECL) system (GE-health care, Little Chalfont, UK). The exposed autoradiography film was developed with automatic developer (Kodak, Rochester, NY).

7.8. Co-immunoprecipitation and GST pull down

For co-immunoprecipitation 22RV1 and VCap prostate cancer cell lines were lysed with cytoplasmic buffer (10 mM HEPES, 10mM KCL, 0.1% NP 40 or Triton X-100) and nuclear buffer (20 mM HEPES (PH 8), 400 mM NACl, 10% glycerol, 1 mm EDTA) in order to fractionate cytoplasmic and nuclear protiens. In both buffers protease inhibitors such as PMSF (1:200), Amphotercin (1:500), and Pepstatin (1:500) were added. Nuclear lysates were diluted into 1:4 in dilution buffer (20 mM, HEPES (pH 8), 0.1 Triton X-100)). Equal amounts of lysates were incubated with primary anti Tudor-SN mouse monoclonal (1:100) and rabbit polyclonal anti androgen receptor antibodies (2) (Santa Cruz Biotechnology) for 1hr and for equilibration protein G-sepharose beads volume of 10 µl was added to each sample. 5% of lysate and lysates incubated with mouse serum (IgG) were used as positive and negative controls, respectively. The antibody, beads and lysate mixture were incubated in +4^{o}C about 1hr. Thereafter, the beads were washed with washing buffer (20 mM HEPES, 100 mM HEPES, 100 mm NACL and 0.1% Triton X-100). Thereafter 20 µl of 2x SDS loading buffer was added and with subsequent heating for 3 minutes co-immunoprecipitated proteins were released from the beads. The proteins were separated by SDS loading buffer and detected by anti Tudor-SN and anti androgen antibodies.

For GST pull down, equal amounts of lysates from 22RV1 and Vcap cells were incubated with GST, GST-SN1-4, GST-Tudor and GST-Tudor-SN full length for 1hrs. Thereafter, neutravidin beads (NeutrAvidin®UltraLinki®Resin, Pierce Biotechnology, Rockford, USA) were added to lysates and incubated for 4hrs in cold room. The beads were washed 4 times by washing buffer and proteins were released by adding 2x SDS loading buffer and detected by anti Tudor-SN and anti androgen receptor antibodies.

7.9. RT-qPCR

RNA samples isolated from liver, lung, muscle, kidney and brain of mice were transcribed to cDNA with M-MULV reverse transcriptase (Fermentas) and quantitative real-time PCR was performed using the cDNA as a template, Tudor-SN primers (CTTTTCCGAGCGTACCTGTG and TCTGCAGCTAGCAGCTCATC) as target gene -specific and TATA binding protein (TBP) primers (GCCTTCCACCTTATGCTCAG *and* TGCTGCTGTCTTTGTTGCTC) as house keeping gene -specific primers with SYBR green reagents (Fermentas) and BioRad CFX96 Real-Time PCR machine.

7.10. Luciferase reporter gene assay and transfection

200,000 PC3 cells were seeded on 12 well for 24 hrs prior to transfection. Thereafter, PCDNA 3.1 Androgen receptor construct with concentration of 0.3 μg, pCl-neo plasmid containing Tudor-SN construct with different concentrations (0, 0.1, 0.2, 0.3 and 0. 5 μg) and CMV-β-galactosidase reporter plasmid were used to transfect PC3. Lipofectamin 2000 (Invitrogen, Carlsbad, CA, USA) transfection reagent was used for transfection. After additional 24 hrs of transfection, the growth medium was changed to charcoal stripped serum free medium. Thereafter, samples were stimulated by dihydrotestosterone (DHT) treatement for 6 hrs and the wells not stimulated by DHT were used as control. Cells were lysed in Promega's Reporter Lysis Buffer (RLB) (Promega, Madison, WI, USA) and luciferase measurement was carried out by using Luciferase Assay Substrate Luminoscent (Thermoelectron Corporation, Finland).

7.11. Polyamine assay

Tissue samples taken from the treated site of the skin were heated in water bath at 55^0C for 20s. Thereafter, dermis and epidermis were separated by scalpel blade with gentle scraping. Separated samples of dermis and epidermis were homogenized under Tris buffer (25 mM Tris, pH 7.4, 1 mM dithiothreitol, 0.1 mM EDTA) and the proteins were precipitated by 5% sulfosalicylic acid (w/v final concentration). The concentrations of the natural polyamines and a-MeSpd were measured with the aid of high-pressure liquid chromatography

8. RESULTS

8.1. Expression level of Tudor-SN is higher in active and rapidly proliferating cells in mouse organs (study I)

8.1. 1. Expression analysis of Tudor-SN in major organs

The expression level of Tudor-SN was analyzed in major organs of mice including liver, brain and lung by using Tudor-SN monoclonal antibodies (Saarikettu *et al.* 2010) for immunohistochemistry and florescent immunostaining. In the liver, the expression level of Tudor-SN was low in hepatocytes; however, the expression level of Tudor-SN was higher in sinusoidal endothelial cells as compared to neighboring hepatocytes. In the brain, Tudor-SN was expressed both in the cortical neuron and some gilial cells. In the kidney, Tudor-SN was robustly expressed in the distal tubules compared to proximal tubules. Relatively higher expression level of Tudor-SN was observed in endothelial cells of glomerulus as compared to neighboring podocytes and nearby messangial cells. In the lung, up-regulated expression level of Tudor-SN was observed in Type I pneumocytes compared to type Type II pneumocytes.

8.1. 2. Expression analysis of Tudor-SN in the organs of the gastro-intestinal system

Tudor-SN was expressed in epithelial lining cells of gastro-intestinal system (GIS); however, the expression level of Tudor-SN protein varied in different cells in a given region of GIS. For, example, in duodenum the expression level of Tudor-SN was the same along the epithelial lining cells of villi whereas the expression level of Tudor-SN was robustly up-regulated in the paneth cells of the crypt of Lieberkühn in ileum compared to the surface epithelial cells and goblet cells. Both in the lamina properia of ileum and duodenum, the expression level of Tudor-SN was higly up-regulated in T lymphoctes and Tudor-SN was co-expressed with CD3. In the colon, up-regulated expression of Tudor-SN was observed in the entroendocrine cells relative to surface absorptive cells and goblet cells. In pancreas, the expression level of Tudor-SN was robustly up-regulated in exocrine cells compared to its expression level in the endocrine part.

8.1. 3. Expression analysis of Tudor-SN in the organs of the reproductive system

Expression of Tudor-SN was analyzed in testis, prostate gland, uterus and ovary. In testis, Tudor-SN was expressed almost in all cells except in terminally differentiated spermatids and spermatozoa cells. However, the expression level of Tudor-SN was up-regulated in spermatogonial cells and the expression level of Tudor-SN showed a decreasing trend as germ cells differentiate and was not expressed in more differentiated and terminally differentiated cells. In ovary, the expression level of Tudor-SN was up-regulated in follicular epithelial cells but it was not expressed in oocytes. In uterus, the expression level of Tudor-SN was higher in uterine epithelium and gland but its expression level was minimal in stromal component of uterus.

8.1. 4. Expression analysis of Tudor-SN in the organs of the lymphoid system

The expression analysis of Tudor-SN in lymphoid organs such as lymph node, spleen and Peyer's patch indicated up-regulated expression of Tudor-SN in T-lymphocytes, and it was also co-

expressed with CD3. In the spleen, up-regulated expression of Tudor-SN was observed in some cells of the red pulp. However, Tudor-SN was not expressed in macrophage of the red pulp of the spleen.

8.2. Tudor-SN in myeloid maturation (study II)

To understand the functional role of Tudor-SN in higher organisms, Tudor-SN -/- mice were generated (study II). Tudor-SN -/- mice as other Tudor-SN model organisms did not show any noticeable abnormalities or functional defect. However, modest decrease in body weight and size was observed in Tudor-SN -/- mice compared to wild type littermates. The comparison between different organs of Tudor-SN -/- mice with their littermates indicated that the weight of some organs most notably liver, kidney, and testis were significantly lower in Tudor-SN -/- mice. Histological analyses of different organs demonstrated that almost all organs were normal except very small necrotic foci were observed in the white pulp of the spleen. The micro-array analysis of splenocytes of Tudor-SN -/- mice showed alterations in the expression of genes involved in the granules of myeloid cells. Hematopoietic cell lineage analysis using cells from bone marrow and spleen showed decrease in granularity and number of granulocytes. Lower numbers of mature eosinophils (0.9%) and neutrophils (13%) in peripheral blood was observed in Tudor-SN-/-mice. There were no noticeable morphological differences between Tudor-SN -/- peripheral neutrophils and eosinophils as compared Tudor-SN +/+ peripheral neutrophils and eosinophils (**Fig. 10, I**). Tudor-SN was expressed in wild type bone marrow myeloid cells **(Fig. 10, II)**. There was no noticeable morphological defect in myeloid lineage cells from bone marrow and mature cells from peripheral blood cells of Tudor-SN null mice compared to the wild type littermates. However, the analysis of hematopoietic cells from spleen showed no significant changes in the T and their subpopulations including CD4 and CD8. The secretion of major cytokines known in the polarization of T cells was not altered. This observation led to conclusion that Tudor-SN is not essential for the development of mouse; however, the surprising phenotype observed in myeloid lineage of Tudor-SN -/- mice suggests that the protein may play a role during infection, allergy and tumorigenesis.

8.3. Role of Tudor-SN in prostate cancer (unpublished data)

Previously it has been reported that Tudor-SN is up-regulated in prostate cancer compared to normal and hyperplasic prostate gland epithelium. The same study suggested that the robust up-regulation of Tudor-SN in castration resistant prostate cancer (Kuruma *et al.* 2009). However, immunohistochemical and fluorescent immunostaining of Tudor-SN from 500 prostate cancer patient samples indicated that Tudor-SN was equally expressed both in normal prostate epithelium and cancerous epithelium in most cases (data not shown). However, the expression level of Tudor-SN was up-regulated in very small number (1%) of prostate cancer patient specimens **(Fig. 11 A).** Previously Tudor-SN has been shown as co-activator of several transcription activators. To check whether Tudor-SN is co-activator of androgen receptor, PC3 cells were co-transfected with a PCDNA3.1 *TMPRSS2* and pCl-neo Turdor-SN with different concentration of plasmid DNA in the presence and absence of DHT. There was no change in the activity of luciferase reporter in the control samples and samples co-transfected with different

concentration of pCl-neoTurdor-SN suggesting that Tudor-SN is not a co-activator of androgen receptor (data not shown).

Furthermore, to check whether Tudor-SN is physically interacting with androgen receptor, Tudor-SN was co-immunoprecipitated with androgen receptor. Co-immunoprecipitation experiment suggests that Tudor-SN did not interact with full length androgen receptor; however, it interacted with splicing variant of the androgen receptor (**Fig. 11 B and C**). To investigate which domain of Tudor-SN was interacting with splice variant of androgen receptor, GST pull down of SN1-4 domains, Tudor domain and full length of Tudor-SN was carried out and immnoblotting was conducted with anti androgen receptor antibody. The results indicated that Tudor-SN interacted with androgen receptor splice variant through SN1-4 domains but not through Tudor domain **(Fig. 11 D).**

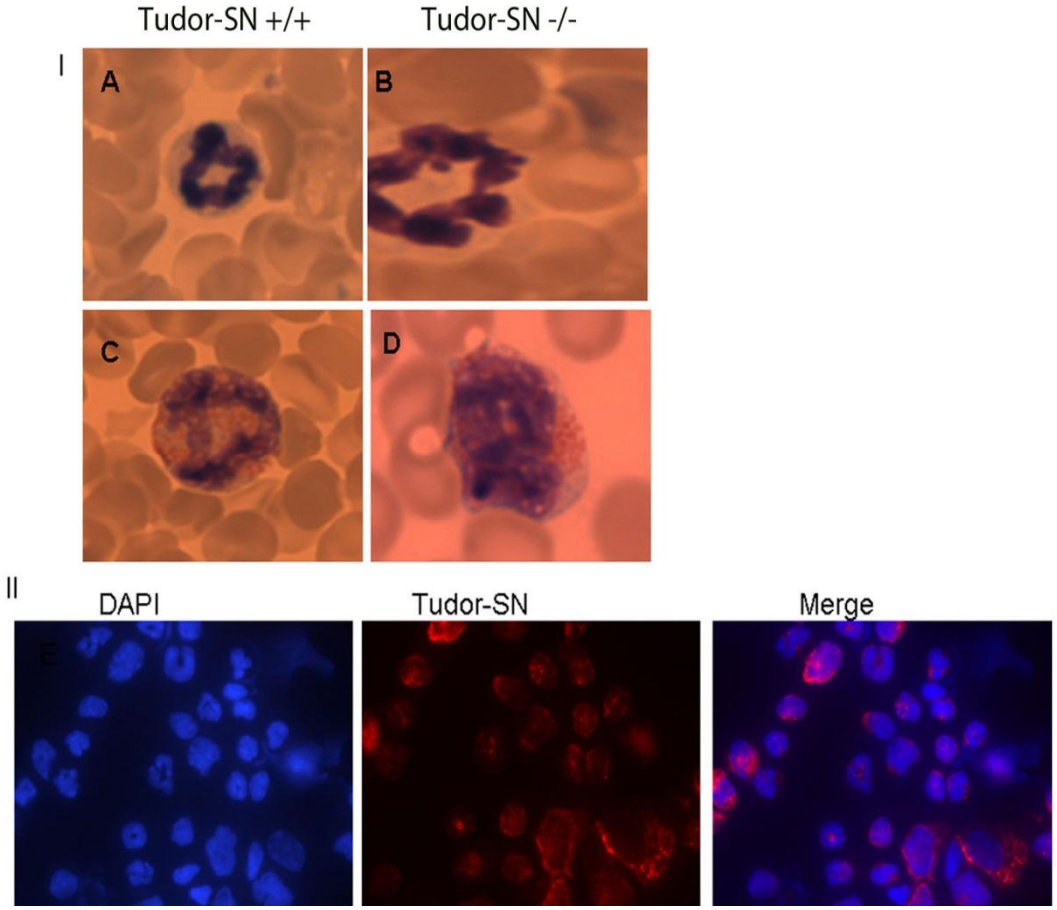


Figure 10. Peripheral polymorphonuclear cells stained by Giemsa and flourescent immunostaing of Tudor-SN in bone marrow cells. I, depicts the morphology of Tudor-SN -/- peripheral polymorphonuclear cells: Tudor-SN +/+ neutrophil (A), Tudor-SN -/- (B), Tudor +/+ eosinophil (C) and Tudor-SN -/- eosinophil (D). II, flourescent immunostaining depicts

expression of Tudor-SN in bone marrow cells.

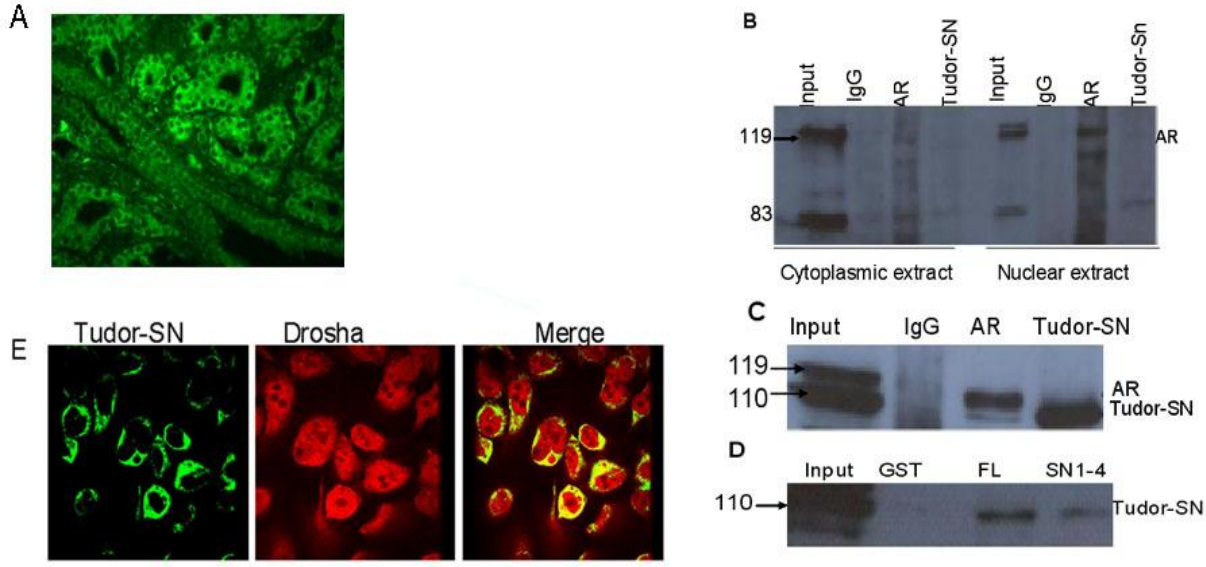


Figure 11. Tudor-SN in prostate cancer specimens and co-localizeation with Drosha in HEK293 cells. Tudor-SN did not interact with full length Androgen receptor (AR); however, it interacted with AR splice variants both in cytoplasm and nucleus of 22RV1 cells (immunoprecipitated by anti-AR and Tudor-SN antibody and detected by anti AR antibody. Interaction band can be appreciated in Tudor-SN lane at 80 kD (B)), whole cell lysates from Vcap cells (C) and GST pull down of different Tudor-SN domain indicated that the interaction between Tudor-SN and AR was mediated by SN1-4 domains of Tudor-SN (D)**.** Confocal image HEK293 cells shows that Tudor-SN was colocalized with Drosha in HEK293 (E). AR, androgen receptor; SN 1-4, staphylococcal nuclease domain1-4; FL, Tudor-SN full length.

8.4. Activation of polyamine regulated genes trigger hair follicle growth (Study III)

Recent studies demonstrated hairless phenotype in both SSAT transgenic and ODC transgenic mice. The biochemical analysis of the skin of ODC and SSAT transgenic mice revealed the accumulation of putrescine and decreased concentration of spermidine suggesting that undisturbed polyamine homeostasis is indespensable for proper hairfollicle development. Furthermore, DFMO treatment of ODC and SSAT transgenic mice corrected the altered polyamine pools of the skin and was able to induce the hair growth. In mammals, the growth of hair follicle is a cyclic phenomenon where hair follicle undergoes three typical phases called anagen, catagen and telogen (Soler *et al*., 1996; Pietilää *et al.* 2001). During the resting phase of telogen, spermidine pool of skin is lower than in the anagen phase (Pietilää *et al.* 2001). Other studies have demonstrated that its analogue, α-methylspermidine can serve as a surrogate molecule for spermidine. Therefore in this study (study III), it was proposed that growth of hairfollicle in the skin of telogen mice can be induced by increasing the concentration of spermidine by application of α-methylspermidine Räsänen *et al*. 2002. Indeed, α-methylspermidine treatment induced hair growth in the application site after two weeks of

application. The growing hair on the application site of skin showed typical attributes of anagen skin including pigmentation, proliferation of follicular keratinocytes and up-regulated expression of β-catenin. Furthermore, natural polyamines were partially replaced by α-methylspermidine and α-methylspermidine was detected in the dermis of the skin suggesting that proteins regulated by polyamines have a key role in the hair cycle.

9. DISCUSSION

9.1. Expression analysis of Tudor-SN (study I)

Tudor-SN is a protein that participates in several cellular functions such as a co-activator of transcription, apoptosis, as a component of the RISC complex, involves in the degradation of hyperedited dsRNA, tumor angiogenesis and cancer metastasis (Tong *et al.* 1995a; Santhekadur *et al.* 2012; Kuruma *et al.* 2009; Kuruma *et al.* 2009; Tsuchiya *et al.* 2007; Blanco *et al.* 2011). Although the protein is well conserved in all metazoan its physiological function has not been fully understood. The protein is expressed in several organs such as mammary gland, pancreatic cells, parotid, anterior pituitary, corpus luteum, ovarian follicular cells, placenta and small intestine both in bovine and mouse. However, by using the recently developed mouse anti-Tudor-SN antibody, comprehensive expression analysis of Tudor-SN in several mouse organs was carried out (in study I) by immunohistochemistry, fluorescent immunostaining, western blotting and RT-qPCR. Except in muscle tissue, the study indicates that Tudor-SN was expressed ubiquitously in all organs. However, higher expression level of the Tudor-SN protein was observed in epithelial cells (with few exceptions) most notably robust expression was observed in a rapidly proliferating and precursor cells.

Expression of Tudor-SN in secretory organs such as mammary gland and liver has been reported (Broadhurst & Wheeler 2001; Broadhurst *et al.* 2005). In agreement with this observation, expression of abundant Tudor-SN was observed in exocrine pancreas. However, the expression level of Tudor-SN was very low in endocrine pancreas. The localization of Tudor-SN to endoplasmic reticulum as well as to lipid droplets in the cytoplasm of milk secreting mammary gland epithelial cells of lactating cow and mice has been demonstrated (Keenan *et al.* 2000). The abundant expression of Tudor-SN in lactating mammary gland epithelial cells has been shown to occur without changes in the expression level of mRNA of Tudor-SN, which suggests the post-transcriptional regulation of the protein (Broadhurst *et al.* 2005). However, comparison of the expression of Tudor-SN protein and its mRNA in liver, lung, skeletal muscle, kidney, and brain with RT-qPCR, immunohistochemistry and western blotting indicated close co-relation between mRNA and protein. Nevertheless, post-transcriptionally modified Tudor-SN protein has been previously reported (Keenan *et al.* 2000). Higher expression level of Tudor-SN in the secretory organs like exocrine pancreas suggests that the protein may have a crucial function in the process of post-transcriptional and or post-translational process of secreted proteins. Indeed Tudor-SN null *Arabidopsis* could not survive under salt stress conditions and Tudor-SN has been shown to stabilize subset of mRNAs that are intended to move to the secretory pathway (dit Frey *et al.* 2010). Nevertheless, it remains to be determined whether the ability of Tudor-SN to stabilize a group of mRNAs intended to secretory pathway is a general phenomenon for all secretory organs.

The expression level of Tudor-SN is also correlated with proliferation behavior of cells in a given organ. High level of Tudor-SN expression was observed in the rapidly dividing and precursor cells such as bulb cells of hair follicle, spermatogonial cells and primary spermatocytes of testis, follicular cells of oocytes and in paneth cells of crypt of Lieberkühn. The expression level of Tudor-SN showed a decreasing trend as cells undergoe further differentiation and Tudor-SN was not expressed in terminally differentiated cells. Previous studies have also demonstrated up-regulated expression of Tudor-SN in cell proliferation, transformation and malignant metastasis

(Santhekadur *et al.* 2012; Tsuchiya *et al.* 2007; Blanco *et al.* 2011). These studies suggested that the expression level of Tudor-SN is high in metabolically active cells.

The higher expression level of Tudor-SN in T cells both in lymphoid organs such as spleen, lymph nodes and Peyer's patch as well as peripheral T cells suggesting that Tudor-SN may have a role to play in immune system. Furthermore, the up-regulated expression of Tudor-SN in paneth cells of crypt of Lieberkühn suggests that the protein can play an important role in innate immunity. Furthermore, the expression level of Tudor-SN in myeloid precursor cells suggest that the expression of Tudor-SN protein is up-regulated in the proemyelocyte stages and decreased expression level was observed in more differentiated segmented cells of myeloid lineage (**Fig. 10**). In myeloid cells it seemed that Tudor-SN is more expressed in granules as compared to other parts of cytoplasm. Taken together, the up-regulated expression of Tudor-SN observed in premyelocyte cells and paneth cells which are known to secrete anti-microbial proteins and peptides indicated that Tudor-SN can play a very important role in innate immunity.

The higher expression level of Tudor-SN protein has been reported most notably in castration resistant and more aggressive prostate cancer (Kuruma *et al.* 2009; Kuruma *et al.* 2009). Kuruma et al. (Kuruma *et al.* 2009) have also suggested that Tudor-SN can be a diagnostic marker for prostate cancer. However, this study suggested that Tudor-SN is expressed both in normal human and mouse prostate gland epithelial cells which challenges the previous study suggesting that Tudor-SN can serve as a diagnostic marker. Apart from few prostate cancer specimens where the Tudor-SN expression was up-regulated, in most prostate cancer specimens (both from hormone sensitive and insensitive prostate cancer patients) the expression level of Tudor-SN was not changed. Nevertheless, through its SN1-4 domains Tudor-SN seems to interact with splice variants of androgen receptor (**Fig 10 B, C and D**).

9.2. Tudor-SN is dispensable for survival but important for myeloid maturation (study II).

Tudor-SN mutant organism including *Schizosaccharomyces pombe* (Decottignies *et al.* 2003), *African trypansomes* (Alsford *et al.* 2010), *C. elegans* (Grishok *et al.* 2005), *Drosophila melanogaster* (Dorner *et al.* 2006), *Arabidopsis* (dit Frey *et al.* 2010) have been generated in the last decade and all Tudor-SN mutant organism have been reported to be viable and fertile. Except in *Arabidopsis,* Tudor-SN homozygous mutant showed a 3-5 fold decrease in the progeny and moderate reduction in the growth of the root in optimal *in vitro* conditions but they are hypersensitive for salt stress condition (dit Frey *et al.* 2010). Tudor-SN -/- mouse (in study II) was also fertile that rules out the role of Tudor-SN in spermatogenesis or oogenesis. Tudor-SN was expected to play an important role in spermatogenesis and oogenesis as the previous study suggested the physical interaction mediated by Tudor domain of Tudor-SN and PIWIL1 protein (Liu *et al.* 2010).

The genome-wide expression analysis of RNA of splenocytes and subsequent validation with RT-qPCR demonstrated decreased expression of genes that are important in neutrophilic granule formation such as myeloperoxidase (Mpo), cathelicidinantimicrobial peptide (Camp), neutrophilic granule protein (Ngp), neutrophil-expressed proteases neutrophil elastase (Ela2) and proteinase 3 (Prtn3). The serine protein inhibitors such as Serpina1b and Serpina1d were also

over-expressed. Serpinas have been shown to be secreted protein (Kuiperij *et al.* 2009) and previously it has been shown that Tudor-SN is stabilizing mRNA of secreted proteins (dit Frey *et al.* 2010) at least in plants. Thus, over-expression of Serpina 1d and 1b in Tudor-SN -/- mice can be a compensatory mechanism to overcome the probable degradation of their mRNA in the absence of Tudor-SN as their stabilizer protein. This study suggests that Tudor-SN is dispensable for normal development of mice at early or late at adult age; however, it is plays a crucial role in the maturation of myeloid lineage. This study generally suggestes that Tudor-SN can be a critical protein in mediating infection, inflammatory process, allergy, innate immunity and cancer.

9.3. α-Methylspermidine activates hair growth (study III)

In mammals, hair follicle undergoes distinct and lifelong cyclic transformation including rapid growth phase (anagen), a short apoptosis driven phase (catagen), and relative long resting phase (telogen)(Stenn & Paus 2001). The factors that trigger hair follicle growth are thought to be originated from dermal papilla. However, the actual regulators which trigger growth of hair follicle have been elusive (Alonso & Fuchs 2006). Nevertheless, several growth factors have been implicated in maintenance of hair cycle. The concentration of polyamine pools has been measured during hair cycle and it correlates with hair cycle phases. For example, an increased concentration of natural polyamines, such as putrescine, spermidine and spermine, was observed in the first 10 days of postnatal life or anagen phase mouse (Pietilä *et al.* 1997). The pool of polyamine in mouse skin has been shown to be increased during anagen (Pietilä *et al.* 2001). Induction of polyamine catabolism or over-expression of antizyme has reduced the susceptibility to chemical induced cutaneous carcinogenesis (Pietilä *et al.* 2001; Feith *et al.* 2007). In study (III) it was proposed that hair growth can be triggered by elevating the cutaneous concentration of polyamines, notably spermidine, in telogen phase of mouse skin. However, the natural spermidine is not stable and therefore it is not a suitable molecule for the proposed study. However, a methylated analogue of spermidine, α-methylspermidine, has been shown to be stable and it can serve as an efficient surrogate for its natural counterpart The application of α-methylspermidine on the skin of the telogen phase mouse induced the growth of hair follicle with typical features of anagen cycle including up-regulated expression of proliferation markers such as PCNA, cytokeratin 6, melanogenesis and up-regulated expression of β-catenin.

The growth of hair follicle through accumulation of α- methylspermidine is achieved even though the concentration of natural spermidine is low in the application site of analogue and this suggests that by replacing intracellular natural spermidine, the stable methylated analogue serves as an efficient and functionally competent substitute for its natural counterpart. Polyamines have been reported to regulate S-phase in cell cycle (Alhonen *et al.* 2002), and the up-regulated expression of PCNA in the growing hair follicle in the application site further reinforces the finding that α-methylspermidine-induced hair follicle growth indeed possesed the characteristic features of anagen. The confined up-regulated expression of cytokeratin 6 in the hair follicle was observed but it was not expressed in the interfollicular epidermis. This further confirms that α-methylspermidine triggered growth of hair follicle like any anagen-triggering molecule as the expression of cytokeratin 6 in the interfollicular epidermis indicates hyperproliferative reaction in response to wound, neoplastic reaction and irritation (Freedberg *et al.* 2001), the unresponsiveness of primary keratinocytes for analogue treatment supports the specificity of anagen-triggering effect of the analogue.

The growth of hair follicle in response of α-methylspermidine treatment occurred with concurrent deposition of melanin pigment. The simultaneous deposition of melanin pigmentation and the growth of hair follicle in the anagen phase of hair cycle have been previously reported (Slominski *et al.* 2004). Melanogenesis, the production of melanin pigment, occurs subsequent to the rapid proliferation of melanocytes and melanocytes transfer their pigments to rapidly dividing keratinocytes during anagen phase of hair cycle (Slominski *et al.* 2004). Both hair follicle morphogenesis and growth are regulated by Wnt/β-catenin/Lef-1 signaling pathway (Millar 2002). Skin specific β-catenin and Lef-1 null mice or Wnt inhibitor over-expressing mice failed to grow hair follicle (Huelsken *et al.* 2001; van Genderen *et al.* 1994; Andl *et al.* 2002). The up-regulated expression of β-catenin in α-methylspermidine application site further supports the conclusion the activated hair growth was indeed regulated by Wnt/β-catenin pathway. The hair cycle has been intensively investigated; however, factors that regulate hair cycle have not been fully comprehended. In this study, it was demonstrated that polyamines can play a crucial role in the regulation of hair cycle and the stable methylated polyamine analogues can be important therapeutic molecules for the treatment of hair loss.

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12. ORIGINAL COMMUNICATIONS

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Expression analysis of Tudor-SN protein in mouse tissues

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ABSTRACT

Tudor-SN (SND1, p100) has been shown to function as a transcriptional coactivator as well as a modulator of RNA metabolism and biogenesis and a component in the RNA-induced silencing complex (RISC). Tudor-SN consists of five repeats of staphylococcus nuclease-like domains (SN1–SN5) and, a Tudor domain implicated in binding to methylated ligands. The protein is highly conserved through evolution from fission yeast to mammals and it exists as a single gene without any close homologs. Tudor-SN is found to be overexpressed in several cancers such as colon adenocarcinomas and prostate cancer. The conservation of Tudor-SN along evolution suggests it may have important functions; however, the physiological function of Tudor-SN has not yet been characterized. In this study we analyzed the expression and localization of Tudor-SN in mouse tissues and organs by immunohistochemistry, fluorescent immunostaining, Western blotting and RT-qPCR. Expression analysis indicated that Tudor-SN is widely expressed in most organs with the exception of muscle cells. Up-regulated expression was observed in rapidly dividing cells and progenitor cells such as in spermatogonial cells in testis, in the follicular cells of ovary, in the cells of crypts of Lieberkühn of ileum and basal keratinocytes of skin and hair follicle when compared to more differentiated or terminally differentiated cells in the respective organs. Moreover, Tudor-SN was robustly expressed in T-cells and Tudor-SN was co-expressed with CD3 in T-cells in the Peyer's patch, spleen and lymph node. The wide expression pattern of Tudor-SN and high expression in proliferating and self-differentiating cells suggests that the protein serves functions related to activated state of cells.
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1. Introduction

Tudor-SN is a 100 kDa protein that was initially identified as a transcriptional co-activator for the viral EBNA-2 protein (Tong et al., 1995). Tudor-SN exists as a single gene and maps to chromosomes 7q31.3 in human, 6 in mouse and 4q23 in rat. In zebrafish Tudor-SN is shown to have two splice transcripts and protein is also shown to be translated from both splice variants (Zhao et al., 2003). The Tudor-SN gene as well as its domain architecture is conserved in mammals, fish, drosophila, *Caenorhabditis elegans*, ciliates and fission yeast, but not in bacteria and *Saccharomyces cerevisiae*. Conservation along evolution suggests Tudor-SN has important physiological functions. Tudor-SN protein consists of four tandem repeats of staphylococcus nuclease like SN domains (SN1–SN4) at the N terminus, followed by a Tudor domain and the fifth SN domain (Ponting, 1997; Selenko et al., 2001; Shaw et al., 2007). The protein has been reported to be localized both in the nucleus and in cytoplasm (Broadhurst and Wheeler, 2001; Zhao et al., 2003; Caudy et al., 2003; Saarikettu et al., 2010). The cellular localization is suggestive of variable functions, and Tudor-SN has been shown to function as a transcriptional co-activator for transcription factors such as c-Myb (Leverson et al., 1998), STAT5 (Paukku et al., 2003), and STAT6 (Yang et al., 2002; Välineva et al., 2005, 2006). The interaction between Tudor-SN and specific transcription factors recruits basal transcription machinery and co-activators such as RNA Pol II, CBP, and RNA helicase A to the promoter to facilitate initiation of RNA transcription (Nakajima et al., 1997; Yang et al., 2002; Välineva et al., 2006).

In addition to its involvement in transcriptional co-activation, Tudor-SN has been shown to function in post-transcriptional regulation including RNA interference, spliceosome assembly and pre-mRNA splicing, and degradation of A to I hyper-edited double-stranded RNA (Serra et al., 2004; Scadden, 2005; Yang et al., 2007). Tudor-SN is detected in the RISC complex and it interacts with Ago-2 (Caudy et al., 2003; Paukku et al., 2008). Both biochemical and structural studies indicate that Tudor-SN functions as a miRNase. I.U. containing double stranded RNA is reported to be a specific target for Tudor-SN mediated degradation (Levanon et al., 2004; Li et al., 2008). Tudor-SN interaction with U5 snRNP (small nuclear ribonucleoproteins) facilitates the assembly of spliceosome and increases the rate of splicing in *in vitro* splicing assays (Yang et al., 2007). Tudor-SN has also been shown to bind to a

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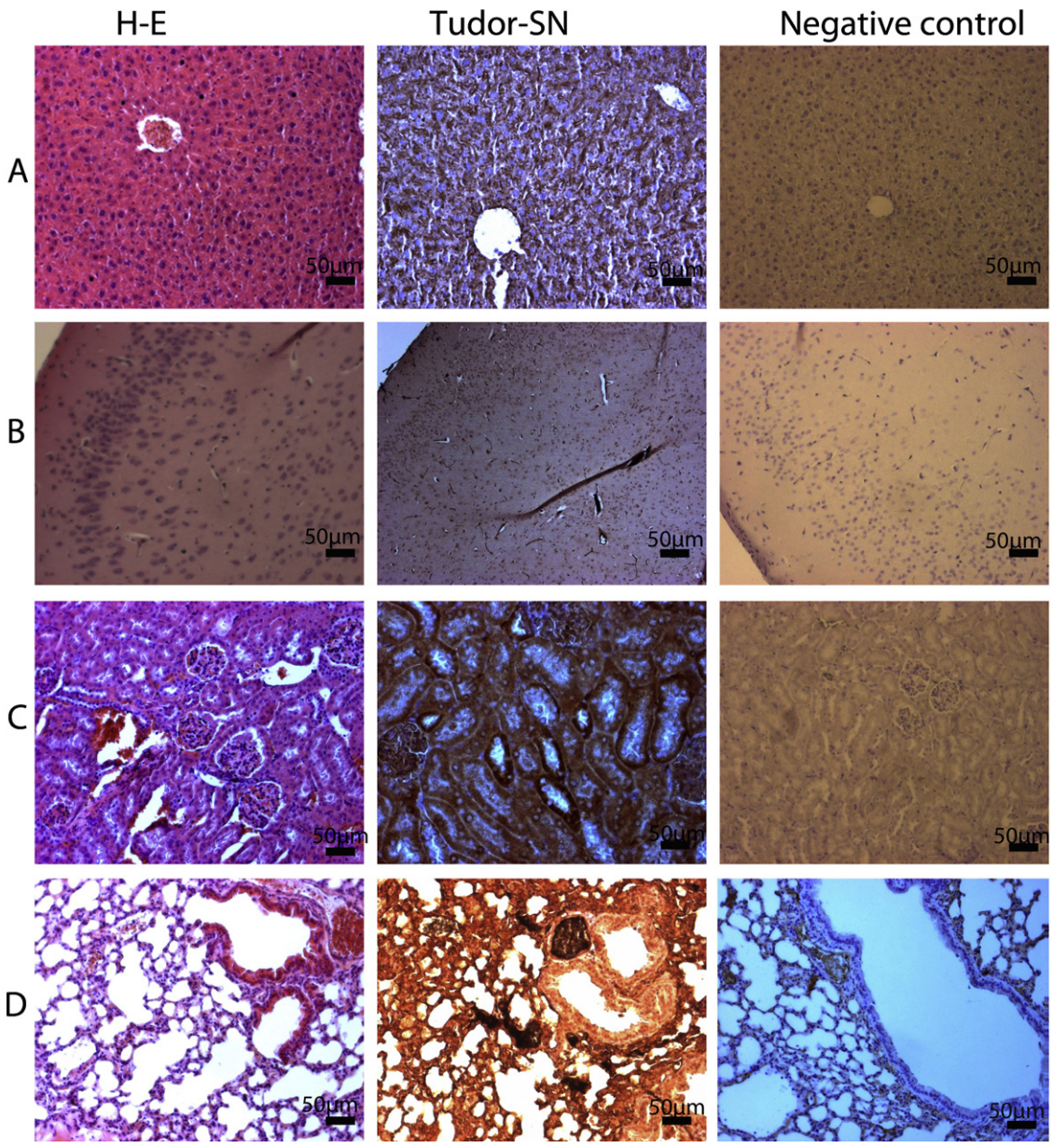

Fig. 1. Immunohistochemical staining of Tudor-SN in formalin fixed major organs of mouse. The figure depicts the immunohistochemical staining of Tudor-SN with mouse anti-Tudor-SN (anti-SN4-1) antibody in liver (A), brain (B), kidney (C) and lung (D). The first column shows ordinary H–E staining of the respective organs; the second column shows Tudor-SN staining and the third column shows negative control (staining without primary antibody). In the hepatocytes of liver, the staining of Tudor-SN shows low expression of protein and the expression level of Tudor-SN is up-regulated in endothelial cells of sinusoids (A). Tudor-SN is expressed both in cortical neurons and in some of glial cells in brain (B). Expression level of Tudor-SN is up-regulated in the distal tubules of kidney compared to nearby proximal tubules. Its expression is also up-regulated in the endothelial cells of glomeruli compared to adjacent cells of podocytes and mesangial cells. Expression level of Tudor-SN appeared to be down-regulated in mesangial cells compared to all surrounding cells of both Bowman's capsule and renal tubules (C). Expression level of Tudor-SN is up-regulated in alveolar cells compared to bronchiolar epithelium (D) (scale bar = 50 μm).

3′-UTR (3′ untranslated region) of a specific mRNA and this binding is suggested to stabilize and increase expression of the gene (Paukku et al., 2008). In summary, these biochemical studies suggest several cellular functions for the Tudor-SN protein but its physiological function has remained elusive. The first *in vivo* Tudor-SN null mutant models have been established in *Schizosaccharomyces pombe* (Decottignies et al., 2003), *C. elegans* (Grishok et al., 2005), *Drosophila melanogaster* (Dorner et al., 2006) and very recently in *Arabidopsis* (dit Frey et al., 2010). All Tudor-SN deficient mutant models were reported to be viable and fertile and without gross developmental defects. In *Arabidopsis* double Tudor-SN homozygous mutant showed a 3- to 5-fold ofunderrepresentation in the progeny, moderate reduction of root growth in optimal *in vitro* conditions. The Tudor-SN mutants showed hypersensitivity to salt stress condition and the authors demonstrated that Tudor-SN is stabilizing a subset of salt stress responsive mRNAs which are intended to secretory pathway (dit Frey et al., 2010).

Tudor-SN protein has been detected in multiple organs such as mammary gland, pancreatic cells, parotid, anterior pituitary, corpus luteum, ovarian follicular cells, placenta and small intestine. Most notably it is highly expressed in actively secreting organs such as lactating mammary gland and pancreatic cells (Broadhurst and Wheeler 2001; Broadhurst et al., 2005). Tudor-SN is also suggested to play a role in cell proliferation as cellular transformation has been achieved via ectopic expression of Tudor-SN (Tsuchiya et al., 2007). This finding was further supported by results showing that cell growth was inhibited by decreased expression of cellular Tudor-SN.

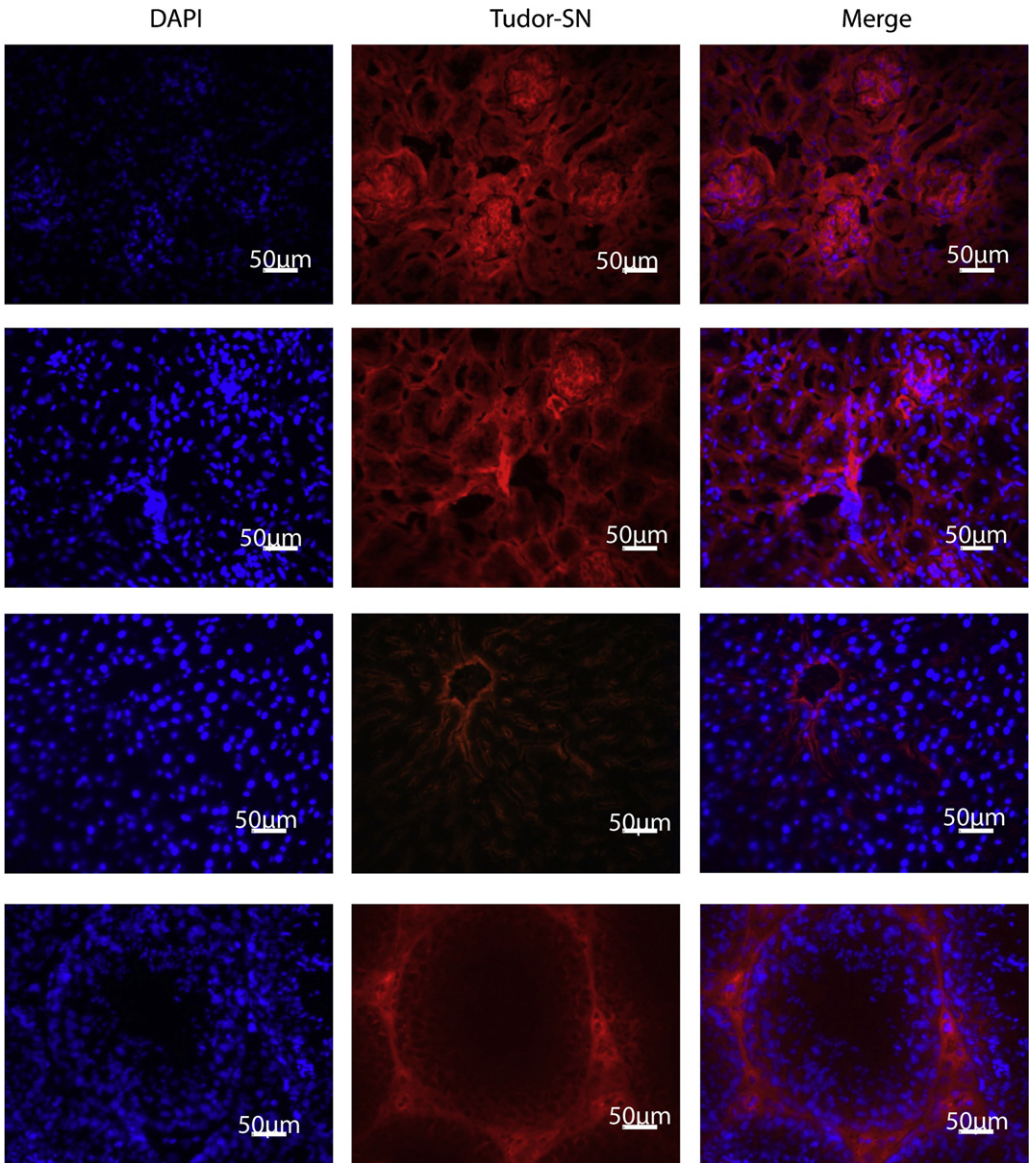

Fig. 2. Fluorescent immunohistochemical staining of Tudor-SN from frozen mouse organs. The figure depicts the fluorescent immunohistochemical staining of Tudor-SN from frozen section of kidney (A and B), liver (C) and testis (D). The first column shows DAPI staining, the second column shows Tudor-SN staining and the third column shows merged image of Tudor-SN and DAPI staining. In kidney there is no appreciable difference in the level of Tudor-SN expression in proximal or distal tubules; however, the expression level of Tudor-SN is slightly higher in the endothelial cells of glomeruli (A and B). In the hepatocytes Tudor-SN expression is low, but Tudor-SN is expressed in endothelial cells of sinusoids and central vein of liver (C). In testis, the expression level of Tudor-SN is higher in Leydig cells and myofibroblast cells followed by spermatogonial cells. However, in spermatocytes and Sertoli cells the expression level of Tudor-SN shows decreasing trend and subsequently its expression is relinquished in terminally differentiated spermatid and spermatozoal cells (D).

Furthermore, Tudor-SN is one of the most overexpressed genes in colon adenocarcinoma and post-transcriptional regulation of APC gene by Tudor-SN is suggested to play a crucial role in colon cancer (Tsuchiya et al., 2007). Tudor-SN gene is also reported to be overexpressed in prostate cancer and the expression level correlated with the grade and aggressiveness of the cancer. Furthermore, knocking down of Tudor-SN suppressed the growth of cancer cells (Kuruma et al., 2009). Recent observations have also linked Tudor-SN with the apoptosis pathway (Sundström et al., 2009), and suggested a role in hereditary autosomal-dominant polycystic kidney disease (ADPKD) (Low et al., 2006).

In this study we used the recently developed MoAbs against Tudor-SN to conduct a comprehensive expression analysis of Tudor-SN in different mouse tissues and compare the expression level of the protein in different cell types and with the functional properties of the tissues. The analysis demonstrated wide expression of Tudor-SN in most tissues and particularly strong expression in epithelial cells. Abundant expression of Tudor-SN was observed in rapidly dividing and progenitor cells, and notably the expression was up-regulated in spermatogonial cells in testis, in the follicular cells of ovary, in the cells of crypts of Lieberkühn of ileum and basal keratinocytes of skin and hair follicle when compared to more differentiated or terminally differentiated cells in the respective organs. Moreover, Tudor-SN was highly expressed in T-cells and co-expressed with CD3 in T-cells in the Peyer's patch, spleen and lymph node.

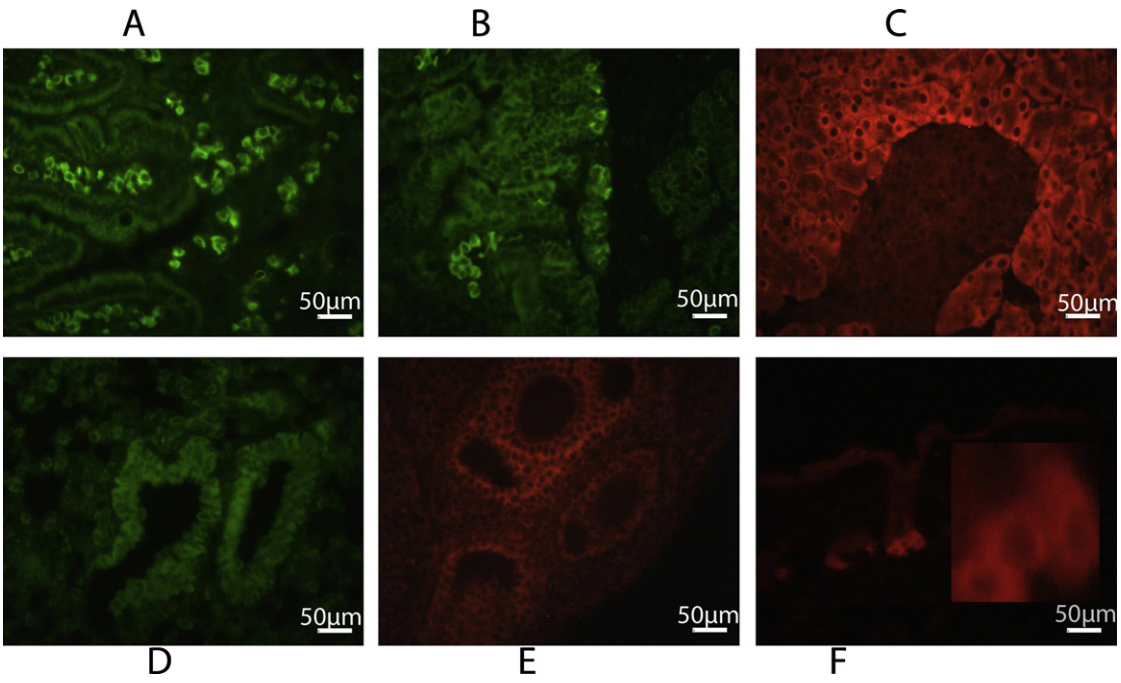

Fig. 3. Fluorescent immunohistochemical staining of Tudor-SN in duodenum, ileum, pancreas, lung, ovary and skin. The figure depicts fluorescent immunohistochemical staining of Tudor-SN in duodenum (A) ileum (B), pancreas (C), lung (D), ovary (E) and skin (F).

2. Materials and methods

2.1. Histology, immunohistochemistry and fluorescent immunostaining

Specimens of organs from both adult female and male C57BL/6 mouse strain were collected. For histology and immunohistochemistry, samples were fixed with buffered formalin (10%) solution for 24 h. Following the fixation, samples were washed with M sodium phosphate buffer (pH 7.4), dehydrated in graded ethanol, embedded in paraffin and cut into 5–8 μm thick sections. The sections were stained with hematoxylin and eosin (H–E). For immunohistochemistry, deparaffinized and dehydrated samples were subjected to 3% H_2O_2 for 10 min to block endogenous peroxidase and washed with Tris buffered saline buffer (TBS). For antigen retrieval samples were boiled in pH 9.0 Tris–HCl buffer. Thereafter, specimens were washed with TBS and blocked by pre-blocking solution for 30 min. Specimens were incubated overnight with primary mouse monoclonal anti Tudor-SN (clone anti-SN4-1, Saarikettu et al., 2010) (1:100) antibody. Specimens were washed with TBS. To visualize the mouse monoclonal primary antibodies, we used Power Vision Poly-HRP Rabbit IgG IHC kit (Immunovision technologies, Leica Microsystems, Wetzlar, Germany). Hematoxylin was used as contrast staining and specimens stained only with secondary antibodies and hematoxylin were used as negative controls. For fluorescent immunostaining, frozen tissues were used. Tissue sections with 4–8 μm thickness was cut with cryostat at −20 °C and before fixation specimens were let to air dry for 10–15 min. Specimens were fixed with 4% paraformaldehyde (pH 7.4) (Merck KGaA, Darmstadt, Germany) for 15 min. After fixation, specimens were rinsed with PBS and blocked with 5% bovine serum albumin (BSA) (Sigma) for 30 min. Thereafter, specimens were incubated overnight with the mouse monoclonal anti Tudor-SN antibody (1:100), monoclonal mouse anti-human-CD3 (Dako, Glostrup, Denmark) (1:10) and monoclonal mouse anti-human CD68 (1:100) (Dako) antibodies. Specimens were washed with PBS and were incubated with anti mouse IgG1-AF555 (Invitrogen, Oregon, USA), anti-mouse IgG2a-AF488 (Invitrogen) and IgG-3a AF488 (Invitrogen) secondary antibodies to detect Tudor-SN, CD3 and CD68 expression, respectively. Specimens were incubated for an hour in dark room. Thereafter specimens were washed with PBS and incubated with DAPI for 15 min in dark room.

2.2. Immunoblotting

For immunoblotting, tissues samples were homogenized using RIPA buffer (Tris 50 mM, glycerol 10%, sodium deoxycholate 0.1 mg/ml (Sigma), NaCl 150 mM, EDTA 1 mM, SDS 0.1% and 1tbl/50 ml) complete protease inhibitor cocktail III (Roche). Equal loadings of protein extract (100 μg) were separated in 10% dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under running buffer (25 mM Tris, 0.19 M glycine and 0.1% SDS). The transfer of separated proteins was carried out onto protran transfer membrane (Whatman). The membrane was incubated with 5% of non-fat milk powder in PBS buffer to block non-specific binding. Thereafter, the membranes were incubated with the mouse monoclonal anti-Tudor-SN antibody (1:1000) and membranes were washed with 0.5% Tween 20 (Sigma)–TBS. Thereafter, membrane was incubated by biotinylated goat anti-mouse secondary antibody (Dako) for half an hour and washed with 0.5% Tween 20–TBS and membranes were washed with 0.5% Tween 20 (Sigma). Thereafter, the membrane was incubated with streptavidin–biotinylated horseradish peroxidase (Little Chalfont Bucking, Hampshire, UK) and the membrane was washed with 0.5% Tween 20–TBS. The membranes were incubated with chemiluminescent substrate, sealed into a plastic bag and placed into a film frame together with an autoradiography film (Kodak, Rochester, NY). The exposed film was developed with automatic developer.

2.3. Real time quatitative PCR

To isolate total RNA 50–100 mg of mouse tissue sample from liver, lung, muscle, kidney and brain were collected in 1 ml TRIzol (Invitrogen) and homogenized with PowerLyzer (Mobio) at

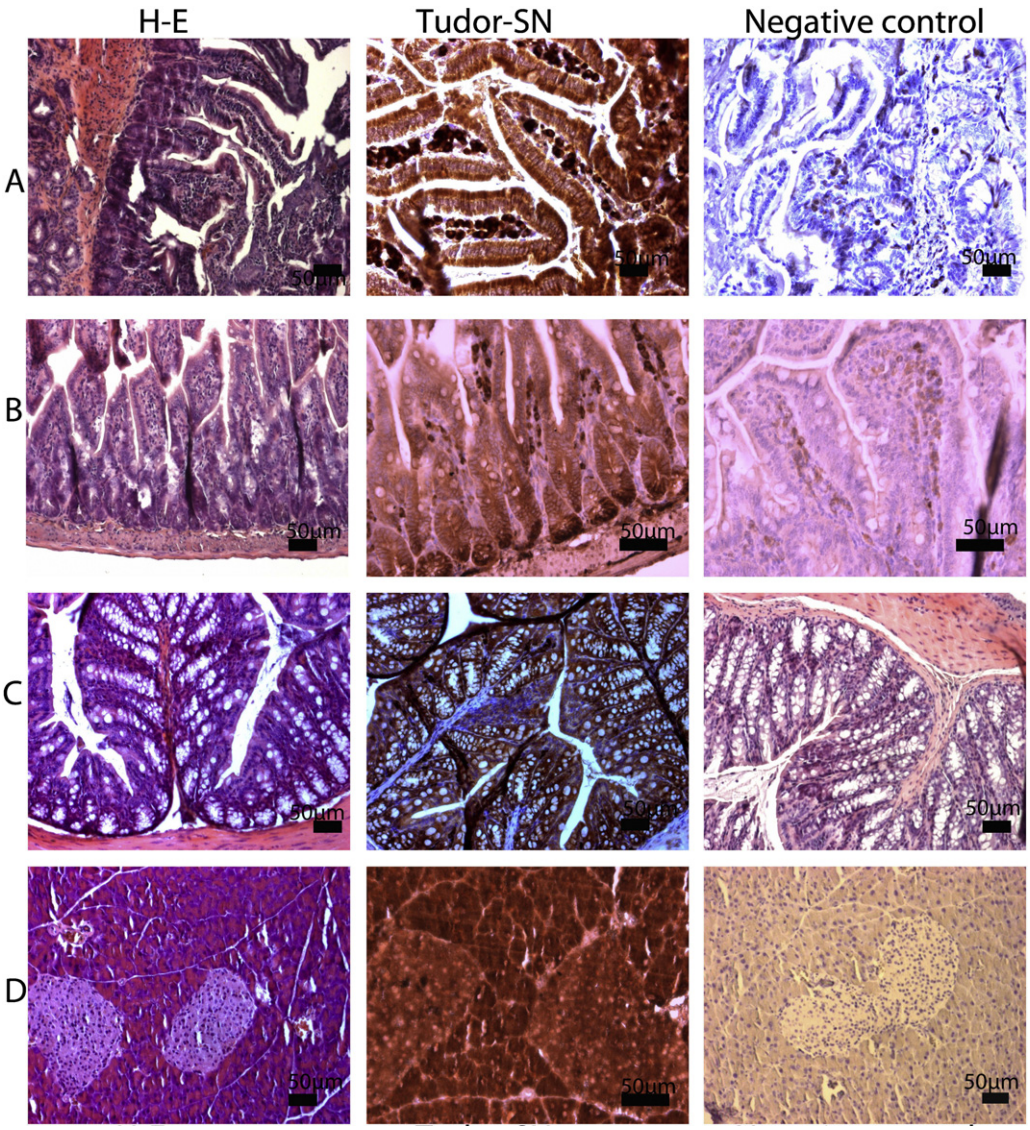

Fig. 4. Immunohistochemical staining of Tudor-SN in formalin fixed organs of gastro-intestinal system of mouse. The figure depicts the immunohistochemical staining of Tudor-SN in duodenum (A), ileum (B), colon (C) and pancreas (D). The first column shows ordinary H-E staining of the respective organs; the second column shows Tudor-SN staining and the third column shows negative control. In duodenum the expression level of Tudor-SN seems equal in all cells of villi (A). Fig. 2B depicts the up-regulated expression level of Tudor-SN in the crypt of Lieberkühn compared to cells lining the villi. Notably the expression level of Tudor-SN is higher in Paneth cells than enteroendocrine cells. In the villi of ileum the expression level of Tudor-SN is higher in scanty cytoplasm of Goblet cells than surface absorptive cells (B). In the colon the expression level of Tudor-SN was up-regulated in enteroendocrine and Goblet cells compared to surface absorptive cells (C). In pancreas the expression level of Tudor-SN is up-regulated in the exocrine part compared to its expression level in endocrine part (D) (scale bar = 50 μm).

speed of 3500 rpm for 30 s and homogenization was repeated twice. Samples were incubated for 5 min at room temperature. Thereafter, 0.2 ml of chloroform was added to each sample and samples were incubated for 5 min. Thereafter, samples were centrifuged for 15 min at 12,000 × *g* at 4 °C. The upper aqueous phase was transferred to fresh test tubes and RNA was precipitated by adding 0.5 ml of isopropyl alcohol. Samples were incubated at room temperature for 5 min and centrifuged at 12,000 × *g* for 10 min at 4 °C. RNA pellet was washed with 1 ml of 75% ethanol and samples were centrifuged at 12,000 × *g* at 4 °C, dried and dissolved in water. Total RNA was transcribed to cDNA with M-MULV reverse transcripitase (Fermentas) and quantitative real-time PCR was performed using the cDNA as a template, Tudor-SN primers (CCGAACGCAGCTCCTACTAC and TAGCAGATCGCTCCTTCTCC) as target gene and TBP primers (TGGTTTGCCAAGAAGAAAGTG and GCCATAAGGCATCATTGGAC) as control gene specific primers with SYBR green reagents and BioRad CFX96 Real-Time PCR machine.

3. Results

3.1. Expression pattern of Tudor-SN in major organs

Immunohistochemical staining of Tudor-SN was performed with specific MoAb against Tudor-SN in major organs of mouse (liver, brain, kidney and lung) (Fig. 1) (Saarikettu et al., 2010). In the liver, the staining of Tudor-SN shows low signal in hepatocytes (Fig. 1A) which is even more apparent in fluorescent immuonohistochemical staining (Fig. 2C). However, both immunohistochemical and fluorescent immunostaining showed up-regulated expression of Tudor-SN in the endothelial cells of sinusoids (Figs. 1a and 2C). We performed also double-staining of liver specimens with anti-Tudor-SN and anti-CD68 antibodies to determine whether Tudor-SN is expressed in Kupffer cells. Kupffer cells are specialized macrophages located in the lining walls of sinusoids. However, Tudor-SN signal was not observed

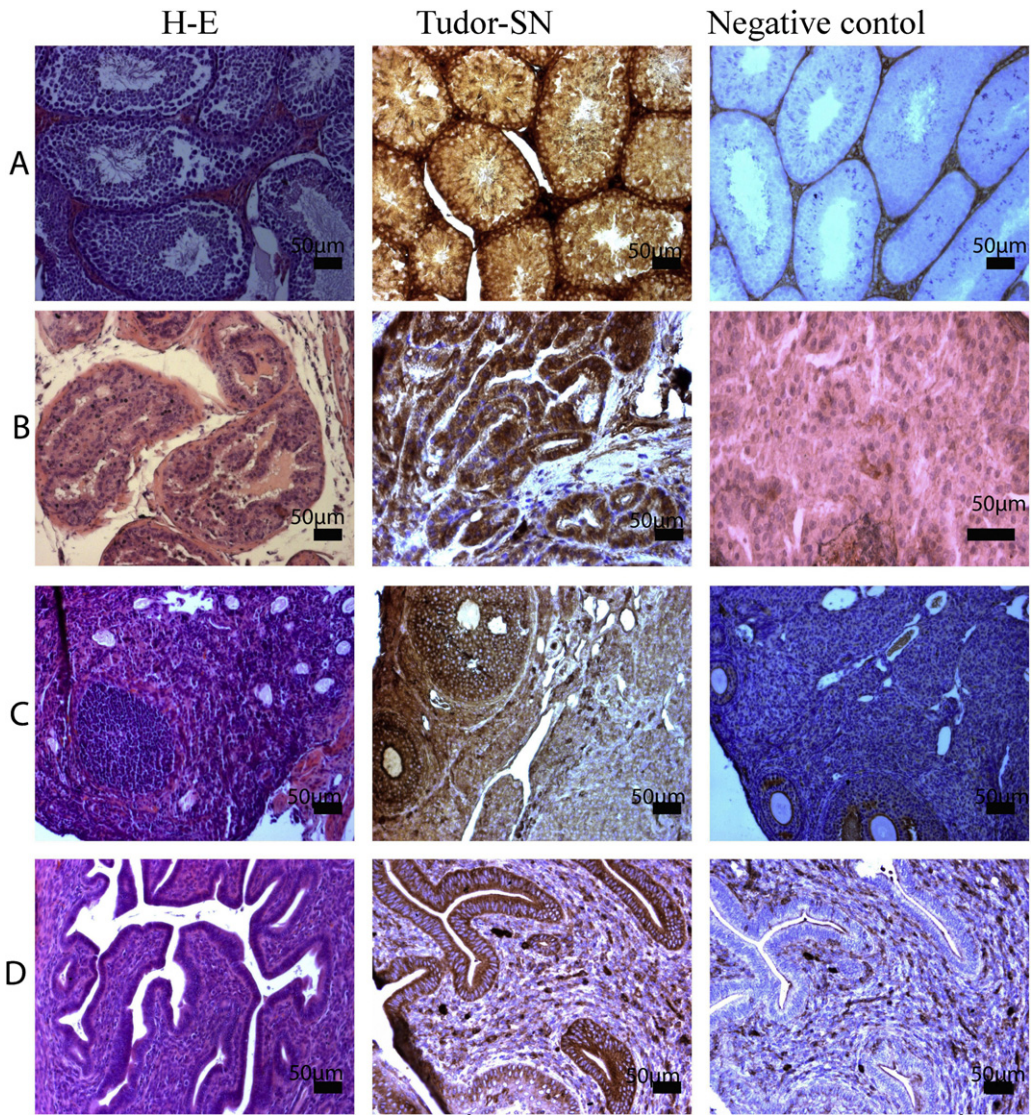

Fig. 5. Immunohistochemical staining of Tudor-SN in formalin fixed organs of reproductive system of mouse. The figure depicts the immunohistochemical staining of Tudor-SN in testis (A), prostate gland (B), ovary (C) and uterus (D). The first column shows ordinary H–E staining of the respective organs; the second column shows Tudor-SN staining and the third column shows negative control. In testis, expression of Tudor-SN is higher in spermatogonial cells. The expression of Tudor-SN shows a decreasing trend in more differentiated spermatocytes and Sertoli cells and is completely absent in terminally differentiated spermatids and spermatozoa cells. Turdor-SN is also expressed in Leydig and myofibroblast cells higher than in spermatogonial cells (A). In prostate gland epithelium the expression level of Tudor-SN is same in all glandular epithelium (B). In ovary, the expression level of Tudor-SN is high in actively proliferating follicular cells; however, Tudor-SN is not expressed in oocytes in any stages of follicular growth (C), which are also terminally differentiated cells. In uterus, the expression level of Tudor-SN is higher both in endometrial and glandular epithelium compared to stromal cells (D) (scale bar = 50 μm).

in Kupffer cells (data not shown). Immunohistochemical staining of Tudor-SN in brain showed the expression of protein in cortical neurons and in some glial cells (Fig. 1B). In the kidney immunohistochemical staining indicated higher Tudor-SN expression in distal tubules compared to the proximal tubules (Fig. 1C); however, the up-regulation of Tudor-SN in distal tubules was not obvious in fluorescent immunostaining (Fig. 2A and B). Both immunohistochemical and fluorescent immunostaining showed up-regulated expression level of Tudor-SN in the endothelial cells of glomerulus compared to the surrounding podocytes and nearby mesangial cells (Figs. 1C and 2A, B). Indeed, in mesangial cells the expression of Tudor-SN was lower compared to all surrounding cells of the Bowman's capsule. In the lung Tudor-SN is expressed both in bronchiolar epithelium and alveolar cells, and the expression of Tudor-SN is up-regulated in cells of bronchiolar epithelium and type II pneumocytes compared to Type I pneumocytes (Figs. 1D and 3D).

3.2. Expression of Tudor-SN in gastro-intestinal system

Immunohistochemical staining of Tudor-SN in organs of gastro-intestinal system (duodenum, ileum, colon and pancreas) is shown in Fig. 4. In duodenum the expression level of Tudor-SN was similar among villus lining cells (Figs. 3A and 4A). In the ileum the expression level of Tudor-SN was up-regulated in the crypt of Lieberkühn compared to cells lining the villi, notably the expression level of Tudor-SN was higher in Paneth cells than in enteroendocrine cells (Figs. 3B and 4B). In the villi of ileum the expression level of Tudor-SN was higher in cytoplasm of Goblet cells than in surface absorptive cells (Fig. 4B). In lamina propria of both duodenum (Figs. 3A and 4A) and ileum (Figs. 3B and 4B) Tudor-SN is up-regulated in T-cells and co-localized with CD3 (data not shown). In the colon the expression level of Tudor-SN was up-regulated in enteroendocrine cells and Goblet cells compared to surface absorptive cells (Fig. 4C). In pancreas, the expression level of Tudor-SN was

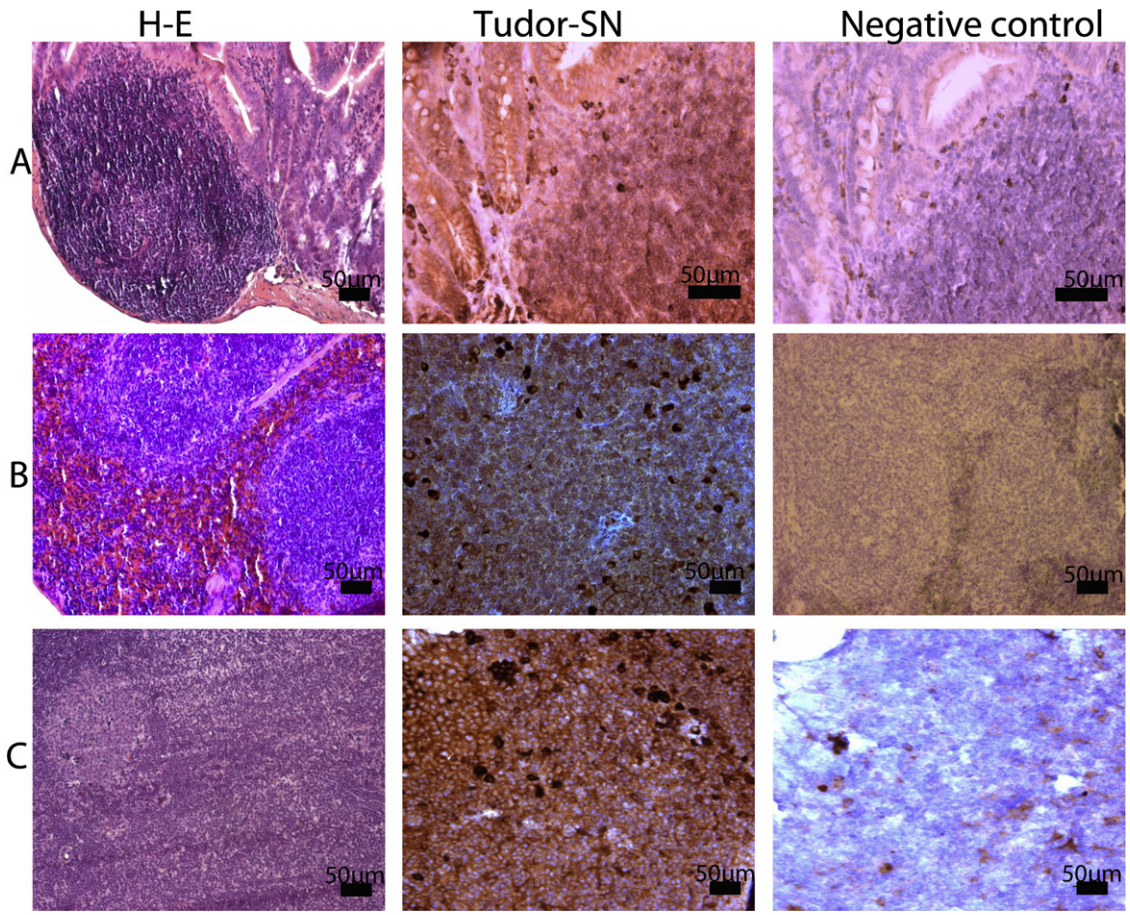

Fig. 6. Immunohistochemical staining of Tudor-SN in formalin fixed lymphoid organs system of mouse. The figure depicts the immunohistochemical staining of Tudor-SN in Peyer's patch (A), spleen (B) and lymph node (C). The first column shows ordinary H-E staining of the respective organs; the second column shows Tudor-SN staining and the third column shows negative control. In Peyer's patch, the expression level of Tudor-SN was higher in sub-epithelial zone than follicle associated epithelium (A). In the spleen Tudor-SN is up-regulated in the cells of red pulp than cells of white pulp (Fig. 4B). In lymph node Tudor-SN is expressed both in cells of cortex and medulla and its expression is up-regulated in lymphocytes outside germinal center compared to cells of the germinal center (Fig. 4C) (scale bar = 50 μm).

up-regulated in exocrine pancreatic acinar cells compared to cells of the endocrine gland (Figs. 3C and 4D).

3.3. Expression of Tudor-SN in reproductive system

Immunohistochemical staining of Tudor-SN in organs of the reproductive system (testis, prostate gland, ovary and uterus) is shown in Fig. 5. Immunohistochemical (Fig. 5A) and fluorescent immunohistochemical (Fig. 2D.) staining of Tudor-SN in testis showed up-regulated expression in spermatogonial cells compared to other cells of seminiferous tubules. However, in spermatocytes and Sertoli cells the expression level of Tudor-SN showed a decreasing trend and the expression was totally absent in terminally differentiated spermatids and mature spermatozoa. However, in Leydig's cells and myoepithelial cells the expression level of Tudor-SN appeared higher compared to nearby spermatogonial cells (Figs. 2D and 5A). In prostate gland the expression level of Tudor-SN appeared to be the same along prostate gland epithelial cells (Fig. 5B). In ovary, our immunohistochemical and fluorescent immunostaining of Tudor-SN showed up-regulated expression in follicular cells compared to stromal cells. Tudor-SN was not expressed in oocytes at any stage of folliculogenesis (Figs. 3E and 5C). In secondary follicles Tudor-SN expression was up-regulated in follicular epithelial cells compared to Thecal cells (Figs. 3E and 5C). In uterus immunohistochemical staining of Tudor-SN showed the expression in endometrial and glandular epithelial cells whereas the expression level of Tudor-SN was minimal in the stromal cells of uterus (Fig. 5D).

3.4. Expression of Tudor-SN in lymphoid organs

Immunohistochemical staining of Tudor-SN in lymphoid organs (Peyer's patch, lymph node and spleen) is shown in Fig. 6. In Peyer's patch the expression level of Tudor-SN was higher in sub-epithelial zone than in follicle associated epithelium (Fig. 6A). In Fig. 7C fluorescent immunostaining shows co-expression of Tudor-SN with CD3 in T-lymphocytes, but also some CD3 negative cells were found to express Tudor-SN. Immunohistochemical staining of Tudor-SN in the spleen showed up-regulated expression in the cells of red pulp compared to cells of white pulp (Fig. 6B). The double staining of spleen with Tudor-SN and CD3 showed co-expression of Tudor-SN with CD3 in T-cells (Fig. 7D). Double staining of spleen with Tudor-SN and CD68 showed that Tudor-SN is not expressed in macrophages (Fig. 8B). Immunohistochemical and fluorescent immunostaining of Tudor-SN showed expression of Tudor-SN in lymph node both in cells of cortex and medulla (Figs. 6C and 7A and B). Furthermore, double staining of lymph node with Tudor-SN and CD3 showed co-expression of Tudor-SN and CD3 in T-cells both in cortex of lymph node (Fig. 7A) and in the cord lymphocytes in the medulla (Fig. 7B). However, in a few high CD3 expressing cortical lymph node T-cells, the expression

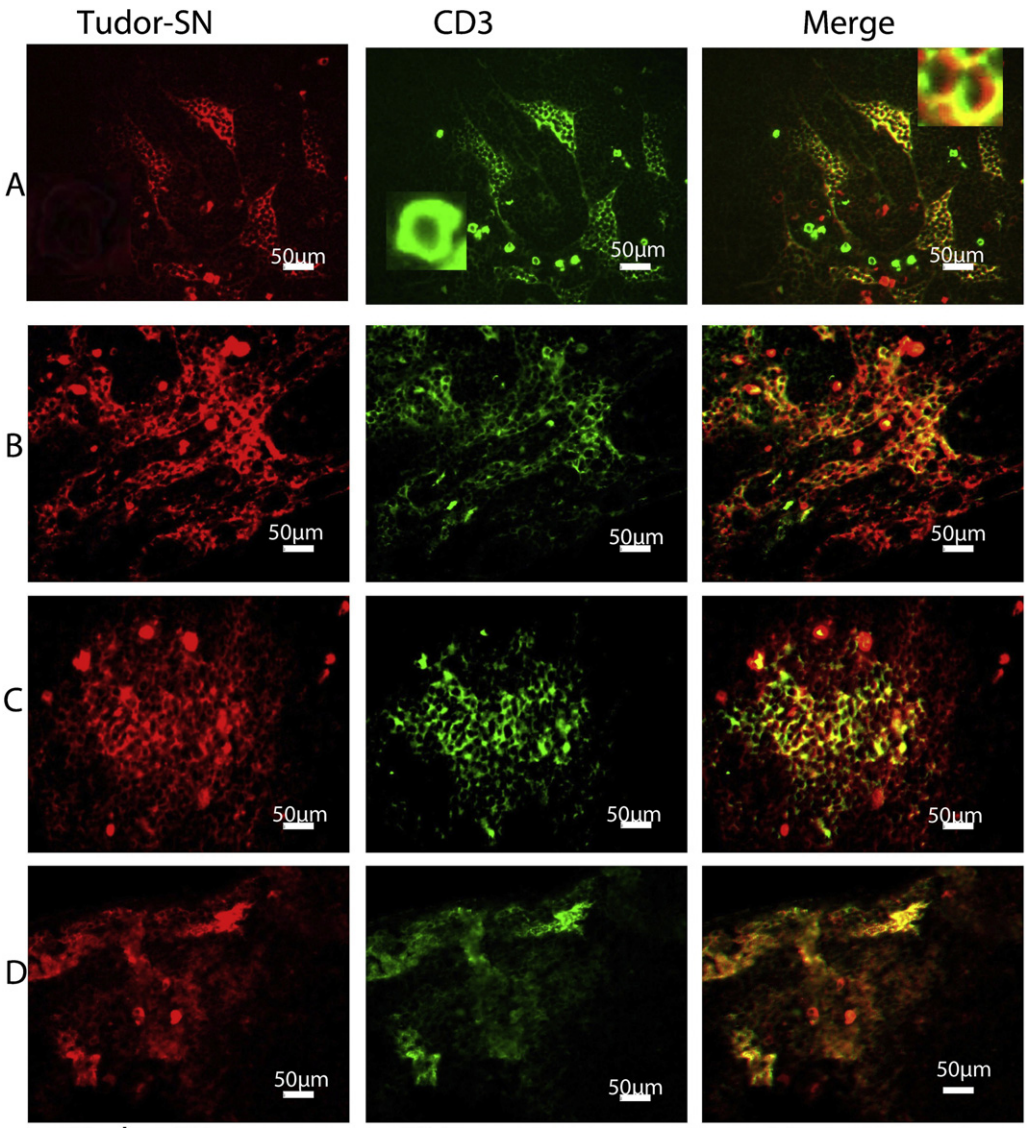

Fig. 7. Double fluorescent immunostaining of Tudor-SN and CD3 from frozen mouse lymphoid organs. The figure depicts the double fluorescent immunostaining of Tudor-SN and CD3 with mouse anti-Tudor-SN and mouse anti-CD3 antibody from frozen section of cortical lymph node (A) and medulla of lymph node (B), Peyer's patch (C) and Spleen (D). The first column shows Tudor-SN staining, the second column shows CD3 staining and the third column shows merged image of Tudor-SN and CD3 staining. Double fluorescent immunostaining of lymph node with anti-Tudor-SN and anti-CD3 antibody shows co-localization of Tudor-SN and CD3 in T-cells of both in the cortex of lymph node (A) and in the cords lymphocytes of the medulla (B). However, in few T-cells of the cortex where CD3 expression is up-regulated the expression level of Tudor-SN was down-regulated (A). Tudor-SN is co-localized with CD3 cells (T-lymphocytes) in Peyer's patch (C) as well as in spleen (D).

level of Tudor-SN was down-regulated (Fig. 7A). Double staining of lymph node with mouse anti-Tudor-SN and mouse anti-CD68 showed that Tudor-SN was not expressed in the macrophages of lymph node (Fig. 8B).

4. Discussion

Tudor-SN is a multifunctional protein implicated in several cellular processes such as a transcriptional co-activation, regulation of splicing and apoptosis, and identified as a component of RISC complex and pre-mRNA 3′ processing complex. The protein is well conserved in metazoans, but the exact physiological function of the protein is still elusive. Tudor-SN has been reported to be expressed in several organs such as mammary gland, pancreatic cells, parotid, anterior pituitary, corpus luteum, ovarian follicular cells, placenta and small intestine both in bovine and mouse, but any comprehensive analysis of tissue and cell expression has not been reported. Here we used our recently developed specific MoAb against Tudor-SN and performed a comparative tissue and cell expression analysis in mouse organs and tissues. The data show that Tudor-SN is almost ubiquitously expressed with the exception of muscle tissues. The expression of Tudor-SN in different cells of mouse tissues and organs is subjectively evaluated and summarized in Table 1. The expression is abundant in epithelial cells and particularly strong in proliferating and precursor cells. The expression profile of Tudor-SN has been analyzed in normal human tissues (Pontén et al., 2008; Uhlén et al., 2005, http://www.proteinatlas.org/). In this study the mouse organ and tissues specimens were stained with the same antibody which allowed comparison between mouse and human expression patterns. The general expression pattern of Tudor-SN in mouse and human organs is similar with only few differences that are mentioned in the following paragraphs. Human and mouse Tudor-SN are highly homologs (97% identity) and it is likely the function of the protein is similar in both species.

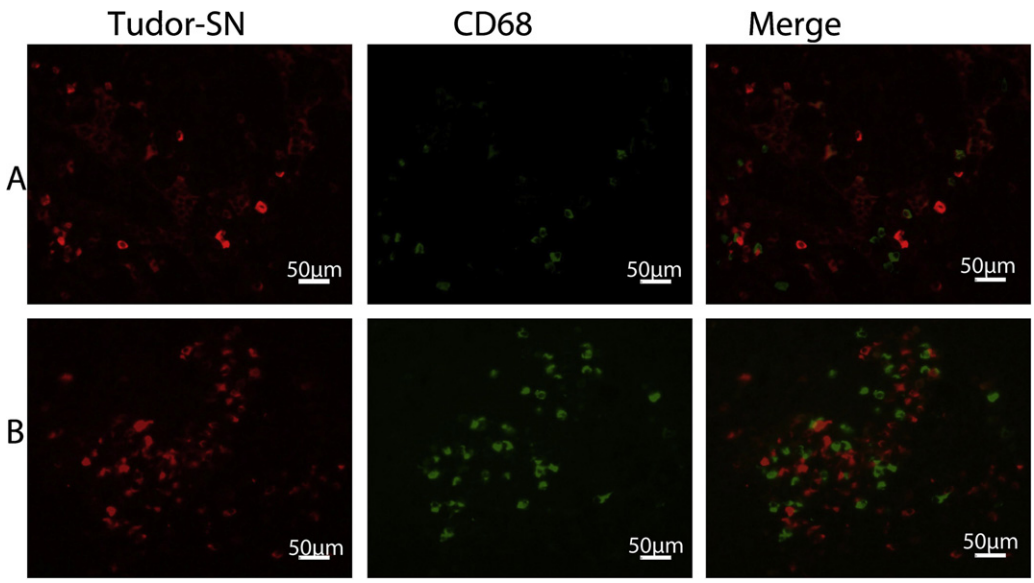

Fig. 8. Double fluorescent immunostaining of Tudor-SN and CD68 from frozen mouse organs. The figure depicts the double fluorescent immunohistochemical staining of Tudor-SN and CD68 from frozen section of cortical lymph node (A) and spleen (B). Tudor-SN is not expressed in macrophages in lymph node or in spleen.

Tudor-SN was abundantly expressed in active secretory organs most notably in exocrine pancreas (Figs. 3C and 4D). Both immunoblotting and immunohistochemical staining confirmed that Tudor-SN was up-regulated in exocrine pancreas in mice while in Langerhans cells we detected much lower expression level. Expression of Tudor-SN in actively secreting organs has also been reported in lactating mammary gland and in liver (Broadhurst and Wheeler, 2001; Broadhurst et al., 2005). Steatogenic condition is reported to promote Tudor-SN localization from other cellular compartments to specific low density lipid droplets (Garcia et al., 2010). Our immunohistochemistry results indicate that the expression level of Tudor-SN is relatively low in hepatocytes, but high expression level is detected in neighboring endothelial sinusoid cells.

Tudor-SN is reported to be localized in the endoplasmic reticulum and lipid droplets in cytoplasm of milk secreting mammary epithelial cells of mouse and cow (Keenan et al., 2000). The increased abundance of Tudor-SN expression during lactation is reported to occur without changes in the level of its mRNA, implying that the protein is regulated post-transcriptionally (Broadhurst et al., 2004). Comparison of the mRNA and protein expression of Tudor-SN by immunoblotting, immunohistochemistry and RT-QPCR in liver, lung, muscle, kidney and brain showed close correlation between mRNA and protein levels (Fig. 9). Besides, Keenan et al. (2000) also demonstrated the appearance of post-translationally modified form of Tudor-SN protein on the surface of lipid droplets in lactating mammary gland epithelial cells. The robust up-regulation of Tudor-SN in secretory organs like pancreas

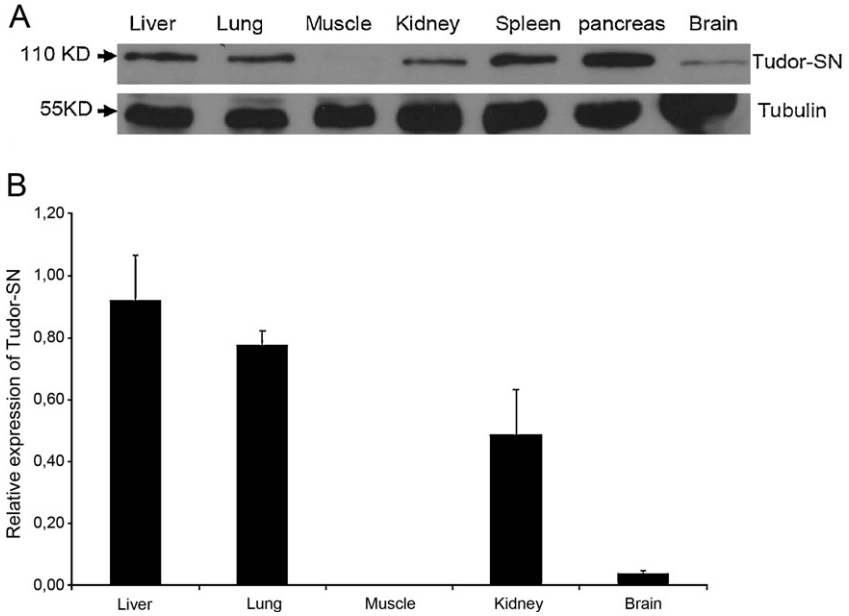

Fig. 9. Expression level of Tudor-SN in selected mouse organs. The figure depicts the expression of Tudor-SN protein in liver, lung, skeletal muscle, kidney, pancreas and brain detected by Western blotting (A) and the relative mRNA expression level of Tudor-SN in liver, lung, skeletal muscle, kidney and brain by RT-qPCR (B).

as demonstrated in this study and its presence on the surface of lipid droplets of lactating mouse mammary gland epithelia (Keenan et al., 2000) suggests that Tudor-SN may have an important role in the posttranslational or posttranscriptional regulation of secreted proteins. Recent studies in *Arabidopsis* provide insights into this question and show that Tudor-SN deficient mutant of *Arabidopsis* failed to survive in salt stress condition and Tudor-SN was able to stabilize subsets of salt responsive mRNAs which are intended to secretory pathway (dit Frey et al., 2010). Whether the ability of Tudor-SN to stabilize subsets of mRNAs of proteins intended to be secreted is a more general on secretory organs remains to be determined.

Previous studies have suggested a role for Tudor-SN in cell proliferation, and cellular transformation has been achieved via ectopic expression of Tudor-SN and cell growth was inhibited by decreased expression of cellular Tudor-SN (Tsuchiya et al., 2007). High expression of Tudor-SN in proliferating and precursor cells and decreased or absent expression in terminally differentiated cells was observed in our analysis. This correlation was obvious in reproductive organs, where the expression level of Tudor-SN was strong both in spermatogonial and Leydig cells and the expression level decreased as cells differentiate and Tudor-SN expression was not detected in terminally differentiated spermatozoa (Figs. 2D and 5A). In ovary Tudor-SN was also strongly expressed in both stromal and follicular cells, moderate in thecal cells and weak in stromal cells (Figs. 3E and 5C). The expression levels of Tudor-SN in human show similar trend and the expression is strong in uterine glandular cells in pre-menopause human but weak in post-menopause uterus. In adult mouse, at equivalent age of human pre-menopause, the expression level of Tudor-SN is strong in endometrial glandular epithelial cells and weak in endometrial stromal cells. Furthermore, Tudor-SN was up-regulated in rapidly dividing cells, in the crypts of Lieberkühn of ileum (Figs. 3A and B and 4B) and basal keratinocytes of skin and hair follicle (Fig. 3F) compared to more differentiated or terminally differentiated neighboring cells in respective organs. In line with these conclusions, our data show that Tudor-SN is not expressed in skeletal muscle. Taken together, these results suggest that Tudor-SN has functions in actively metabolizing cells.

The expression level of Tudor-SN is high in neuronal cells of cerebral cortex and glial cells in mouse brain (Fig. 1B); however, in human brain the expression level of Tudor-SN is moderate in both cells. In the kidney of human the expression level of Tudor-SN is moderate both in glomerular and tubular cells. The expression level of Tudor-SN is up-regulated in distal tubules compared to proximal tubules in mouse (Fig. 1C). The expression level of Tudor-SN is moderate in glomerular cells and renal tubules. In the mouse lung, the expression level of Tudor-SN is up-regulated in bronchiolar epithelial cells and type II pneumocytes, but low in type I pneumocytes (Figs. 1D and 3D). In human lung the expression level of Tudor-SN is moderate in higher epithelial cells and moderate in macrophage, but low in pneumocytes. However, in mice Tudor-SN was not expressed in alveolar macrophages (data not shown). The wide tissue distribution of Tudor-SN from neuronal to epithelial cells tempts us to speculate that the protein may participate in general physiological functions.

The expression level of Tudor-SN is moderate in glandular cells of duodenum and the expression is strong in T-cells in lamina propria (data not shown). The expression level of Tudor-SN in glandular cells of ileum was moderate; however, the expression level of Tudor-SN was strong in Paneth cells in the crypts of Lieberkühn (Figs. 3B and 4B) and in T-lymphocytes of lamina propria of small intestine (Figs. 3A and B and 4B). However, the expression level in the ileum surface epithelium was weak. In the mouse colon, the expression level of Tudor-SN was moderate in enteroendocrine and Goblet cells; however, the expression level of Tudor-SN was lower in surface absorptive cells than enteroendocrine and Goblet cells (Fig. 4C). Presently we do not have any evidence to suggest that the differences in expression levels of Tudor-SN between human and mouse are due to physiological differences.

In mice Tudor-SN is up-regulated in most cells residing in surrounding follicles. Tudor-SN is also co-expressed with CD3 in lymph node, spleen and Peyer's patch. The expression of Tudor-SN was found to be down-regulated in few T-cells in lymph node which express high levels of CD3 (Fig. 7A). This finding suggests that

Table 1
Summary of expression of Tudor-SN in cells of mouse organs.

Organ system	Cells	Tudor-SN expression level subjective scores
Liver	Hepatocytes	**
	Sinusoidal endothelial cells	**
	Kupffer cells	*
Cerberum	Cortical neurons	***
	Glial cells	***
Kidney	Proximal tubular cells	***
	Distal tubular cells	****
	Cells lining loop of Henle	**
	Glomerular endothelial cells	***
	Podocytes	**
	Mesangial cells	*
Lung	Epithelial cells lining bronchioles	***
	Alveolar macrophages	*
	Type I pnuemocytes	**
	Type II pnuemocytes	***
Duodenum	Surface absorptive cells	**
	Enteroendocrlne cells	***
	Paneth cells	****
	Cells of Brunner glands	***
	Lymphocytes in lamina propria	****
Ileum	Surface absorptive cells	**
	Enteroendocrlne cells	***
	Paneth cells	****
	Goblet cells	***
	Lymphocytes in lamina propria	****
Colon	Surface absorptive cells	**
	Goblet cells	***
Pancreas	Acinar cells	****
	Cells lining pancreatic ducts	***
	Cells of islets of Langerhans	**
Testis	Spermatogonial cells	****
	Primary spermatocytes	***
	Sertoli cells	***
	Spermatozoa	*
	Leydig cells	****
	Myoepithelial cells	****
Prostate gland	Prostate gland epithelial cells	***
Ovary	Follicular cells	****
	Thecal cells	**
	Oocytes	*
	Stromal cells	**
Uterus	Endometrial epithelial cells	***
	Uterine glandular cells	***
	Uterine stroma cells	**
Lymphoid organs (Peyer's patch, lymph node and spleen)	T lymphocytes	****
	Macrophages	*
Skin	Basal keratinocytes	***
Hair follicle	Cells in the bulb of hair follicle	****

The table shows summary of Tudor-SN expression in different cells of mouse organs. Data were summarized by subjective scoring and the scores refer to an approximate estimate of strength of staining signals.
* Absent.
** Low.
*** Medium.
**** High.

Tudor-SN expression is down-regulated in more differentiated T cells of cortex which are characterized by up-regulated expression of CD3. Tudor-SN is not expressed in macrophages in lymphoid organs (spleen, lymph node and Peyer's patch) or in tissue residing macrophages such as alveolar macrophage in the lung and Kuppfer's cells in the liver.

Tudor-SN is also reported to be up-regulated in prostate cancer, more specifically in castration resistant prostate cancer (Kuruma et al., 2009). Though the exact function(s) of Tudor-SN in mammal is yet to be revealed, our current study sheds light on tissue and cellular expression and localization of Tudor-SN in different organs of mouse. Expression of Tudor-SN in rapidly dividing cells, its absence in terminally differentiated cells as well as its up-regulation in aggressive human neoplasm suggests that the protein may mediate imperative physiological functions and participate in human diseases such as cancer. Furthermore, the expression of Tudor-SN in T-cells as well as its co-expression with CD3 leads to speculation that the protein may have some role in regulation of immunity, and deserves further investigation.

Acknowledgements

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